

UNIVERSITÀ DEGLI STUDI DI MILANO

PhD Course in Veterinary and Animal Sciences (Cycle XXXIV)
Department of Veterinary Medicine and Animal Sciences

Nutrition and Immunity: Molecular Approaches

GABRIELA ÁVILA MORALES
R12345

SUPERVISORS: PROF. FABRIZIO CECILIANI
PROF. ARMAND SÁNCHEZ BONASTRE
DR. MURIEL BONNET

PHD PROGRAM COORDINATOR: PROF. FABRIZIO CECILIANI

Academic Year 2021-2022

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Abbreviations

ALA: α-linolenic acid

APC: antigen-presenting cells

ARA: arachidonic acid

cDNA: copy DNA

CFU: colony-forming units

CLA: conjugated linoleic acid

CP: citrus pectin

DAMP: damage-associated molecular patterns

DC: dendritic cells

DHA: docosahexaenoic acid

2-DE: two-dimensional gel electrophoresis

DIGE: differential imaging gel electrophoresis

DPA: docosapentaenoic acid

EPA: eicosapentaenoic acid

EV: extracellular vesicles

FA: fatty acid

FAE: Follicle-associated epithelium

FAO: Food and Agriculture Organization

FO: fish oil

GALT: gut-associated lymphoid tissues

GPR120: G protein-coupled receptor 120

GO: gene ontology

H_2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate

H_2O_2: hydrogen peroxide

HPLC: high liquid chromatography

HRP: horseradish peroxidase

IAV-S: influenza A virus in swine

IEC: intestinal epithelial cells

IEL: intraepithelial lymphocytes

IFN: interferon

Ig: immunoglobulins

IL: interleukin

LA: linoleic acid

LP: lamina propria

LPS: lipopolysaccharide

LTA: lipoteichoic acid

MACS: Magnetic-Activated Cell Sorting

M cell: microfold cells

MHC: Major Histocompatibility Complex

miRNA: micro RNA

mRNA: messenger RNA

MTT: dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

MS: mass spectrometry

MyD88: myeloid differentiation primary-response 88

NADPH: nicotinamide adenine dinucleotide phosphate

NFκB: nuclear factor kappa B

NK: natural killer

NLR: NOD-like receptor

NOD: nucleotide oligomerization domain

PAMP: pathogen-associated molecular patterns

PBMC: peripheral blood mononuclear cells

PGE_2: prostaglandin E2

PI3K: phosphoinositide 3-kinase

PMN: polymorphonuclear cells

PPARγ: peroxisome proliferator-activated receptor gamma

PP: Peyer's patches

PRR: pattern recognition receptors

PRRSV: porcine reproductive and respiratory syndrome virus

PUFA: polyunsaturated fatty acids

RNAseq: RNA sequencing

ROS: reactive oxygen species

SCFA: short-chain fatty acids

SED: subepithelial dome

SPM: specialized pro-resolving lipid mediators

sRNA: small RNA

TLR: Toll-like receptor

TNF-α: tumor necrosis alpha

Treg: regulatory T cells

TRIF: TIR-domain-containing adaptor inducing IFNβ

ZAS: Zymosan Activated Serum

Abstract

Nutrition plays an important role in modulating livestock species immunity. Therefore, this thesis aimed at evaluating the effects of different dietary molecules used in animal nutrition on mammalian and avian immunity. Both, *in vitro* functional analyses and OMIC technologies (proteomics and miRNAomics) were implemented herein for an integral characterization of the molecules' impact on the animals' immune response. Specifically, in this thesis the *in vitro* impact of the n-6 conjugated linoleic acid (CLA), citrus pectin (CP), and porcine milk exosomes and n-3 polyunsaturated fatty acids (PUFA) on bovine, chicken and porcine mononuclear cells immune response was evaluated, respectively.

In the first study, the *in vitro* activity of CLA on bovine monocytes apoptosis and immune activities, including chemotaxis, phagocytosis, killing capability, and extracellular reactive oxygen species (ROS) production was assessed. Anti-apoptotic effects and an increase in extracellular ROS production during experimental pro-inflammatory conditions were observed, only when using the mixture of the two main isomers of CLA in equal proportions (50:50). The present results demonstrated for the first time that CLA does have immunomodulatory effects on some functions of bovine monocytes *in vitro* and that the CLA (50:50) mixture is more effective than the CLA isomers individually. The proteomics analysis performed on bovine peripheral blood mononuclear cells (PBMC) revealed that CLA (50:50) mixture does modulate bovine PBMC proteome, supporting the antiapoptotic and immunomodulatory effects observed in the previous *in vitro* study on bovine monocytes, and propose a potential cytoprotective role of CLA (50:50) mixture against oxidative stress.

In the second study, the *in vitro* activity of CP on chicken monocytes viability, apoptosis, chemotaxis and phagocytosis was assessed. The study demonstrated for the first time that CP inhibits monocytes' chemotaxis and phagocytosis *in vitro*, suggesting a potential anti-inflammatory activity. The proteomics analysis carried out on chicken PBMC provided a proteomics background to the anti-inflammatory activity of CP, demonstrating that the *in vitro* reduction of phagocytosis and chemotaxis is associated with changes in proteins related to the actin cytoskeleton.

In the third study, the *in vitro* activity of porcine milk exosomes on porcine monocytes viability, apoptosis, chemotaxis, phagocytosis, killing capability and extracellular ROS production was assessed. Milk exosomes were successfully purified from sows' milk and characterized using their size, concentration, morphology, and exosome protein markers. This study reported for the first time that porcine milk exosomes can be internalized by porcine monocytes *in vitro* and that they can modulate the cell's immune response, by decreasing their chemotaxis and

phagocytosis; and increasing their ROS production under resting and pro-inflammatory conditions. The proteomics analysis performed on porcine PBMC demonstrated for the first time that porcine milk exosomes can modulate porcine PBMC proteome *in vitro*. Moreover, the gene ontology (GO) functional analyses revealed that porcine milk exosomes enrich biological processes related to innate immune-related processes and exosome uptake processes, supporting the immunomodulatory effects and the exosome internalization observed in the previous *in vitro* study.

In the last study, the *in vitro* activity of the n-3 PUFA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), on porcine monocytes viability, apoptosis, chemotaxis, phagocytosis, and intracellular, extracellular and total ROS production was assessed. The results of the study showed that DHA and EPA at the highest concentration (200 μM) decreased porcine monocytes' viability. In addition, it was reported for the first time that DHA and EPA can exert differential *in vitro* immunomodulatory effects in pigs, by dampening monocytes' chemotaxis and potentiating their intracellular oxidative burst, respectively. The proteomics and miRNAomics analyses were not performed for this study. Instead, a first glance on the results from the bioinformatic analyses of the miRNAomics data of all the rest of the studies is presented herein.

In conclusion, this thesis provides both, a phenotypical and molecular characterization of the *in vitro* impact of these dietary molecules on bovine, porcine and chicken immune responses.

Chapter 1. The importance of livestock: cattle, pigs and poultry

Livestock includes terrestrial and domesticated animals that are raised with the sole purpose of providing a wide range of goods and services, including meat, milk, eggs, fibres, feathers and traction. Livestock species include, for example, cattle, water buffaloes, pigs, poultry (chickens, ducks, geese and quails), horses and small ruminants (goats and sheep) (https://www.fao.org). Livestock is the main source of animal protein (milk, meat and eggs) worldwide (Gilbert et al., 2018), corresponding to 34% of the global food protein supply and one-third of all the protein consumed by people. In addition, livestock production contributes to the global food systems by contributing to crop productivity through draught power and manure – organic matter often coming from the animals' faeces that is used as fertilizer in agriculture – and to the nutrition and subsistence of households from developed countries, but especially in those from developing countries. Indeed, as reported by the Food and Agriculture Organization (FAO), livestock accounts for approximately 40% of the total agricultural output in developed countries and around half of it in developing ones, supporting the subsistence of more than one billion people around the world.

Cattle, pigs and chickens are among the most widely used for food production and distributed livestock species worldwide. In Figure 1, their worldwide production profiles are presented.

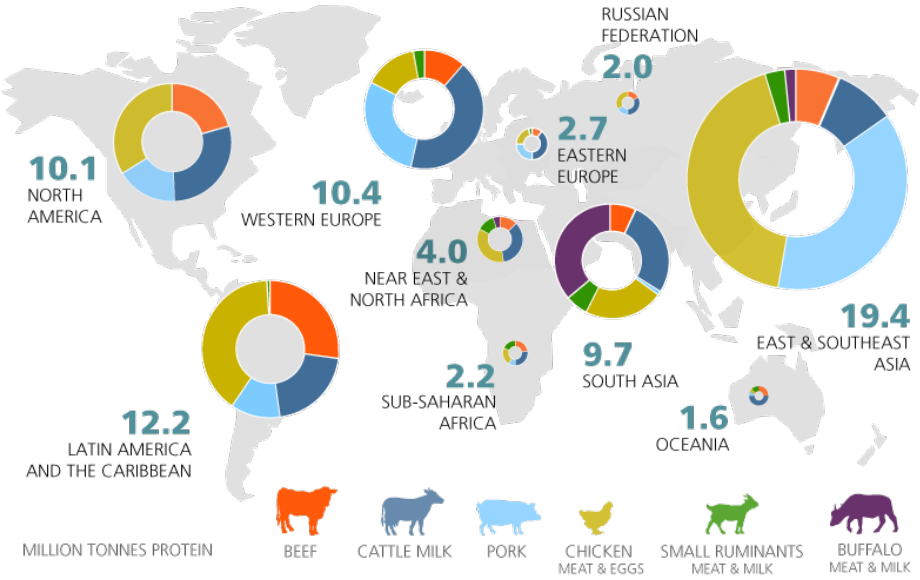


Figure 1. Total production (millions of tonnes of protein) of milk, meat and eggs from cattle, pigs, chicken and their global distribution.

Source: https://www.fao.org

1.1 Cattle

Cattle - domesticated bovids - are the most common and widespread species of large ruminant livestock and they can be classified into two subspecies, *Bos taurus* and *Bos indicus* (Utsunomiya et al., 2019). They are mainly raised to produce milk, meat and hides (leathers) and to provide draft power. Indeed, cows are the biggest contributors to milk production in the world, accounting for over 90%, and the countries where the most cow milk is produced are the United States of America, India and China (https://www.fao.org).

The estimated worldwide cattle standing population was around 1.5 billion by the end of 2021. *Bos taurus* breeds are commonly found in developed and temperate countries, while *Bos indicus* ones are mostly found in developing countries and humid tropics. India, Brazil, China, Ethiopia and Pakistan are the countries with the highest numbers of dairy cows in the world, being mostly *Bos indicus* (https://www.fao.org). Within the *Bos taurus* group, the Holstein-Friesian is the most widespread cattle dairy breed in the world as it is present in more than 150 countries (McGuffey and Shirley, 2011), and the most predominant throughout western Europe (Mayne et al., 2011). In European countries, around 76 million cows were estimated at the end

of 2020. France constituted the country with the highest (23.3 %) bovine population (https://ec.europa.eu/eurostat) as shown in Figure 2.

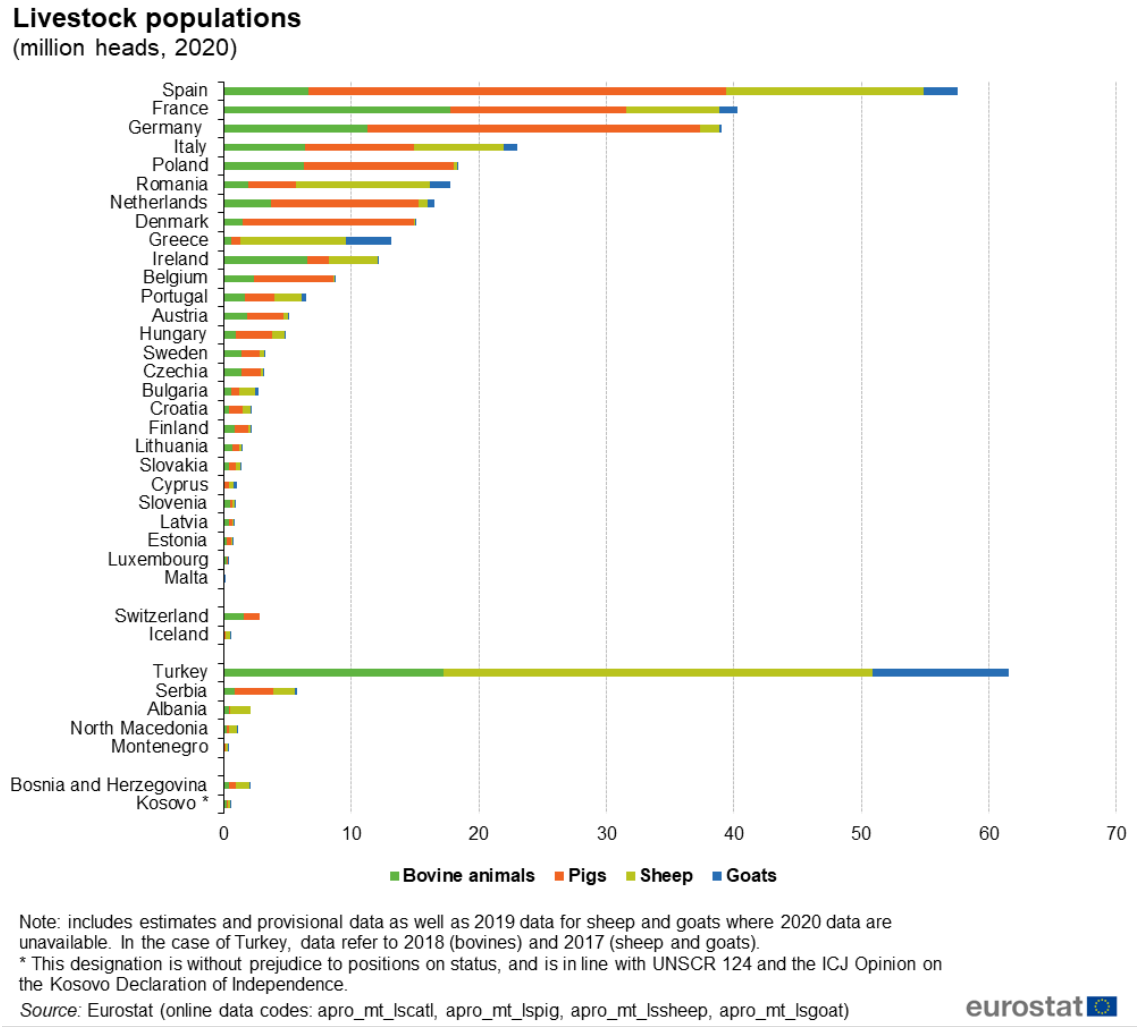


Figure 2. Livestock population (millions of heads) in European countries.
Source: https://ec.europa.eu/eurostat

Italy is one of the most important European countries for milk production. Indeed, in 2019 Italy was among the top five European countries with higher yields in milk production, delivering a total of 1,208,647 thousand tons. The milk production within the country comes mainly from Italian Holstein cows and it is concentrated mostly in the regions of Lombardy, Emilia-Romagna, Veneto and Piemonte, as these regions produced approximately 80% of the total milk in 2020 (Masi et al., 2021). Most of the milk (80%) produced is then allocated to cheese production, one of the main assets of the Italian dairy industry at both national and international levels (Ablondi et al., 2021).

1.2 Pigs

Sus scrofa domesticus, the domesticated pig, is bred for meat production. The pig sector, together with the poultry, is the fastest-growing livestock subsector and its production is global except in some regions where is prohibited due to cultural and religious reservations (https://www.fao.org). Worldwide, there are around 1 billion pigs, China harbouring almost half of them. In Europe, around 150 million have been estimated, representing the largest livestock category before cows. Moreover, in the EU pig meat production is one of the principal as it accounts for nearly half of total EU meat production. Countries like Germany, Spain and France are the major producers of pig meat in the EU (https://www.europarl.europa.eu). According to Eurostat (2020), the EU pig meat production had a new rise peak in 2020, where 23 million tons of pig meat were produced, producing Germany and Spain the largest amounts (around 5-5.1 million). In Figure 3, the total quantity of meat production of the most commonly used livestock species (cattle, pig, poultry and other small ruminants) in the EU in 2020 is shown in detail.

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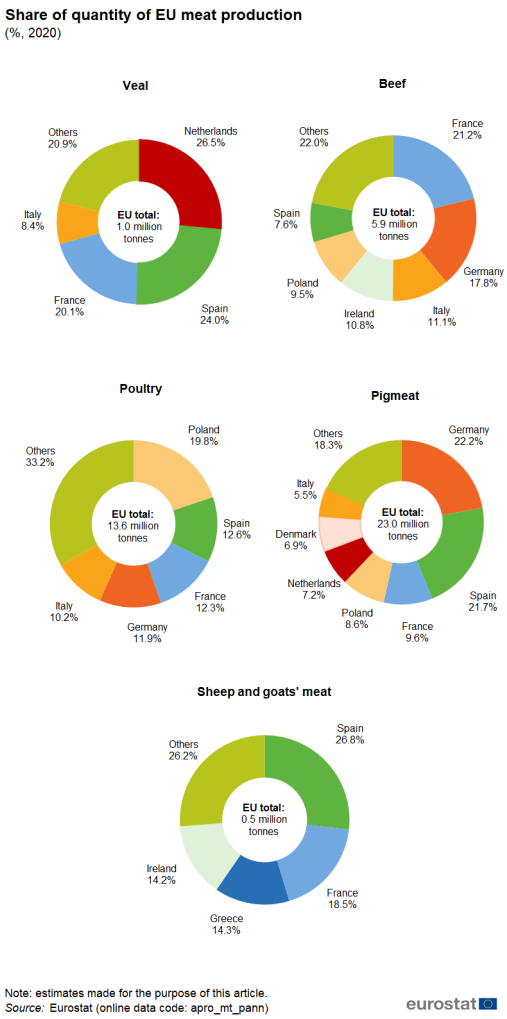


Figure 3. Total quantity of meat production (millions of tons) of the most commonly used livestock species in the EU, and the contributing percentages of the biggest EU meat producers' countries in 2020.

Source: https://ec.europa.eu/eurostat

Finally, pigs are extremely versatile and easy to adapt, which allows to raise them in very different systems that go from small and labour-intensive family units to massive, capital-intensive production units (Pedersen, 2018). This versatility makes the pig meat production sector, a fundamental one to address the increasing demand in the world for animal-source foods.

1.3 Chickens

Chickens (*Gallus gallus domesticus*) are the most important and numerous poultry species worldwide, accounting for 93% of the world's poultry population in 2019. They are specifically bred for meat or egg production, indeed contributing 90 % of world poultry meat production and 93 % of egg production. However, in small scale production systems they could also have a dual purpose. They are ubiquitously distributed, but more than half (56%) of the total worldwide chicken population is found in Asia. In 2017, the global chicken population was over 22 billion birds (https://www.fao.org), from which a vast majority corresponded to broilers– chickens raised specifically for meat (Bennett et al., 2022). Most of the commercial companies that use modern, industrialized and intensive production systems, have focused on the production of meat from broilers, mainly due to their high feed-meat conversion ratio, allowing them to rapidly adapt to the increasing demand for food worldwide (Rowe et al., 2019). Remarkably, in the EU approximately 13.6 million tons (Figure 3) of poultry meat were produced in 2020, showing an increase of 2.3% respect to 2019. In the same year, the principal poultry meat producers in the EU included Poland (2.7 million tons), Spain and France (1.7 million tons), Germany (1.6 million tons) and Italy (1.4 million tons), the latter showing a rise of 1.7% respect to the previous year (https://ec.europa.eu/eurostat). Finally, as chickens exhibit a great potential to adapt to diverse environmental conditions, have short lifecycles and high feed conversion ratios, which allow breeding improvement, they represent valuable livestock and genetic resource that could help us face and address the challenges of food shortage and security (Lawal and Hanotte, 2021).

1.4 Challenges in livestock production

In the past decades, the increase in the worldwide population and the high demand for animal-source food has caused an important increase in livestock production systems, making extremely necessary the development of novel strategies that could allow us to meet those needs without leaving aside livestock health and welfare (Scholten et al., 2013). Indeed, the improvement of livestock health, immunity, welfare and at the same time productive performance remains one of the major challenges in the livestock industry. Specifically, the rise in production of diseases and indiscriminate use of antibiotics have both affected animals' productivity, causing substantial economic losses worldwide (Hafez and Attia, 2020). For

example, in dairy cows, production diseases such as mastitis, external and internal parasites often reduce the efficiency of the production system, by decreasing milk yield and quality, animals' fertility and feed conversion, among others. Moreover, zoonotic diseases represent a high risk also for human health (e.g. tuberculosis and brucellosis) (Hoischen-Taubner et al., 2021) (https://www.fao.org). In chickens, the genetic pressure that has been put to improve their performance has shown to adversely affect the animals' welfare, immunity and consequently their disease tolerance. Common poultry enteric disorders, due to infection by rotavirus, coronavirus enteritis, parasitic infestation and *E. coli*; and respiratory diseases have caused substantial losses to the poultry industry (Hafez and Attia, 2020). Furthermore, viral diseases such as Newcastle disease and avian influenza are economically important diseases that cause high mortality and morbidity in chickens and have cost billions of dollars worldwide (Hafez and Attia, 2020) (https://www.fao.org). Lastly in the case of pigs, even if in the past decades an improvement in pig performance and productivity has been observed, the animals' health has not shown to increase parallelly. Indeed, many endemic, caused by important respiratory, intestinal, and systemic pathogens, (e.g. porcine reproductive and respiratory syndrome virus (PRRSV), influenza A virus in swine (IAV-S), pathogenic *E. coli* strains and *Streptococcus spp.*), epidemic (African swine fever), foodborne zoonotic diseases (human salmonellosis) are still prevalent in many pig herds worldwide, affecting the animals' health, welfare and productivity (Maes et al., 2020).

In conclusion, immunity is key for the animals' protection, disease control and for consequently assuring their overall health. Therefore, the improvement of livestock species' immunity is fundamental to control the spread of infectious diseases between the animals, prevent transmission of zoonotic diseases, minimize the use of antibiotics, enhance the animals' productivity and improve food security. As highlighted by the FAO, protecting livestock against diseases and preventing disease spread has a pivotal role in fighting hunger, malnutrition and poverty worldwide.

Chapter 2. The mammalian and avian immune system

In the first part of this chapter, the general principles of the mammalian and avian immune systems will be described together. However, at the end of this chapter, a separate subsection will be dedicated only to the avian immune system to further describe and focus on its differential aspects when compared to mammalian immunity.

2.1 The mammalian immune system

The host immune system is composed of a group of cells and molecules specialized to identify and destroy potentially dangerous microorganisms or their products, toxins, cancer and dead cells. The immune system besides protecting our body from invading agents is also responsible for tolerating and maintaining the symbiosis with the beneficial microbiota living in our bodies; and for being involved in tissue repair, wound healing and resolution of inflammation (Cohen, 2000; Swiatczak and Cohen, 2015).

To unleash an immune response against invading agents, it is first necessary that the host immune system sorts out self from non-self-entities, and from the non-self-ones to know which represent danger and which does not (Chaplin, 2010). According to the type of mechanisms implemented to recognize these non-self-entities, the immune system can be further classified into innate and adaptive immunity, as shown in Figure 1. Briefly, innate immunity corresponds to our first line of defense and it is characterized for being a non-specific and rapid response, while adaptive immunity is a slow, but highly specific response (Calder and Kew, 2002).

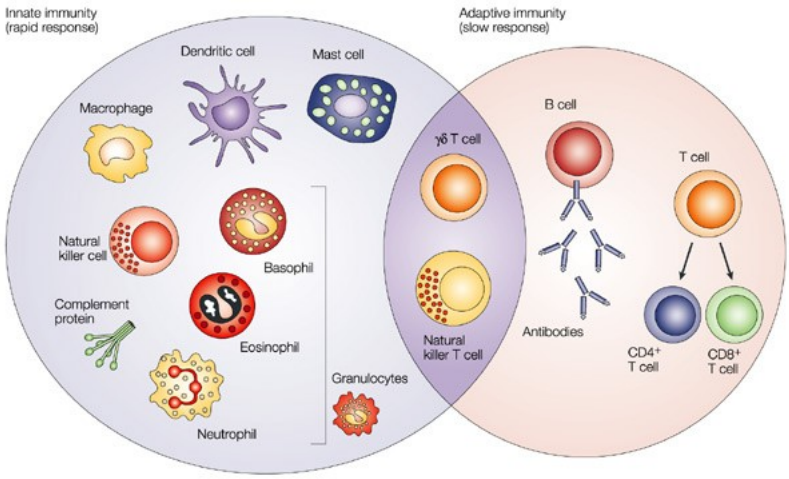


Figure 4. Overview of the major cellular and molecular players in innate and adaptive immunity.

Source: (Dranoff, 2004)

2.1.1. Innate Immunity

The mammalian and avian immune systems in response to potential pathogens unleash the first set of responses that activates the innate immunity, which serves as the first line of defense against infection. Innate immunity is mainly composed of: 1) physical barriers, including the epithelial cell and mucosal layers, blood-brain barrier, and molecular or chemical barriers, including soluble proteins and other bioactive molecules such as the complement proteins, cytokines, chemokines, reactive oxygen species (ROS), pattern recognition receptors (PPR) found in innate immune cells and antimicrobial peptides that bind pathogen-associated molecular patterns (PAMP); 2) innate immune effector cells (Chaplin, 2010). Among the innate immune cells, we can find polymorphonuclear cells (PMN) or also known as granulocytes (e.g. neutrophils, basophils and eosinophils), monocytes, macrophages, dendritic cells, mast cells and a group of lymphocytes called natural killer (NK) and gamma-delta T cells ($\gamma\delta$T), which function at the intersection of innate and adaptive immunity (Vlasova and Saif, 2021). Neutrophils, monocytes and macrophages are specialized phagocytes that kill bacteria and fungi after phagocytosis (Klasing, 2007). Usually, pathogens are first recognized by monocytes/macrophages through TLR, starting an inflammatory response by releasing inflammatory mediators that at the same time recruit neutrophils to the affected tissue for them further kill the pathogens (Yang et al., 2014). Eosinophils and basophils on the other side

correspond to the antiparasitic cells of the innate immune system, while NK cells are involved in viral infections and destroying cancer cells (Li et al., 2010; Stone et al., 2011).

These innate immune responses are known to be very rapid, as no preliminary cloning step is needed as in adaptive immunity, and PPR that is widely expressed by different immune cells and have been selected over evolutionary time, quickly detect PAMP, triggering different inflammatory reactions (Dranoff, 2004; Chaplin, 2010).

The PPR are class of receptors expressed by both immune cells, such as mast cells, monocytes, macrophages, dendritic cells, innate lymphoid cells, and basophils; and some non-immune cells, such as epithelial cells (Gong et al., 2020; Zindel and Kubes, 2020). The two most commonly known PPR in the innate immune system of vertebrates are Toll-like receptors (TLR) and the cytoplasmic nucleotide oligomerization domain (NOD)-like receptors (NLR), found in the surface or inside of antigen-presenting cells (Fritz et al., 2006). On the other hand, PAMP is specific and highly conserved molecular structures found in the same type of microorganisms, which include lipids, proteins, and nucleic acids such as Gram-negative bacteria lipopolysaccharides (LPS), Gram-positive bacteria lipoteichoic acid (LTA), and bacterial DNA, among others (Janeway, 2001; Zindel and Kubes, 2020). It is important to highlight that PPR can also recognize molecular structures from apoptotic, damaged senescent and tumorigenic cells, which are often known as the damage-associated molecular pattern (DAMP) (Gong et al., 2020). As shown in Figure 5, once the PPR recognize and bind their associated PAMP or DAMP they recruit adaptor molecules (e.g. myeloid differentiation primary-response 88 (MyD88) and TIR-domain-containing adaptor inducing IFNβ (TRIF)) that help to initiate downstream signaling pathways (e.g. NFκB) to exert anti-infection, antitumor, and other immunoprotective functions (Gong et al., 2020; Li and Wu, 2021).

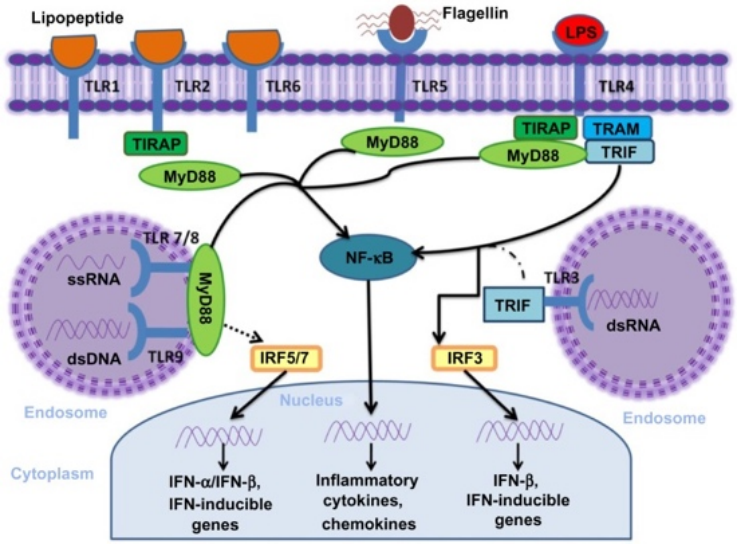


Figure 5. The recognition of pattern pathogen-associated molecular patterns (PAMP) by Toll-like receptors (TLR), and their downstream signaling pathways.

Abbreviations: *LPS, lipopolysaccharide; TLR, toll-like receptors; NF-κB, nuclear factor kappa- B; MyD88, myeloid differentiation factor 88; IFN, interferon; ssRNA, single-strand ribonucleic acid; dsDNA, double-strand deoxyribonucleic acid; dsRNA, double-strand ribonucleic acid; IRF, IFN regulatory factor; TRAM, TRIF-related adaptor molecule.*

Source: (Vallés et al., 2014)

Specifically, the activation of the immune cells expressing those PRR leads to the production of pro-inflammatory cytokines and chemokines, and IFN-α and IFN-β inducible genes, which start the inflammatory response and help in recruiting professional phagocytes and other immune cells in the site of infection or injury and activate adaptive immune responses (Mogensen, 2009; Chen and Nuñez, 2010) (Figure 6).

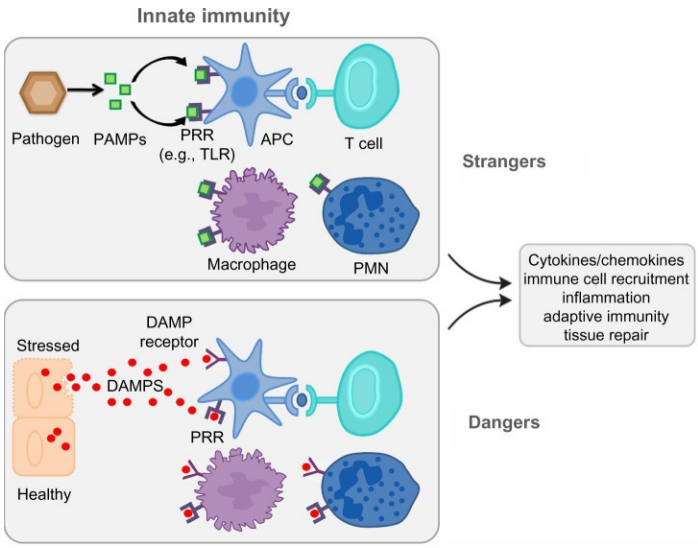


Figure 6. Recognition of PAMP and DAMP by PPR of different immune cells, and their downstream effects.

Abbreviations: *TLR, toll-like receptor; APC, antigen-presenting cells; PMN, neutrophils; PPR pattern recognition receptors; PAMP, pathogen-associated molecular pattern; DAMP, damage-associated molecular pattern.*

Source: (Vallés et al., 2014)

Specialized phagocytes such as neutrophils, monocytes, macrophages migrate towards the site of infection through a process known as chemotaxis – the ability of the cells to migrate in response to a chemical signal or chemoattractant gradient concentration –, and once there they exert different inflammatory functions like production of pro-inflammatory cytokines, ROS and eventually phagocyte and kill the engulfed microorganism (WU, 2005; Rendra et al., 2019). Finally, these professional phagocytes are also responsible for presenting the foreign antigens to lymphocytes to activate an adaptive immune response, suggesting an important crosstalk and close relationship between both types of immunity (Rendra et al., 2019).

2.1.2. Adaptive immunity

Adaptive immunity provides the second line of defense and it unfolds when the innate immune system fails to resolve an infection. The adaptive immune system, just like the innate immune system, relies on both molecular humoral (e.g. immunoglobulins (Igs) and major

histocompatibility complex (MHC)) and cellular responses (Smith et al., 2019). Adaptive immunity is characterized by the slow response, as it is composed of a fewer number of cells that need to undergo first under proliferation after recognizing their specific antigen to be able to have enough cell numbers for an effective immune response. Despite, the adaptive immunity mounts a slower response than innate immunity, it is greatly specific for any individual pathogen, toxin or allergen, as it depends on antigen-specific receptors (e.g. T and B cell receptors) that are tailored and selected through somatic recombination of a large set of gene segments, allowing the formation of millions of different antigen receptors, each with potentially unique specificity for a different antigen. Another key feature of adaptive immunity is the immunological memory that it provides to the host as it produces long-lived cells that can re-express effector functions rapidly when they find again their specific antigen (Bonilla and Oettgen, 2010; Chaplin, 2010).

Cells involved in this type of immunity are T and B lymphocytes. T and B cells are involved in cellular immunity, exerting cytotoxic functions and humoral immunity by producing antibodies, respectively. It is through T cells, in secondary lymphoid organs, that adaptive responses start. First, antigen-presenting cells (APC) recognize, take up and process pathogens and their antigens, later they present them through surface MHC molecules to antigen-specific T cells for their further activation to function either as a helper (CD4+) or cytotoxic (CD8+) T cells (Fujihashi et al., 2013). After T cells activation and clonal expansion, they also recruit and give out signals to B cells that differentiate into antibodies-producing plasma cells. The antibodies - also known as immunoglobulins (Igs) - that are produced present a high affinity against foreign antigens and are key players of the so-called humoral immunity (Smith et al., 2019). Moreover, B cells can also act as APC to further activate T cells, creating a positive feedback loop. After antigen recognition, T and B cells can become long-lived memory cells that persist in the host for life-giving in this immunological memory and the ability to be activated and generate a more rapid and robust response in case of reinfection (Chaplin, 2010). Although, both innate and adaptive immunity present clear differences in the mechanisms used to protect us from non-self-entities and even though often they are thought to be opposite to each other, they work closely together and complement each other (Calder and Kew, 2002).

2.2 The avian immune system

Even though the avian immune system is genetically simpler than the one of mammals, they share the same basic structure and function (Ferreira Júnior et al., 2018). For example, TLR is

largely conserved between mammalian and avian species, so they mount a similar innate immune response. However, as it diverged from that of mammals over 200 million ago some differences can be found. Specifically, birds possess only one polymorphonuclear leucocyte, the heterophil, which is the analogue to the mammalian neutrophils (Wigley, 2017). Similar to mammals, avian heterophils exert phagocytic activities against microbes or foreign particles (Kogut et al., 2005). Moreover, the avian red blood cells and thrombocytes – analogues to mammalian non-nucleated platelets – are both nucleated, and the thrombocytes might play an important role in innate immunity as they may act as phagocytes and APC (Wigley, 2017). Thrombocytes constitute 70% of the peripheral blood mononuclear cells (PBMC) fraction, therefore confirming their critical role in the overall avian immune response (Désert et al., 2016). In addition, since avian thrombocytes are nucleated, have sizes and densities similar to lymphocytes and monocytes, and have the adherent capacity, technical challenges to isolate PBMC or monocytes alone are often encountered (Mudroňová et al., 2014).

In the case of their adaptive immunity, chickens have less polymorphism of major receptor families, present a 'compact or minimal' MHC complex as it expresses only two Class I and Class II genes, compared to the hundreds of genes of mammals, which limits chickens to develop an effective immune response against certain pathogens (Ferreira Júnior et al., 2018). They also present only three immunoglobulin classes (IgY, IgM and IgA) instead of the five found in mammals, possess three T-cell receptor types (one more than mammals), and they lack structured peripheral or mesenteric lymph nodes. Despite these differences, the adaptive immune system function is similar to that of mammals, as both cellular and humoral immunity are present (Wigley, 2017).

Chapter 3. The intestinal immune system

Generally, immune response takes place throughout the body. However, it is well known that the intestine is the largest immune organ in both mammalian and avian species, and where most of the immune interactions and shared signal pathways are produced between the mucosal immune system and the intestinal bacteria (Takiishi et al., 2017; Ma et al., 2018). Indeed, the intestine is the first site of interaction between the nutrients, microbiota and host immune response. Approximately, the lumen of the human gastrointestinal tract possesses trillions of commensal bacteria, a number that exceeds the number of cells from the host (Ramakrishna, 2013). This large number of bacteria is essential for influencing the host's health, as they are the ones responsible for the development and function of the immune system, among other roles (Ramakrishna, 2013). The intestinal immune system also plays an important role in communicating, tolerating and maintaining the homeostasis with the beneficial symbiotic microbiota there residing to maintain host health. Disruptions in this homeostasis can cause the onset of different acute and chronic intestine inflammatory diseases (Strober et al., 2007).

3.1 The mammalian intestinal immune system

3.1.1. Components and organization of the intestinal immune system: mucosal barrier and gut-associated lymphoid tissues (GALT)

The gut-associated lymphoid tissues (GALT) are distributed along the intestinal tract and contain one of the largest lymphoid cell populations in the body, which is organized as lymphoid tissues (Coker and Madan, 2020). Within the intestine, the intestinal mucosa separates the commensal bacteria from the GALT. This mucosa is a dynamic interface, containing a monolayer of intestinal epithelial cells (IEC) composed of enterocytes, goblet and Paneth cells, which generates physical and chemical barriers to maintain this separation (Okumura and Takeda, 2017). Goblet cells release mucus, a viscous fluid enriched in mucin glycoproteins that form large net-like polymers, which serves together with the tight junctions strongly linking IEC as a physical barrier (Okumura and Takeda, 2017). Additionally, as chemical barriers it is also possible to find within the mucus layer, anti-microbial peptides (e.g. defensins) that are produced by Paneth cells and immunoglobulin A (IgA) - an antibody isotype specialized in mucosal protection- produced by B cells found in the GALT and that collaborate with IEC to release it to the mucosal surface (Fagarasan et al., 2010; Bevins and Salzman,

2011; Gutzeit et al., 2014). Therefore, these physical and chemical barriers constitute the first line of intestinal defense against pathogens (Ma et al., 2018).

The second line of defense corresponds to the immune cells present in the GALT. The GALT is mainly organized: 1) as individual and scattered immune cells in the lamina propria (LP), underlying the intestinal epithelium; 2) as immune cells interlinked with the intestinal epithelium (e.g. intraepithelial lymphocytes (IEL)); and 3) as organized lymphoid structures or follicles (Peyer's patches) (Murch, 2021). The main components and organization of the intestinal immune system are shown in Figure 7.

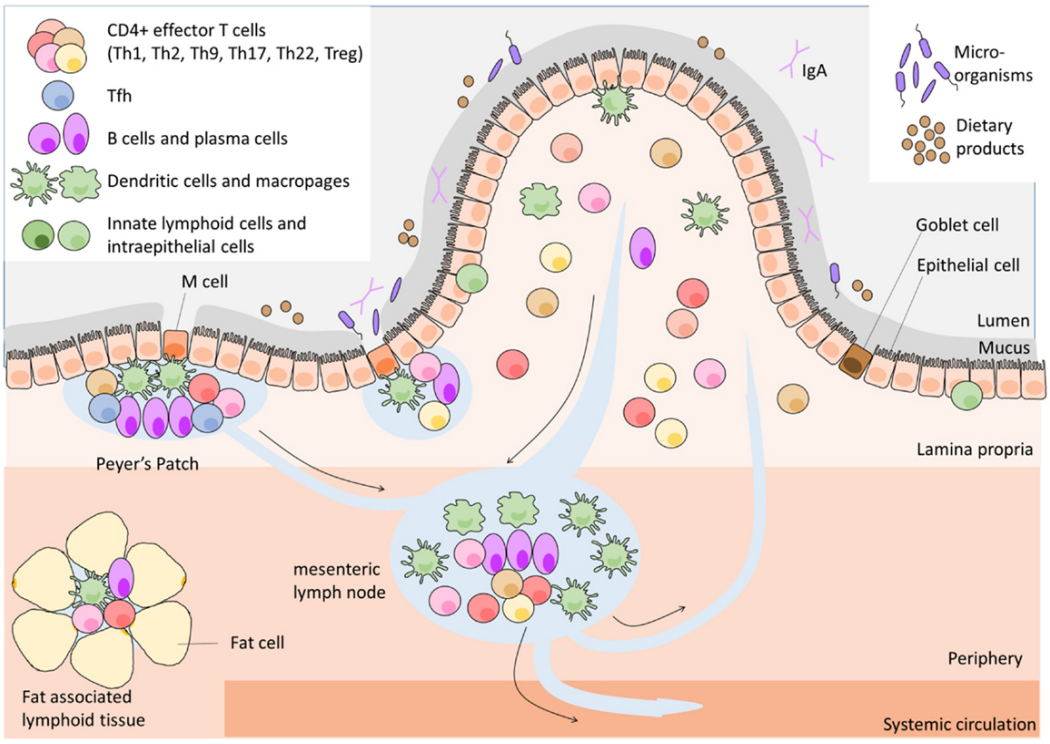


Figure 7. Composition and organization of the mammalian intestinal immune system.

Abbreviations: *Tfh, T follicular helper cells; IgA, immunoglobulin A; M cell, microfold cell.*

Source: (Brucklacher-Waldert et al., 2014)

Inside the LP is possible to find immune cells from both innate and adaptive immune systems such as IgA-producing B cells, T cells, mostly CD4+ T helpers and T regulatory (Treg) cells, and APC like DC and macrophages. DC, macrophages and Treg are of great importance in maintaining the intestinal immune homeostasis, and the first two specifically have been implicated in the dual role of eliciting a robust immune response against pathogens, but remaining tolerant to beneficial bacteria and dietary antigens (Denning et al., 2007). However,

under inflammatory conditions and upon antigen presentation to T cells by DC and macrophages, the increased production of pro-inflammatory cytokines and chemokines leads to the recruitment of additional circulating innate immune cells, including monocytes, which are mucosal DC and macrophages precursors (K. et al., 2006; Varol et al., 2006), PMN, mast cells, and other T cells and B cells, for further defensive roles (Cominelli et al., 2003).

The organized lymphoid structures are located in anatomically defined microcompartments distributed all along the intestine. Among them, we can find the Peyer's patches (PP) in the small intestine, cecal patches in the cecum, colonic patches in the colon, isolated lymphoid follicles in the large intestine and rectum, mesenteric lymph nodes and fat associated lymphoid tissues, which are farther away from the lumen (Brucklacher-Waldert et al., 2014; Silva-Sanchez and Randall, 2020). In general, all the GALT types share a similar cellular structure and basic function that allows the interaction of APC with B and T cells, their activation and the consequent mounting of adaptive immune responses. However, it is known that these GALT also present differential functions, as their location varies along the intestine, together with the microbiota composition and load (Takiishi et al., 2017).

3.1.2. Peyer's patches (PP)

The PP are the best-characterized of all the organized tissues of the GALT and are macroscopically visible domelike structures that extend into the lumen of the ileum (Mowat and Agace, 2014). As the other GALT, they are fundamental sites for the induction of the adaptive immune response within the intestine, being indeed considered as the immune sensors of the intestine. They are composed of an overlying layer of follicle-associated epithelium (FAE) that contains specialized epithelial cells called microfold cells (M cells). Their function is to interact and transport, through endocytosis, phagocytosis, pinocytosis and micropinocytosis, antigens from the lumen into the subepithelial dome (SED) where resident APC - mainly DC although macrophages are also present in lower densities-, further process and present the antigens to B and T cells, unleashing an adaptive immune response or of immune tolerance towards the gut commensal bacteria and dietary antigens (Jung et al., 2010; Lycke, 2012). Moreover, PP is mostly composed of large areas containing B cells (follicles or germinal centres), often surrounded and flanked by T and DC, which converts them into important centres of IgA production within the gut, as shown in Figure 8 (Jung et al., 2010). Then, the long-lived IgA producing plasma cells and memory B cells generated in the germinal

centres can leave the PP and migrate to the blood through the mesenteric lymph nodes, or to effector sites in the LP of both the large and small intestine (Lycke, 2012). Finally, studies have shown that PP also could play an important role in innate immunity, as monocyte-derived DC and macrophages from PP of mice also exerted strong antibacterial and antiviral gene signatures and only the macrophages were able to phagocyte naïve T helper cells (Jung et al., 2010; Bonnardel et al., 2015).

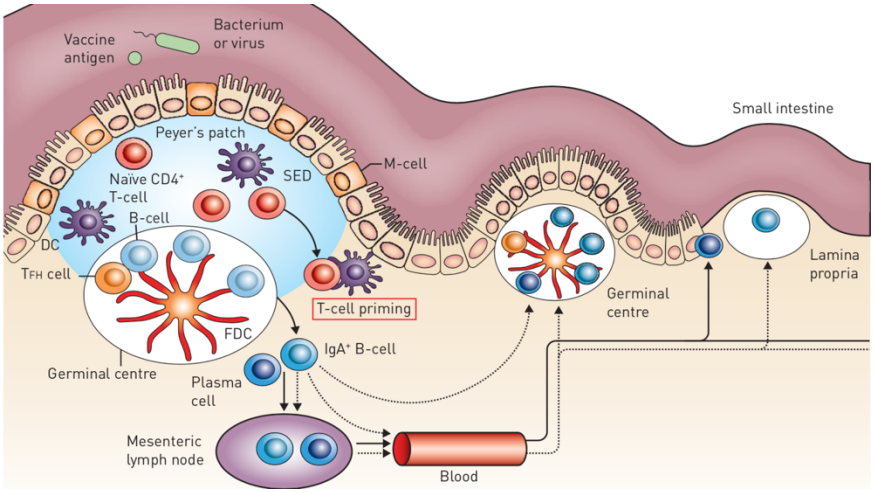


Figure 8.Peyer's patches (PP) structure and organization.

Abbreviations: *T_{FH}, T follicular helper cells; DC, dendritic cell, SED, subepithelial dome; IgA, immunoglobulin A; M-cell, microfold cell; FDC, follicular dendritic cell.*

Source: (Lycke, 2012)

3.2 Avian intestinal immune system

The GALT, and consequently the immune responses that take place within them, are only found in mammals and birds (Fagarasan et al., 2010). Like in mammals, the avian GALT is composed of scattered immune cells in the LP, immune cells intermingled within the intestinal epithelium (IEL such as NK or other non-common T cells subsets) or as organized lymphoid tissues (Casteleyn et al., 2010) as shown in Figure 9. Among the avian intestinal immune cells, we can find heterophils -analogues of mammalian neutrophils-, macrophages, DC, NK, and different subsets B and T cells (Ijaz et al., 2021).

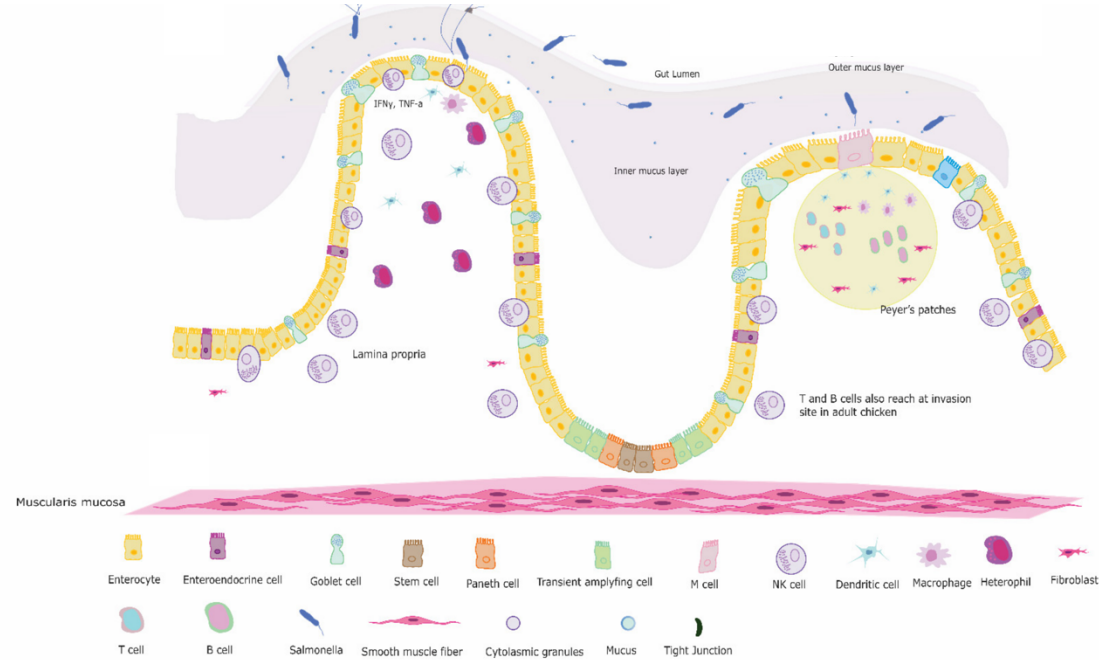


Figure 9. Schematic representation of chicken intestinal immune system structure and organization.

Source: (Ijaz et al., 2021)

However, some aspects of avian GALT distribution, shape and size can differ among species and also within organisms of the same specie (Sinkora et al., 2011). The main lymphoid tissues within avian GALT are PP, cecal lymphoid aggregates (tonsils) -the largest lymphoid aggregates of the GALT-, and the Meckel's diverticulum and the Bursa of Fabricius, the latter exclusively found in birds (Heidari et al., 2015).

In both, cecal tonsils and PP, protective immune responses are carried out to protect the chicken against bacterial, parasitic and viral pathogens in the gut (Heidari et al., 2015; Kuper et al., 2017). The most abundant cells in cecal tonsils at hatch are CD4+ T cells that get replaced by B cells and some CD8+ T cells as it matures into a secondary lymphoid aggregate (Alqazlan et al., 2021). Avian PP, are very similar to those in mammals, as they consist of lymphoid follicles that mainly contain B lymphocytes and are separated by interfollicular regions rich in T lymphocytes, and M cells located in the overlying epithelium are the ones in charge of sampling antigens to present them to APC which start eliciting the adaptive immune response (Befus et al., 1980; Alqazlan et al., 2021). In chicken, up to 6 PP have been identified in chicken

jejunum and 1 in the ileum; and they are known to develop very quickly as after 10 days they can be seen macroscopically (Casteleyn et al., 2010).

The bursa of Fabricius is a spherical lymph epithelial organ, located adjacent to the cloaca, and predominates in young birds as it undergoes involution with time. It is divided into follicles that contain B cells that produce specific antibodies, which circulate in the bloodstream (Hafez and Attia, 2020). B cells are first produced in the bone marrow, embryonic liver, and yolk sac and then to the bursa of Fabricius, where they mature to B lymphocytes. The Meckel's diverticulum on the other hand is the embryonic remnant of the yolk stalk on the small intestine. Among the immune cells that can be found in it are: heterophils, monocytes and large numbers of plasma cells, however, no erythrocytes or thrombocytes are found (Oláh and Glick, 1984; Olah et al., 1984). It is thought that its main functions include the nourishing of the neonatal bird, during the first days of life, and acting as a myelopoietic organ in later stages. Lastly, another difference between the immune system of avian and mammalian species is that the first ones lack structured lymph node follicles such as mesenteric lymph nodes (Casteleyn et al., 2010).

Finally, it is important to highlight that both, mammalian and avian GALT, are not only necessary for triggering an adaptive immune response against pathogens but are also critical to managing the immune response to dietary antigens, making the difference between them and pathogenic antigens and inducing immune tolerance towards them (Chase, 2018). Indeed, when this tolerance is not maintained it leads to the development of several food-induced gastrointestinal pathologies such as food allergies and celiac disease (Pabst and Mowat, 2012).

Chapter 4. Nutrition and immunity

4.1 Nutrition: mechanisms of immunomodulation

As it was discussed in the previous chapter, the intestine is the largest immune organ and it is the first site of interaction between the host immune system, the ingested nutrients and the resident microbiota. Thus, feeding can modulate animals' immunity, and it can do it through both direct and indirect mechanisms. For example, one of the most common indirect mechanisms by which nutrition can affect the host immune response is through changes in the microbiota composition, load and/or their metabolic products (Shen et al., 2012; Ma et al.,

2018). As previously mentioned, the gut microbiota plays a critical role in shaping the development and function of the immune system, and indeed many immune-associated pathologies can be prevented by modifying the immune response via the modulation of the microbiota (Ma et al., 2018). Changes in the diet of young calves greatly influenced microbiome development and composition (Dill-McFarland et al., 2019). In humans, *in vitro* supplementation of dietary fibres increased the abundance of beneficial colonic microbiota (Shen et al., 2012). Moreover, one of the ways by which the microbiota can modulate gut immunity is through the production of microbial metabolites with immunomodulatory effects, like short-chain fatty acids (SCFA). Metabolites such as SCFA can be obtained whether by bacterial fermentation of dietary fibres or by their direct supplementation in the animal's diet (Corrêa-Oliveira et al., 2016).

On the other hand, it is well known that the immune system directly depends on adequate nutrients to function properly, like any other system in the organism (Wu et al., 2019). Indeed, the nutritional status seems to be closely related to the capacity of the host resistance to infection and in general immune competence (Field et al., 2002). Some types of feeds, besides helping in maintaining a well and adequate function of the immune system, could also enhance or boost some of the host immune responses, and/or counteract excessive and uncontrolled ones that could be detrimental to the organism. The functions that different classes of dietary supplements and feed additives can exert to enhance the host immunity are inhibition of pro-inflammatory cytokines, chemokines and other inflammatory mediators, increase in the production of anti-inflammatory mediators, direct modulation of immune cells activities (e.g. chemotaxis, phagocytosis, ROS production, proliferation, differentiation, activation), and enhance the crosstalk between the innate and adaptive immune systems, among others (Salman et al., 2008; Lecchi et al., 2011; Wu et al., 2019; Ávila et al., 2021). A schematic representation of how some dietary supplements (macro and micronutrients) or feed additives (probiotics and green tea polyphenolic phytochemicals) can directly affect immune cells functions is shown in Figure 10.

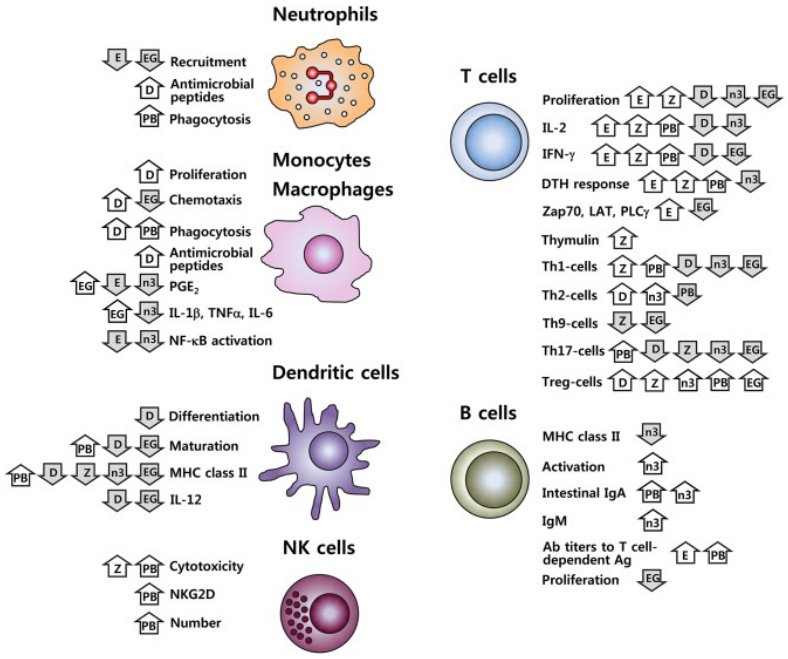


Figure 10. Immune cell functions affected by micronutrients (vitamins D, E and zinc); macronutrients (n-3 PUFA); and other feed additives (probiotics, and EGCG a polyphenolic phytochemical of green tea).

Abbreviations: *D, vitamin D; E, vitamin E; Z, zinc; n-3, n-3 PUFA; PB, probiotics; EG, EGCG;*

Source: (Wu et al., 2019)

4.2 The role of nutrition on livestock species immunity

The role of nutrition on mammalian and avian immunity has been widely studied over the last decades, and different nutrition strategies have been implemented to improve the animals' immune status, and ultimately their overall health (Bobeck, 2020). As previously described in Chapter 1, according to the FAO, livestock species such as cows, pigs and chickens have shown to be extremely important as they provide most of the animal-source food that is consumed worldwide, therefore, having a great economic and social impact. One of the main urgent challenges that the cattle, pig and poultry industries face is the increase in infectious diseases and antimicrobial resistance, which ultimately may affect the animals' health, welfare and productivity, as it has been reviewed elsewhere (Hafez and Attia, 2020; Maes et al., 2020; Vlasova and Saif, 2021). The improvement of immunity represents the most obvious way to overcome those challenges, and to do it through nutrition has become a preferred strategy (Sordillo, 2016; Hafez and Attia, 2020). For example, nutrition intervention with omega-3

polyunsaturated fatty acids (n-3 PUFA) attenuated the effects of subclinical inflammations and improved the energy balance in periparturient dairy cows (Trevisi et al., 2011). Moreover, not only FA diet supplementation has influenced the inflammatory status of lactating cows, but also has been shown to enhance lactation performance (Greco et al., 2015). In weaned piglets fed with antibiotic-free diets, dietary fibre has been shown to influence their growth, immune response, gut barrier function and microbiota. Specifically, the authors observed a decrease in serum pro-inflammatory cytokines, an increase in antibodies, and a reduction in diarrhea rate, which is one of the major causes for reduced growth performance during weaning (Heo et al., 2015; Shang et al., 2021). Lastly, in broiler chickens, the supplementation of organic acids mixed with dietary fibres also enhanced the humoral immune responses, and only supplementation with organic acids improved their performance (Sabour et al., 2019). Additionally, supplementation of laying hens' diets with fish oil, which is rich in n-3 PUFA, enhanced the antibody response, without affecting different reproductive parameters of the animals (Ebeid et al., 2008). In conclusion, based on what has been reported in the previous studies diet complementation with different food components or dietary integrators such as FA and dietary fibres could not only improve the animals' immune competence, but also affect positively other equally important parameters such as growth and reproductive performance.

4.3 Common and novel dietary supplements used on livestock species nutrition

As previously discussed, the supplementation of animals' diets with micro and macronutrients, including vitamins, minerals, lipids, proteins and carbohydrates, has been a widely used strategy in animal nutrition. This supplementation not only aims to maintain adequate cell homeostasis and function, but also to enhance some aspects of the animal's immunity, health, welfare and performance (Wu et al., 2019; Bobeck, 2020; Hafez and Attia, 2020). Moreover, in the past years, a whole research area has been dedicated to identifying novel feed supplements that could help to cope with some of the major challenges faced in the different livestock animals' industries.

The immunomodulatory effects that some common (n-3 and n-6 PUFA and dietary fibres) and novel feed supplements (milk exosomes) used in bovine, porcine, and chicken nutrition will be presented and further discussed in the following subsections.

4.3.1. n-6 PUFA: Conjugated linoleic acid (CLA)

Dietary lipids are important energy-providing macronutrients and especially essential n-6 and n-3 PUFA, and their metabolic products are important mediators of immune cell functions (Wu et al., 2019). It is known that the immune system, both innate and adaptive, require high energy utilization and especially under inflammatory conditions where innate immune cells such as monocytes, macrophages and PMN carried phagocytosis, production of ROS and cytokines for pathogen killing; and lymphocytes upon antigen presentation undergo proliferation and secretion of cytokines and antibodies (Brand, 1985; Newsholme et al., 1986; Sordillo, 2016).
The n-6 and n-3 essential PUFA, linoleic acid (LA) and α-linolenic acid (ALA), respectively, cannot be synthesized by mammals, including ruminants, so they need to be obtained from the diet (e.g. fresh pasture and vegetal oils) (Kaur et al., 2014; Haubold et al., 2020). n-6 PUFA has been shown to alter the FA profile of cell membranes phospholipids, with increased LA and arachidonic acid (ARA) proportions, which are known precursors of eicosanoids and other inflammatory mediators (Calder, 2012).
CLA is a group of naturally occurring positional and geometrical isomers of LA that are characterized by having conjugated double bonds in either cis or trans configuration, and that is naturally present in milk and meat of ruminants (Bhattacharya et al., 2006). In ruminants, CLA can be synthesized both, as an intermediate product during the biohydrogenation of LA by rumen bacteria (Churruca et al., 2009) or through the endogenous conversion of trans-vaccenic acid by desaturase-9 in the mammary gland, as previously demonstrated (Griinari et al., 2000) (Figure 11).

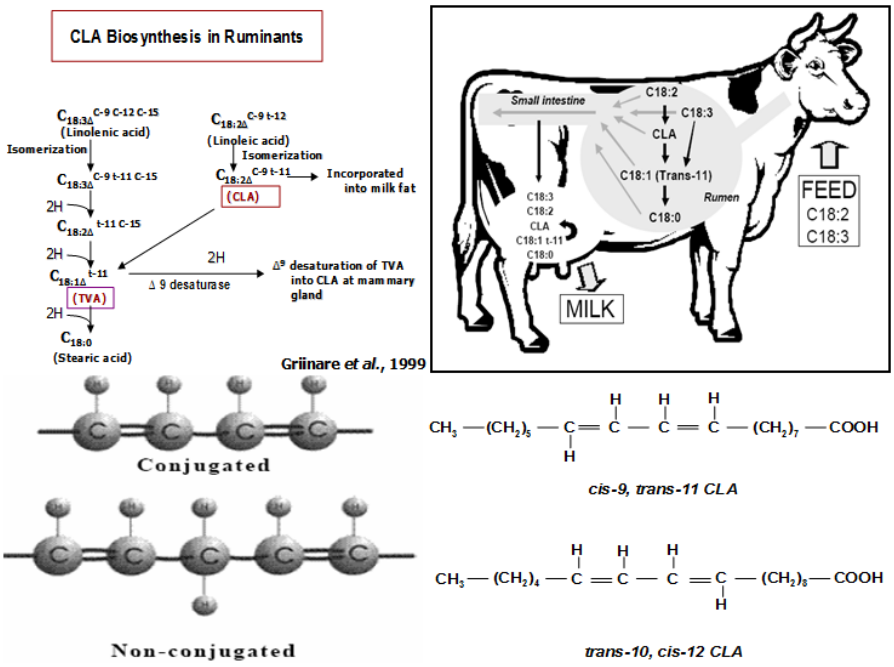


Figure 11. Biosynthesis of conjugated linoleic acid (CLA) in ruminants.

Source: (Chinnadurai, 2011)

The two most important and biologically relevant CLA isomers are the cis-9, trans-11 and the trans-10, cis-12 CLA isomers, being the first one the most abundant in nature and animal dietary products (Collomb et al., 2006). They have been shown to exert differential effects, and synergic and enhanced effects have been reported when they are given to the animals in a mixture, containing equal amounts of each (Pariza et al., 2001; Viladomiu et al., 2016).

In cows, CLA dietary supplementation has been a common nutritional practice, as it has shown to exert beneficial properties, including an increase in milk yield, reduce milk fat and glucose production, enhancing whole-body energy utilization, among others (Selberg et al., 2004; Galamb et al., 2017). Moreover, CLA isomers have exhibited several immunomodulatory properties in different animal and humans' studies. For example, CLA isomers both, individually and in the mixture have been shown to decrease the expression of pro-inflammatory cytokines (e.g. IL-6, IL-1β and TNF-α) on humans macrophages *in vitro* (De Gaetano et al., 2015); inhibit human monocytes migration *in vitro* (McClelland et al., 2010); increase of porcine PMN phagocytosis and chemotaxis, and canine PMN phagocytosis and oxidative burst *in vitro* (Kang et al., 2007; Kang, Ji-Houn and Yang, 2008; Paek et al., 2010).

The effects of CLA isomers have also been limitedly investigated on some bovine immune cells. The supplementation of dams with a commercial CLA preparation (Lutrell Pure, BASF

SE, Ludwigshafen, Germany), which contains the mixture of both isomers in roughly equal proportions (50:50), enhanced the *ex vivo* stimulation ability of bovine PBMC (Dänicke et al., 2012). Moreover, c9,t11 and t10,c12 CLA isomers separately, have been shown to inhibit bovine PBMC mitogen-activated proliferation, while affecting marginally their cytokine expression *in vitro* (Renner et al., 2013). Altogether, these results suggest that CLA might play pleiotropic functions as both, immune-enhancing and dampening effects have been observed and that these effects might vary between species, cell types and how CLA is supplemented, with the isomers separately or in the mixture.

Finally, so far there is no information on how CLA isomers, separately or in the mixture, can affect other bovine mononuclear cells' (e.g. monocytes) critical defensive functions, including chemotaxis, phagocytosis, ROS production and killing capability. Therefore, further research in this field is needed.

4.3.2. n-3 PUFA: Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA)

n-3 PUFA is among the most recognized immunomodulating supplements even over n-6 PUFA (Wu et al., 2019; Bobeck, 2020). n3- PUFA are FA that naturally contain more than one double bond in their structure and the first double bond is on carbon number 3, counting the methyl carbon as carbon number 1 (Calder and Yaqoob, 2009; De Caterina, 2011). They can be divided into 1) short-chain n3-PUFA: ALA, and 2) the longer-chain n3-PUFA: EPA, DHA and docosapentaenoic acid (DPA) (Calder, 2013). EPA and DHA are most abundant in seaweeds and fish oil (FO) (Calder, 2015), but they can be also synthesized from ALA, following several steps of desaturation and elongation (Domenichiello et al., 2015) (Figure 12). However, it has been demonstrated that the conversion of EPA and DHA in mammals is quite limited, being therefore the diet the main source of these long-chain n-3 PUFA (Calder, 2013).

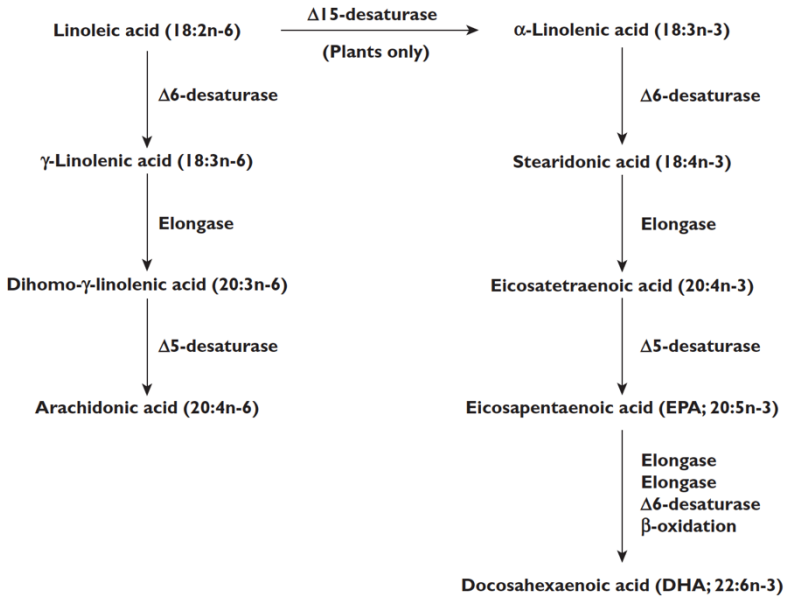


Figure 12. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) biosynthesis in mammals.

Source: (Calder, 2013)

In animal and human nutrition studies, EPA and DHA have been demonstrated to enhance antioxidant defense mechanisms, growth, fertility, and immunity (Calder, 2007; Lee et al., 2019; Fu et al., 2021). Especially, they are well known for their anti-inflammatory properties as reviewed elsewhere (Savoini et al., 2019). Different mechanisms have been proposed to explain EPA and DHA's immunomodulatory and anti-inflammatory effects. As they are both structural and functional components of immune cells' membrane phospholipids, they can alter the immune cells' functions by changing the cells' membrane FA composition, which consequently impacts the cells membrane's fluidity, lipid raft formation, cell signaling and consequent gene expression (Gutiérrez et al., 2019). Moreover, both PUFA is major precursors of anti-inflammatory eicosanoids, and specifically of specialized pro-resolving lipid mediators (SPM) that mediate the resolution (turn off) of the inflammation (Chiang and Serhan, 2020). Finally, they are natural ligands of the intracellular and surface receptors, peroxisome proliferator-activated receptor γ (PPARγ) and G protein-coupled receptor 120 (GPR120), respectively. These two receptors are known to inhibit the NF-κB signaling pathway that is involved with the expression of inflammatory cytokines (Calder, 2017; Al-Khalaifah, 2020).

These immunomodulatory and anti-inflammatory effects have been both reported in different *in vivo*, *in vitro* and *ex vivo* studies. *In vivo*, FO – which is rich in EPA and DHA – has been shown to increase different leukocytes subsets counts (e.g. T cells and PMN) in the blood of periparturient goats during the transition period, suggesting that some aspects of cell-mediated immune response may be improved around this critical period in the animals (Agazzi et al., 2004; Bronzo et al., 2010). Besides showing an effect on the phenotype and counts of immune cells, further defensive actions of immune cells can be affected by EPA and DHA. *In vitro*, EPA and DHA induced the polarization of murine macrophages towards an anti-inflammatory M2 phenotype (Chang et al., 2015); increased goat monocytes phagocytosis and ROS production (Lecchi et al., 2011); and increased goat neutrophils phagocytosis and downregulated their ROS production (Pisani et al., 2009). Moreover, increasing EPA and DHA in the human diet reduced monocytes and neutrophils chemotaxis *ex vivo* (Schmidt et al., 1992).

In the past few years increased attention has been put to DHA and EPA supplementation in other mammals' diets, including pigs, to exploit their beneficial immunomodulatory and anti-inflammatory properties. Indeed, in previous studies, n-3 PUFA decreased the gene expression of pro-inflammatory cytokines (e.g. IL-6 and TNF-α) in growing-finishing barrows, and the production of pro-inflammatory cytokines and eicosanoids (e.g. PGE_2, TNF-α and IL-8) in porcine alveolar macrophages *ex vivo* (Møller and Lauridsen, 2006; Huang et al., 2008). In addition, EPA and DHA have also exhibited *in vitro* cytoprotective and proliferative effects on porcine enterocytes (IPEC-J2) submitted to different biological and chemical insults (Sundaram et al., 2020). Altogether, these results confirm the potential of EPA and DHA in maintaining pigs' gut health, integrity and immunity, especially under pro-inflammatory conditions. However, most of the studies have mainly focused on EPA and DHA effects on the production of inflammatory mediators and gut health and integrity; and no studies of their *in vitro* impact on other inflammatory functions of porcine mononuclear cells (e.g. phagocytosis, chemotaxis, oxidative burst) are available yet. Therefore, it would be of great value to further investigate it with the aim at covering this gap.

4.3.3. Dietary fibres: Citrus Pectin (CP)

It has been widely recognized that the consumption of dietary fibres provides several health benefits for both humans and animals (Anderson et al., 2009; Sabour et al., 2019). Dietary fibres refer to several plant-based non-digestible food components, including non-

starch polysaccharides, oligosaccharides, lignin, and analogous polysaccharides. Generally, dietary fibres are classified by their solubility in water, microbial fermentation in the large intestine, and viscosity. Among the soluble fibres, pectin is one of the most recognized and used for dietary integration (Papathanasopoulos and Camilleri, 2010). Pectin is a family of complex polysaccharides, primarily composed of repeating units of galacturonic acid (Ridley et al., 2001). It is an important component of all plants' cell walls but is most abundant in citrus fruits (Sahasrabudhe et al., 2018).

CP has been widely used as dietary fibre in both human and animal nutrition, as it has shown to have antioxidative (Sanders et al., 2004), anti-diabetic (Liu et al., 2016), anti-cancer (Glinsky and Raz, 2009; Salehi et al., 2018), anti-inflammatory and other immunomodulatory effects (Chen et al., 2006b; Salman et al., 2008; Sahasrabudhe et al., 2018).

It has been proposed that pectin can affect intestinal immune cells' response through both, indirect and direct mechanisms, which are summarized and illustrated in Figure 13. Pectin can indirectly alter the immune response, by changing the composition and function of the microbiota; favouring the adhesion of commensal microbiota to epithelial cells, while preventing pathogens adhesion; and promoting the production of SCFA, which are microbial immunomodulatory metabolites produced by the fermentation of dietary fibres in the large intestine. In weaning pigs, pectins have indeed shown to shape the colonic microbiota, which potentially could have health-promoting effects (Tian et al., 2017). On the other hand, pectin can exert direct effects on the cell's immune functions by interacting with PRR such as galectin-3 or TLR (Beukema et al., 2020).

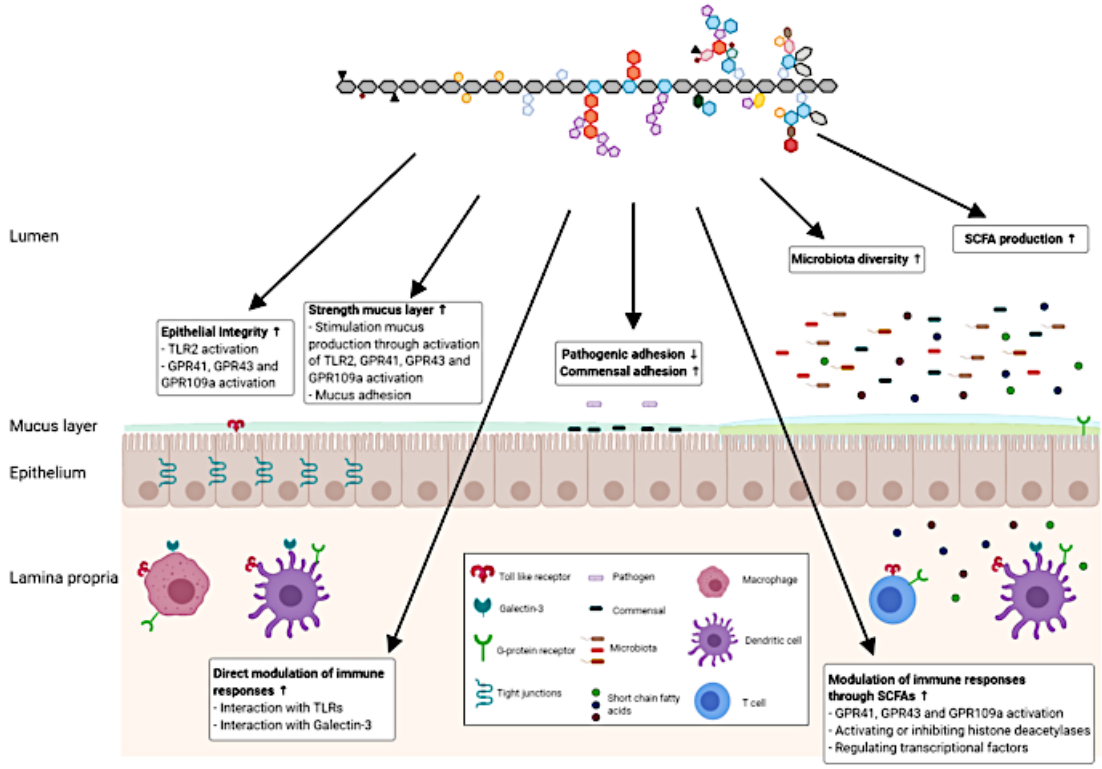


Figure 13. Potential indirect and direct immunomodulatory effects of pectins on intestinal immune cells.

Abbreviations: *TLR, toll-like receptor; NF-κB, Nuclear factor-kappa B; P3CSK4, synthetic lipopeptide (TLR ligand).*

Source: (Beukema et al., 2020)

Specifically, it has been proposed that pectin can inhibit TLR 1 and 2 activation in murine macrophages and human DC, which consequently can inhibit the downstream NF-κB pro-inflammatory pathway, as shown in Figure 14 (Sahasrabudhe et al., 2018). *In vitro* studies carried out in human PBMC also demonstrated that CP causes inhibition of proinflammatory cytokines (IL-1β), while increasing the secretion of the anti-inflammatory cytokines (IL-1ra and IL-10), confirming CP's potential anti-inflammatory role (Salman et al., 2008). Moreover, it has been reported that CP could modulate mice's intestinal innate and adaptive immune response *in vivo*, by interacting and activating immune cells from the PP, after being transported by M cells (Suh et al., 2013; Ishisono et al., 2017). As previously described herein, the immunomodulatory effects of CP, and consequently its mechanisms of action, have been mainly studied and observed in human and mice, and no specific reports on its direct immunomodulation on other species is available so far.

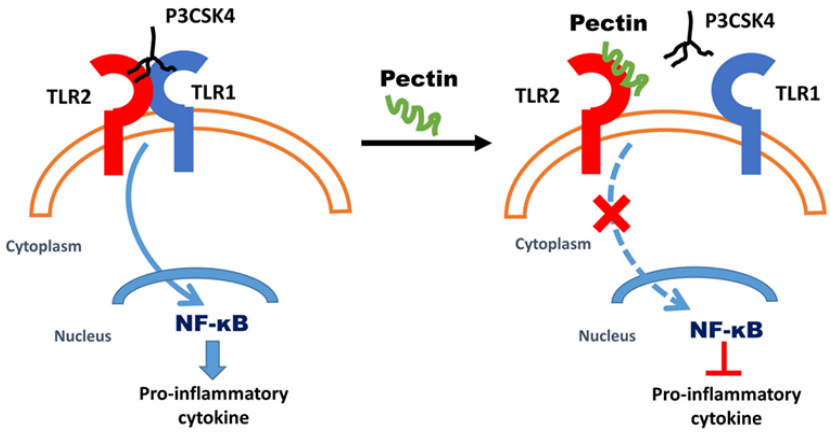


Figure 14. Schematic representation of a proposed mechanism of the anti-inflammatory effects of pectin on immune cells.

Abbreviations: *TLR, toll-like receptor; NF-κB, Nuclear factor-kappa B; P3CSK4, synthetic lipopeptide (TLR ligand).*

Source: (Sahasrabudhe et al., 2018)

In poultry nutrition, there has been an increased interest in integrating the animals' diets with dietary fibres, including pectin (Jha and Mishra, 2021). Pectin has been demonstrated to exert some protective effects against a parasitic infection in broilers chickens (Wils-Plotz et al., 2013). Specifically, CP improved broilers' energy utilization and nutrient digestibility, impacting positively their growth performance (Silva et al., 2013). However, there is limited information on CP effects on chicken immunity, and most specifically on different immune functions of chicken mononuclear cells, which are critical players in both types of immunity. Therefore, to cover this gap more research should be conducted to examine CP immunomodulatory capacity on chicken mononuclear cells' defensive functions.

4.3.4. Milk exosomes

In the past few years, special attention has been focused on novel potential bioactive food components that could also exert beneficial and immunomodulatory effects on the animals, just as the traditionally used macronutrients. Specifically, exosomes have shown to be a promising option for novel food-derived components (Munir et al., 2020).

Exosomes are nano-sized extracellular vesicles (30 - 160 nm) that mediate intercellular communication in both physiological and pathological conditions (Becker et al., 2016; Kalluri and LeBleu, 2020). They are produced by all cell types, including immune cells, and are released to the extracellular space through exocytosis (Wen et al., 2017). Exosomes can carry proteins, lipids, DNA, RNA (mRNA, miRNA, non-coding RNA) and metabolites. Some of the exosomes' surface and intracellular proteins have been used as exosomes biomarkers for their identification, as shown in Figure 15. Once released by the donor cells, they mediate near and long-distance cell-to-cell communication signaling events, by carrying and transferring their cargo or content into recipient cells. The transfer of exosomes' cargo can ultimately alter the recipient cells' function, as it has been demonstrated by immune cell-derived exosomes that modulated recipient immune cells' gene expression by transferring their miRNA (Mittelbrunn et al., 2011; Montecalvo et al., 2012). It is important to highlight that exosome content and its amount seems to vary depending on the type of the donor cell, its physiological and activation state, among other environmental factors (Kalluri and LeBleu, 2020).

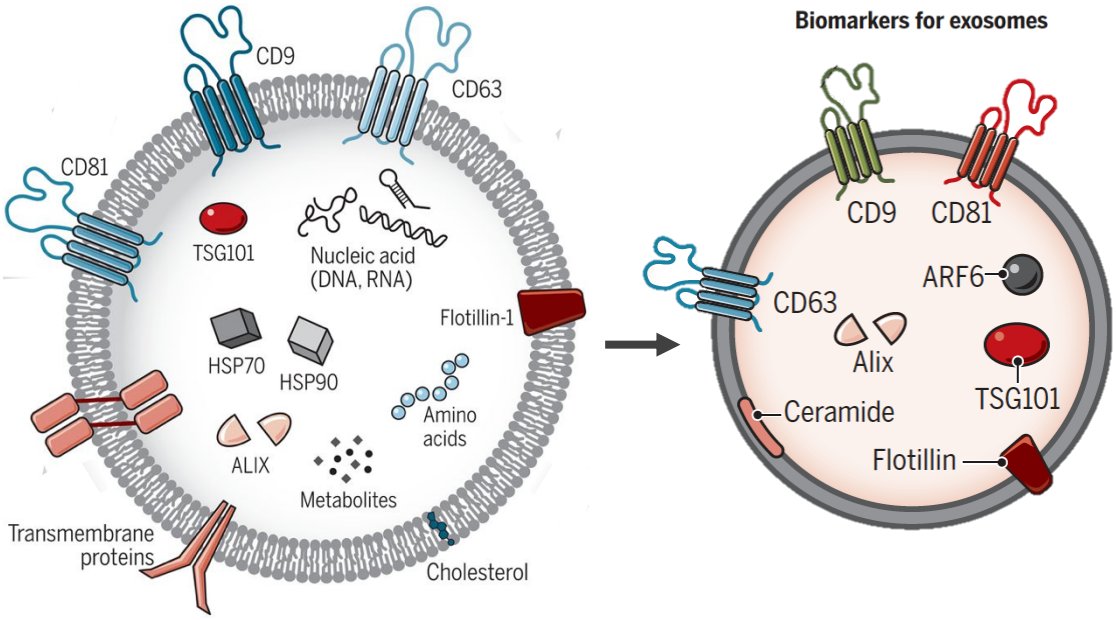


Figure 15. Graphical representation of an exosome and its content: transmembrane and cytosolic proteins, amino acids, nucleic acids, metabolites, lipids.

Source: (Kalluri and LeBleu, 2020)

Accumulating evidence has demonstrated that exosomes can induce, amplify and/or modulate both innate and adaptive immune responses, including NK activation, macrophage differentiation and monocyte chemotaxis induction (Dalvi et al., 2017), antigen presentation, T cell activation and differentiation (Raposo et al., 1996). However, the immunosuppressive and anti-inflammatory roles of exosomes have also been described (Chaput and Théry, 2011). Exosomes can be isolated from different body fluids, including milk (Admyre et al., 2007). Moreover, it has also been possible to isolate exosomes from fruits, vegetables, and other animal-derived sources, showing similar sizes and morphology to mammalian exosomes (Raimondo et al., 2015).

The particular intention has been focused on milk exosomes, as they are thought to play an important role in the transmission of immunity from the mother to the offspring, by transferring immunoregulatory molecules (e.g. miRNA). Therefore, they may play an essential role in the development of the newborn immune system and growth (Zhou et al., 2012). Human milk exosomes have been shown to resist digestion *in vitro,* be internalized by intestinal cells and affect their gene expression (Lonnerdal et al., 2015). Similarly, bovine milk exosomes were taken up by human and rat intestinal cells, where they released their miRNA cargo (Wolf et al., 2015). Both, bovine and porcine milk exosomes, and their cargo, can also accumulate in suckling piglets and mice peripheral tissues rich in immune cells such as the liver, spleen, lung and small intestine (Manca et al., 2018). Therefore, after reaching the intestinal tract, milk exosomes could modulate the animals' immunity, as shown *in vitro* (Ascanius et al., 2021). Furthermore, immune cells like human and murine macrophages are also capable of taking up *in vitro* breast and bovine milk exosomes, containing functional RNA(Lässer et al., 2011; Arntz et al., 2015; Izumi et al., 2015). Figure 16, illustrates how food-derived exosomes after being ingested can be taken up by intestinal epithelial cells and macrophages, where they transfer their regulatory cargo (miRNA and mRNA). Moreover, it shows how these exosomes can later travel through the bloodstream to reach target organs (e.g. liver, lungs, spleen); and how in the large intestine (colon) they can also be taken up by the microbiota, which can consequently affect the host immune defense (Teng et al., 2018).

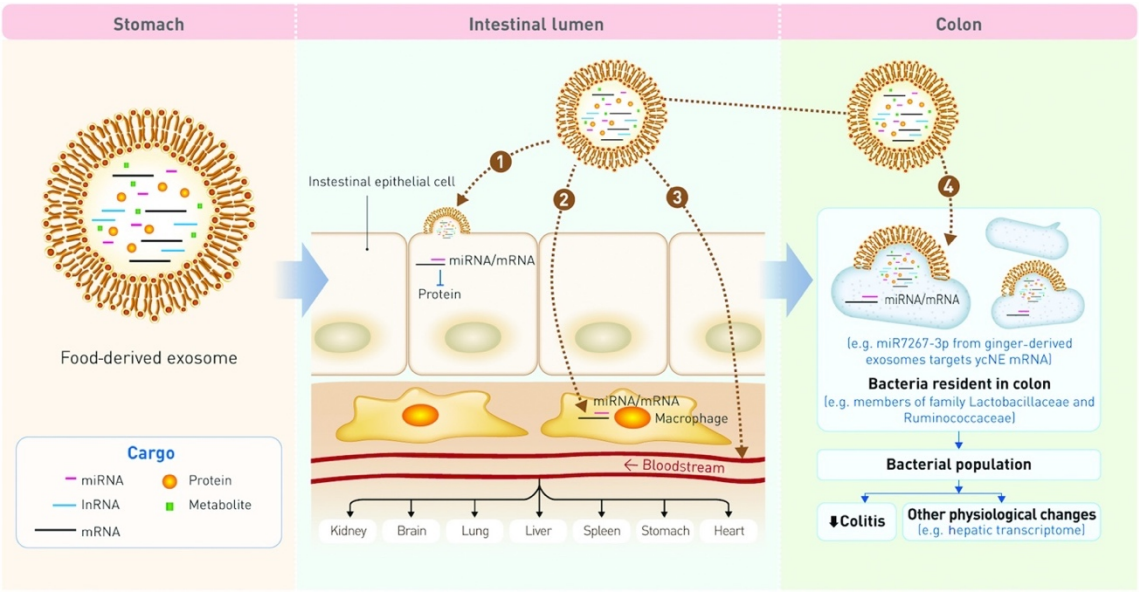


Figure 16. Potential mechanisms of food-derived exosomes internalization by intestinal immune cells (e.g. epithelial and macrophages), and their regulatory effects.

Source:(Munir et al., 2020)

Multiple mechanisms have been proposed to contribute to the exosomes internalization or uptake, including (1) ligand-receptor interaction-induced intracellular signaling, (2) direct fusion of the exosomes membrane with the plasma membrane, (3) phagocytosis and macropinocytosis, (4) clathrin-mediated endocytosis, (5) caveolae-mediated endocytosis, (6) receptor-mediated endocytosis, (7) lipid raft- mediated endocytosis, among others (Figure 17) (Kalluri, 2016; Huang-Doran et al., 2017). Concretely, in professional phagocytes like monocytes and macrophages, exosome uptake occurs mainly through phagocytosis, in a cytoskeleton and PI3-kinase (PI3K) phagocytic pathway-dependent manner (Feng et al., 2010; Parada et al., 2021). However, previous studies highlight the possibility that several mechanisms of exosome uptake could occur in parallel and that these depend on the signaling status of the recipient cell, among other factors (Svensson et al., 2013).

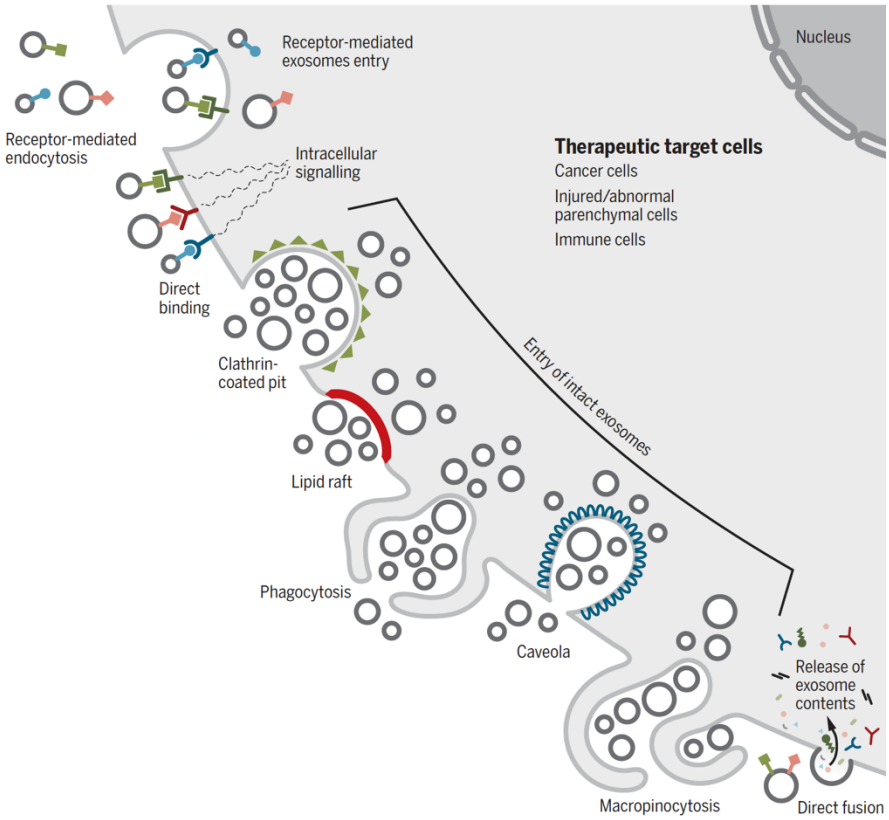


Figure 17. Mechanisms of exosomes internalization or uptake by target cells.

Source: (Kalluri and LeBleu, 2020).

Finally, porcine milk exosomes have also been shown to promote porcine intestinal epithelial cells proliferation *in vitro* and *in vivo* in mice (Chen et al., 2016), but their effects on porcine immunity are unknown so far. Therefore, it might be of great interest to further investigate if they can be internalized by porcine mononuclear cells and consequently alter their immune activities.

Chapter 5. *In vitro* models: characterization of the impact of nutrition on immunity

Nowadays, many of the studies to assess the immunomodulatory role of different dietary supplements in animal science have been carried out *in vitro* (Pisani et al., 2009; Lecchi et al., 2011; Dipasquale et al., 2018). *In vitro* models provide a cheap and reliable tool to obtain relevant information on cell biology, in a simpler and more controlled way. In an *in vitro* approach, it's possible to see the intrinsic response of a specific cell type when stimulated with the homogeneous and individual form of a nutrient in the culture. The information collected at this stage is of great importance, as it provides an initial idea of the potential effects of these molecules on the cells, thus proving a description of their phenotype, and more importantly allows for further elucidation of their mechanisms of action (Calder and Kew, 2002). Therefore, *in vitro* assays represent a first, but reliable approach that opens the door to answering more complex questions in advanced *in vivo* nutrition studies.

To characterize the impact of nutrition on immunity *in vitro* there are different cellular models that can be used, including PBMC and monocytes. PBMC are a heterogeneous population of blood mononuclear cells including monocytes, lymphocytes (T cells, B cells, NK) and dendritic cells, which are critical players in both innate and adaptive immunity (Deng et al., 2014). PBMC can be considered a valuable biological sample to investigate the immunomodulatory effects of dietary molecules, as they can closely reflect the animals' overall immune response. Moreover, PBMC can be obtained rapidly from routine animal blood collections and isolated with simple *in vitro* techniques, such as Ficoll gradient centrifugation, yielding a large number of cells (Ceciliani et al., 2007; Silva et al., 2016; Li et al., 2019). As previously mentioned monocytes are found within the PBMC, and they can be easily isolated from the PBMC through different techniques, including those that rely on the adhesive capacity of monocytes and the sorting of the cells by magnetic-activated cell sorting (MACS, Miltenyi Biotec, Germany), specifically those of the CD14+ fraction (Ceciliani et al., 2021). The preferred technique for isolating mammalian monocytes, including ruminants and porcine, has been the MACS, as it has been reported previously (Lecchi et al., 2008, 2013a; Auray et al., 2010); while in chickens is most common to use the adherence methods due to the lack in availability of antibodies for chicken monocytes cell surface markers (He et al., 2007).

Monocytes are circulating blood leucocytes derived from the bone marrow that play a pivotal role in the innate immune reaction against infections and injuries, and are involved in almost

all the phases of the immune response as they also support the adaptive immune responses and participate in tissue homeostasis (Lauvau et al., 2014; Hussen and Schuberth, 2017). They fulfil their defensive roles by trafficking throughout the body and migrating rapidly into inflamed tissues, where they produced pro-inflammatory cytokines and reactive oxygen species (ROS), and eventually phagocyte and kill engulfed pathogens. Upon tissue entry, monocytes can differentiate into resident macrophages and some subsets of DC, which converts them into major precursor cells critical in replenishing the pool of these immune cells in different tissues, including the intestine (Murray and Wynn, 2011). The adequate display of their functions is critical for effective immune response (Chávez-galán et al., 2015). Therefore, given their critical role, monocytes represent a suitable *in vitro* model to assess the impact that nutrition exerts on mammalian and avian immunity.

Exists a wide range of *in vitro* functional assays that can be implemented to measure different monocytes' immune-related activities such as chemotaxis, phagocytosis, ROS production, and killing capability. Moreover, there are *in vitro* assays that can assess the cells' viability and their capacity to undergo different types of cell death such as apoptosis. These assays can be highly useful to discard any potential cytotoxic effects of the dietary molecules and to set up their optimal working concentrations for further functional assays. All of these activities are excellent indications of the cells' health state and function, so to assess if nutrition affects them *in vitro* is highly informative.

5.1 Viability and apoptosis

Identifying the cells' viability is fundamental when working *in vitro* studies, as it evidences the overall health of the cells and the potential cytotoxic effects of a specific drug or chemical. Cell viability assays can be mainly be classified as dye exclusion assays (Trypan-blue), colorimetric, fluorometric and luminometric assays, and flow cytometric assays. The simplest methods correspond to the ones based on the use of dyes that are excluded by the living cells and not the dead cells, which can be detected using light microscopy. The colorimetric is based on the measurement of a biochemical marker to determine the cell's metabolic activity, using spectrophotometry. The classic and most widely used colorimetric method is the one based on the reduction of Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) into the colored product formazan. Fluorometric and luminometric assays can be also used providing

higher sensitivity than the colorimetric ones (Zaritskaya et al., 2010; Arndt et al., 2014; Kamiloglu et al., 2020).

Apoptosis is an ATP-dependent type of programmed cell death, necessary for maintaining tissue development and homeostasis (Zamaraeva et al., 2005). It is considered an integral a critical feature of the immune system, as it controls immune cells' activity by modulating their abundance in the inflammatory environment (Feig and Peter, 2007).

The *in vitro* measurement of the activity of the two main effectors enzymes in apoptosis, namely caspase-3 and −7, is among the most common approaches to measure this type of cell death in animal studies. Indeed, it has been already reported and validated in bovine monocytes (CD14+) and other small ruminants (Ceciliani et al., 2007; Lecchi et al., 2013a). In these studies, the activation of the two enzymes was determined through a fluorometric detection assay kit that contains a pro-fluorescent peptide substrate that upon cleavage by the effector caspases it releases a green fluorophore (rhodamine-110) that when excited at a certain wavelength produces a fluorometric signal that can be measured using a fluorometer.

Caspases activity can be also measured through colorimetric assays, as it has been demonstrated for bovine monocytes infected with *Mycobacterium bovis* (Mulongo et al., 2014). Lastly, other *in vitro* methods to measure apoptosis and other cell death types in monocytes and macrophages, specifically in bovine, have been extensively reviewed previously (Ceciliani et al., 2021).

5.2 Chemotaxis

Chemotaxis refers to the capacity of cells to migrate towards a chemoattractant or chemical signal. Innate immune cells, including monocytes, migrate and are recruited to affected tissues, where they fulfil their phagocytic and killing functions against invading pathogens (WU, 2005; Lecchi et al., 2008).

The most common way to measure immune cells' chemotaxis *in vitro*, is by using a Boyden chamber that is composed of an upper and a lower compartment separated by a porous membrane (Gomez-Lopez et al., 2011). It mainly consists in adding the cells with migratory capacity in the upper chamber and monitoring their migration through the porous membrane towards a chemoattractant (e.g. Zymosan Activated Serum or ZAS), which is added in the lower compartment of the chamber. Then, the cells that migrated to the other side of the porous membrane are stained and counted through light microscopy (Guan, 2005). This technique has

been previously validated in bovine monocytes and macrophages (Walter and Morck, 2002; Lecchi et al., 2008).

In addition, many studies have assessed the *in vitro* immunomodulatory effect of several dietary compounds on the chemotactic capacity of monocytes. In most of them, alternative or modified Boyden chambers are used (e.g. Transwell migration assay), which follow the same principle, but offer additional technical facilities to the user. In a previous study, the effects of CLA isomers on human monocytes' chemotaxis using this technique were reported (McClelland et al., 2010).

Finally, chemotaxis can be also evaluated with flow cytometry, which seems to be in most cases a more simple and rapid approach, which additionally allows the phenotypic characterization of the cells, which is necessary when working with mixed cell populations like PBMC (Gomez-Lopez et al., 2011).

5.3 Phagocytosis

Monocytes, among other innate immune cells, exert their protective functions by recognizing invading pathogens through their PRR, phagocytizing and killing them. Phagocytosis is defined as the cellular process of ingesting and eliminating particles larger than 0.5 μm in diameter, including microorganisms, foreign substances, and apoptotic cells. Phagocytosis is also an important process that bridge both types of immunity, as phagocytic cells can then present the antigens of the ingested pathogens to the lymphocytes, triggering an adaptive immune response (Uribe-Querol and Rosales, 2020).

Typically, phagocytosis can be measured *in vitro* using both, fluorometric methodologies using microplate readers and flow cytometry. In the first case, the fluorescein-labelled *E. coli* bioparticles – as well other microorganisms bioparticles - internalization assay is among the most commonly used in animal studies. It is based on the co-incubation of professional phagocytes such as monocytes, macrophages and neutrophils with previously opsonized fluorescein-labelled inactivated *E. coli* bioparticles, and then measuring the intracellular fluorescence emitted by the engulfed bioparticles(Pisani et al., 2009). For this type, of technique is of great importance to quench the extracellular fluorescence of non-internalized bioparticles with dyes such as Trypan Blue, to be sure of only measuring the intracellular fluorescence. This technique has been previously used to evaluate the effects of n-3 PUFA (DHA and EPA) in caprine monocytes and PMN (Pisani et al., 2009; Lecchi et al., 2011); and

of heat stress on bovine PMN (Lecchi et al., 2016b). Lastly, to measure the *in vitro* impact of CLA on porcine PMN phagocytic capacity, a flow cytometry approach was used. In this study, the cells were co- incubated with latex beads labelled with the fluorophore FITC, and the fluorescence of ingested particles was then measured by a flow cytometer (Kang et al., 2007). A similar protocol, but using FITC-conjugated and heat-killed *S. aureus* or *E. coli*, has been reported for bovine monocytes.

5.4 ROS production

The production of ROS by monocytes is a critical step for their antimicrobial activities. Once the pathogen, is phagocytosed and activation of NADPH oxidase takes place and several highly toxic ROS (e.g. superoxide anion, hydrogen peroxide (H_2O_2)) are produced, which can be detected both intracellularly and extracellularly (Chew and Park, 2004; Rinaldi et al., 2008). The overall and simultaneous (total) detection of both types of ROS is also possible, as previously described for bovine PMN (Rinaldi et al., 2007).

Different techniques have been developed to measure ROS production *in vitro*, those implementing coulometry, fluorescence and chemiluminescence. For example, the Cytochrome C assay is one classical used one, and it relies on the reduction of cytochrome c, by the extracellularly generated superoxide anion and its quantification by spectrophotometry (Chen and Junger, 2012). The cytochrome C reduction assay has been previously used in caprine monocytes and PMN to assess the effects of n-3 PUFA on their oxidative burst (Pisani et al., 2009; Lecchi et al., 2011). In addition to colorimetric assays, chemiluminescent based ones can also be used for the detection of extracellular superoxide anion. For example, the isoluminol-dependent chemiluminescent assay in the presence of horseradish peroxidase (HRP) – an enzyme that amplifies the chemiluminescent signal - has been implemented previously in bovine PMN (Grob et al., 2020).

For the detection of total ROS production (intracellular and extracellular) on bovine PMN, it has been quite common to use the luminol-dependent chemiluminescent assay. Luminol is a redox-sensitive compound that is oxidized by total ROS, causing the emission of blue luminescence that can be detected using a luminometer (Rinaldi et al., 2008; Bedouhène et al., 2017; Grob et al., 2020).

Lastly, the intracellular ROS can also be measured by using intracellular ROS indicators such as the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), which gets reduced by ROS and

become fluorescent, allowing their measurement through fluorescence plate readers. This approach has also been used on bovine PMN (Wang et al., 2020).
Additionally, to these traditional methods, the use of flow cytometry has also been possible, making measurements easier, more rapid and efficient, as thousands of cells can be measured by using less amount of the starting material such as blood (Chen and Junger, 2012).
Finally, excessive ROS production may damage the host tissue (Pisani et al., 2009). Therefore, is very important also to assess if dietary supplements not only influence the cells killing capability against invading pathogens, through oxidative burst but also if they also can potentially control or mitigate their cytotoxic effects.

5.5 Killing capability

The killing capability of phagocytes like PMN, monocytes and macrophages against pathogens can also be measured. It is important to highlight that even if a pathogen is phagocytosed by these immune cells, it might not succeed in killing it. Therefore, phagocytosis and killing capability assays should be performed separately (Campbell et al., 1994). The most common way to elucidate these cells' killing capacity is through the implementation of intracellular bacterial killing assays, in which the phagocytes are first incubated with live and opsonized bacteria or other microorganisms for 1 h, in the case of monocytes. Then all the unbound and extracellular bacteria are eliminated by washing and treating the cells with antibiotics, cells are lysed and the surviving bacteria are plated on MacConkey agar plates for colony forming units (CFU) counting. This technique has been previously used on bovine monocytes and PMN (Lecchi et al., 2013b).

In conclusion, despite *in vitro* studies can present some limitations as they often don't provide an exact representation of the *in vivo* conditions (Calder and Kew, 2002), they do represent a useful and reliable first step in the way to try to understand the potential immunomodulatory effects that some dietary supplements exert on animals, and to identify the phenotype of the cells. Moreover, they allow the assessment of a wide range of key immune functions that could correlate well with what can be seen *in vivo.* Therefore, it is of great importance later to integrate the data coming from different approaches, including *in vivo* studies that could help us elucidate the biological significance of these dietary supplements.

Chapter 6. OMIC technologies: system biology approach

The implementation of OMIC technologies – the set of high-throughput technologies that detect genes (genomics), RNA (transcriptomics), proteins (proteomics), lipids (lipidomics) and metabolites (metabolomics) – has become a widely used approach in livestock nutrition as it helps to understand better the molecular effects of novel feed additives comprehensively and systematically (Lippolis et al., 2019). Specifically, they represent a great tool for further elucidating the molecular mechanisms underlying the impact observed in previous *in vitro* or *in vivo* studies.

The integration of the data coming from different OMIC technologies, together with the ones observed in 'phenotypical' studies such as *in vitro* or *in vivo* ones would allow us to understand better and holistically what these dietary supplements are doing in our complex biological system (Kato et al., 2011). The systems biology approach can be indeed defined as the combination of data coming from these different technologies to create large molecular data sets that are later integrated using statistical and computational modelling approaches to provide predictive pathways, complex networks, identification of novel molecules and biomarkers. Therefore, the ultimate goal of that systems biology approach is to explain how our biological system behaves after dietary intervention (Badimon et al., 2017). Figure 18 exemplifies how the systems biology approach can be used in nutritional research.

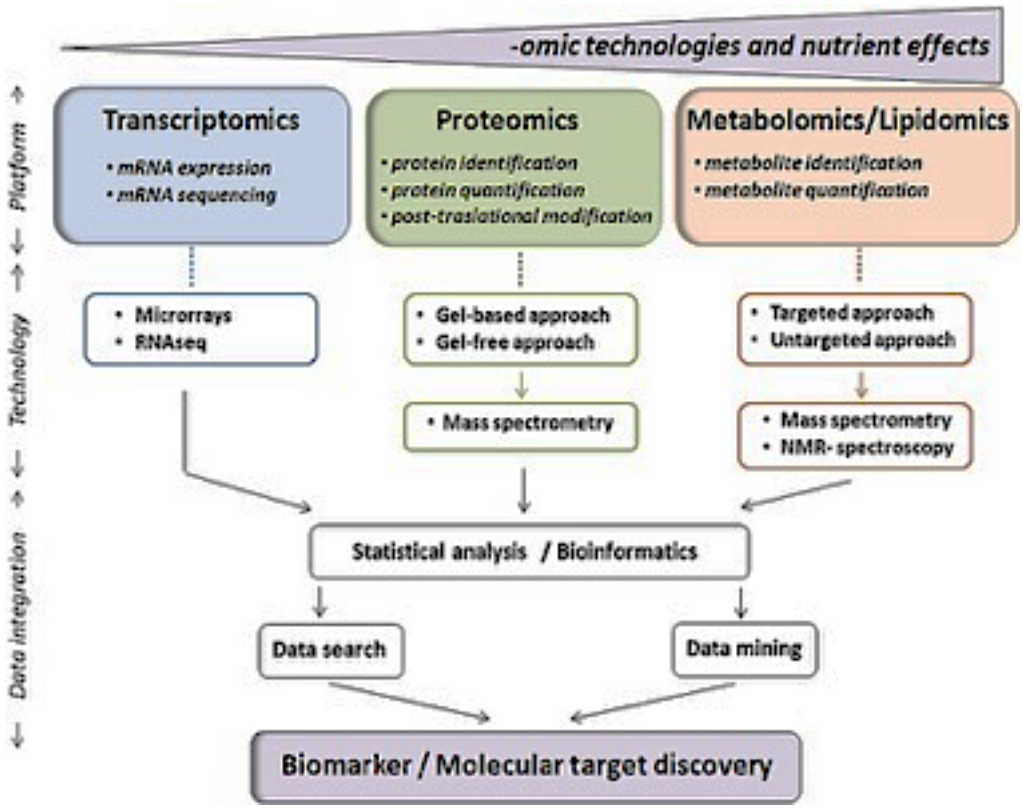


Figure 18. Workflow of a systems biology approach in nutritional studies.

Source: (Badimon et al., 2017)

6.1 Transcriptomics

Transcriptomics is the study of the complete set of RNA transcripts within a cell or tissue (transcriptome) (Martyniuk, 2020). Specifically, transcriptomics analysis studies the gene expression (mRNA), alternative splicing events, long non-coding RNA and small RNA (miRNA) (Ritchie et al., 2015). The development and advances of high-throughput next-generation DNA sequencing (NGS) technologies have been of great utility for the growth of the transcriptomics field, making RNA-sequencing (RNA-seq) the method of choice for it (Martyniuk, 2020). RNA-seq allows RNA analysis through cDNA sequencing at a massive scale, supporting both the discovery and quantification of transcripts (Ozsolak and Milos, 2011; Wang et al., 2019).

Generally, the main steps followed in RNA-seq includes sample collection, high-quality nucleic acid extraction, library preparation, clonal amplification, and sequencing (Figure 19). After sequencing, data processing and analysis are required and includes data cleaning,

filtering, assembly, alignment with a reference genome (de novo or reference-based), variant calling, differential expression analysis and bioinformatic analysis (e.g. Gene Ontology (GO), enrichment pathway and network analysis) (Misra et al., 2019).

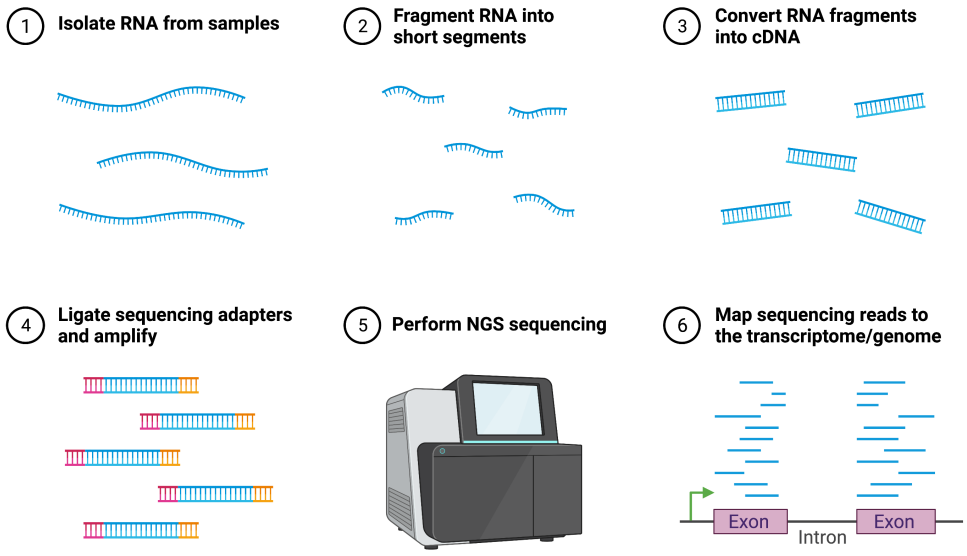


Figure 19. Schematic representation of RNA sequencing workflow.

Source: created with BioRender.com

The mRNA profiling is of great importance for nutrition studies and indeed corresponds to the most commonly used technique among the OMIC technologies (Kato et al., 2011). It has the potential to identify specific changes in mRNA level that respond to a given nutrient or diet, and it serves to obtain a global idea of the pattern of expressed mRNA after the dietary intervention. However, this mRNA expression might not linearly correlate with the changes seen at a protein level (Koussounadis et al., 2015). This could be partially explained by the existence of a type of small RNA (sRNA), the microRNA (miRNA), that are known to play a key role in gene expression.

6.1.1. miRNAomics

The miRNA are small non-coding endogenous RNA (19-25 nucleotides) involved in post-transcriptional gene expression regulation, by either degradation of a target mRNA or the inhibition of translation (Plotnikova et al., 2019). Indeed, in mammals miRNA is thought to

control the activity of approximately 50 % of all protein-coding genes (Krol et al., 2010). They are known to play important regulatory roles in cell biological processes and pathways such as cell cycle, proliferation, apoptosis, and immune response, among others. Thus, dysregulation of miRNA may lead to the development of different pathologies such as cancer (Giovannetti et al., 2012; Plotnikova et al., 2019).

The regulatory mechanism of miRNA towards mRNA is exerted through complementary binding of seed sequences of 2–8 nucleotides of miRNA and 3′ untranslated regions (UTR) of mRNA (Huntzinger and Izaurralde, 2011).

Nowadays, sRNA profiling, including miRNA, mostly relies on NGS platforms, however, some preparation strategies differ from those for longer RNA (>200 nucleotides) are not suitable for sRNA so alternative and adapted preparation strategies have been developed (Ozsolak and Milos, 2011).

miRNAomics analysis has been previously and extensively implemented in veterinary and animal husbandry. For example, they could serve as potential biomarkers of livestock species' health and welfare as it has been previously reviewed (Miretti et al., 2020). Moreover, several studies have identified miRNA as potential biomarkers of transport-related stress in turkeys (Lecchi et al., 2016a), of tail docking and castration-related pain in pigs (Lecchi et al., 2020), and Brucella infection in water buffaloes (Lecchi et al., 2019).

As it is clear that miRNA play important roles in different biological processes, including the immune response, and thus in the maintenance of the animals' health, there is no doubt that miRNAomics analysis could provide meaningful information on the effects of nutrition on the animals' immunity.

6.2 Proteomics

Proteomics is the large scale study of the protein profile in a given biological system (e.g. cells, tissues, organs) (Pardanani et al., 2002). Besides the identification and quantification of the proteins, proteomics analysis aims at identifying and characterizing protein-protein interactions and protein post-translational modifications (Mann and Jensen, 2003). As proteins are the effector molecules of the biological functions encoded by genes, the study of the proteome allows us to better understand both, physiological and pathological phenotypes (Pardanani et al., 2002; Diz et al., 2012).

Proteome analyses are generally comprised of separation, quantification, and identification of proteins (Kussmann et al., 2008). Separation of proteins can be done through gel-based or gel-free methods. Gel-based methods depend on separation using two-dimensional gel electrophoresis (2-DE), in which separated proteins are visualized and quantified after staining with reagents for silver stain or fluorescent stain. A similar, but more sophisticated strategy, the differential imaging gel electrophoresis (DIGE) method, in which proteins of different samples are pre-labelled with different fluorescent dyes has also been applied (Swatton et al., 2004). On the other hand, gel-free methods rely on protein separation before their identification through chromatography, such as high liquid chromatography (HPLC). Nowadays, for the identification and quantification of proteins mass spectrometry (MS) is the most widely used technique. MS measures mass-to-charge ratio (m/z), yielding the molecular weight and the fragmentation pattern of peptides derived from proteins (Mann and Jensen, 2003). MS-based proteomics techniques can be used for the quantification of proteins in multiple samples using targeted or shot-gun (untargeted) approaches. The former, aims at the identification of specific proteins of interest, while the latter aims through a large-scale analysis the identification of all proteins present within a given sample (Borràs and Sabidó, 2017).

In untargeted proteomics, the entire set of extracted proteins is directly digested, and the resulting peptides are prepared, concentrated, separated with HPLC and analyzed by MS (Soares et al., 2012).

The development of shot-gun proteomics would have not been possible without the development and the advances in techniques such as mass spectrometry, which provides increased sensitivity while decreasing the amount of needed sample, allowing still the detection of minimal differences in protein abundances and identification of post-translational modifications (Aebersold and Mann, 2016). Therefore, these advances have made easier the identification and quantification of large numbers of candidate protein biomarkers or molecular signatures from complex biological systems (Yates 3rd, 2019). In addition, the development of novel bioinformatics and functional annotation tools has also made simpler the retrieval of meaningful biological information about the function of the protein by associating them with biological processes and molecular pathways (Kaspric et al., 2015; Manzoni et al., 2018).

Protein quantification can be done through label-free and chemical and metabolic label-based methods (Ning et al., 2011). Despite if a label-based or label-free approach is used, both approaches follow the same overall steps, including sample collection, protein extraction, enzymatic digestion of proteins into peptides, peptide concentration and/or separation using liquid chromatography, followed by MS analysis and peptide and protein identification and

quantification (Soares et al., 2012; Misra et al., 2019). Additionally, to retrieve biological information out of the list of identified and quantified proteins, statistical and bioinformatics analyses (GO enrichment and pathway analyses and network analyses) should be performed as shown in Figure 20.

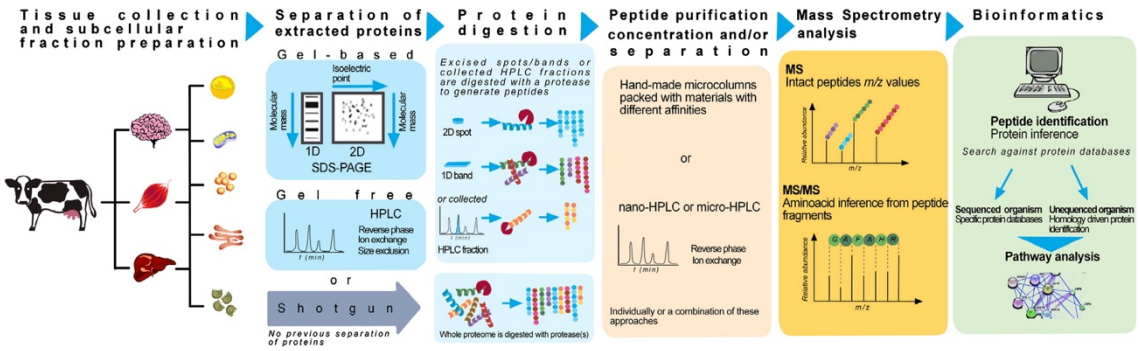


Figure 20. Schematic representation of proteomics workflow.

Source: (Soares et al., 2012)

Proteomics analyses have been extensively used in animal and veterinary science. Some studies have used it to assess the effects of infectious diseases, and characterization of specific immunological pathways on livestock species immune cells (e.g. PBMC) (Deng et al., 2014; Deeg et al., 2020). In addition, also other studies have assessed the effects of dietary supplements (n-3 PUFA) on the *ex vivo* bovine PBMC proteome (Kra et al., 2021).

In conclusion, proteomics represents a great tool for identifying the molecular signature of different dietary supplements, which could help us explain the molecular basis of the effects of nutrition on animal immunity.

Chapter 7. Aim of the thesis, specific objectives and experimental design

The main aim of this thesis was to determine the *in vitro* activity of some molecules that are used in animal nutrition on mammalian (bovine and porcine) and avian (chicken) immune defenses.

To achieve this aim, *in vitro* functional studies were first performed to elucidate the effect of these nutrition molecules on bovine, porcine and chicken monocytes' immune-related activities, including chemotaxis, phagocytosis, killing capability and reactive oxygen species (ROS) production. Proteomics and miRNAomics analyses were then performed to further elucidate the molecular impact of these molecules on bovine, porcine and chicken peripheral blood mononuclear cells (PBMC), to have a system biology perspective.

The specific objectives of this thesis were the following:

1. To set up the protocols for bovine, porcine and chicken PBMC and monocytes (CD14+) isolation.
2. To evaluate the *in vitro* impact of conjugated linoleic acid (CLA) on bovine monocytes' immune response.
3. To evaluate the *in vitro* impact of sows' milk exosomes on porcine monocytes' immune response.
4. To evaluate the *in vitro* impact of the n-3 PUFA, DHA and EPA, on porcine monocytes' immune response.
5. To evaluate the *in vitro* impact of citrus pectin (CP) on chicken monocytes' immune response.
6. To determine the *in vitro* impact of CLA, milk exosomes and CP on bovine, porcine and chicken PBMC protein (proteomics) profile, respectively.
7. To determine the *in vitro* impact of CLA, milk exosomes and CP on bovine, porcine and chicken miRNA (miRNAomics) profile, respectively.

It is important to highlight that for pigs, only the effects of porcine milk exosomes on porcine PBMC were investigated with proteomics and miRNAomics analyses.

The experimental design followed to achieve all of the scientific objectives of this thesis is illustrated in a more detailed manner in Figure 21.

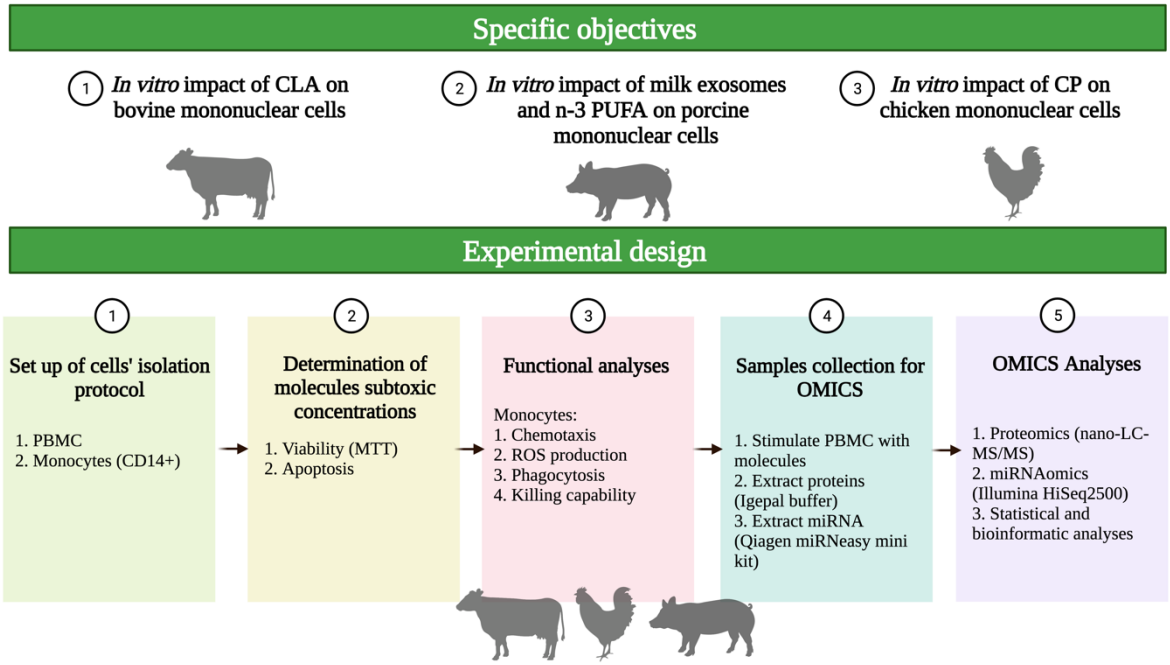


Figure 21. Specific objectives and experimental design followed in the PhD project.

Chapter 8. Results (See papers)

Paper 1: *In vitro* effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes

The following results have been published in Journal of Dairy Science (2020),
https://doi.org/10.3168/jds.2020-18659

In vitro effects of conjugated linoleic acid (CLA) on bovine monocytes' inflammatory functions

G. Ávila, C. Catozzi, D. Pravettoni, G. Sala, P. Martino, G. Meroni, C. Lecchi, F. Ceciliani, Department of Veterinary Medicine, Università Degli Studi di Milano, Via dell'Università 6, 26900, Milano, Italy*

*Corresponding author:
Fabrizio Ceciliani
Department of Veterinary Medicine, Università Degli Studi di Milano, Via dell'Università 6, 26900, Milano, Italy
Tel: 0039 02 50318100
E-mail address: fabrizio.ceciliani@unimi.it

HIGHLIGHTS

- CLA exerts immunomodulatory effects in some in vitro functions of bovine monocytes
- CLA mixture reduces bovine monocytes' apoptosis
- CLA mixture also increases ROS production under pro-inflammatory conditions
- The mixture of the two CLA isomers is more effective than the individual isomers

ABSTRACT

The conjugated linoleic acid (CLA) isomers, a group of naturally occurring isomers of the essential fatty acid (FA) linoleic acid, have received special attention in animal and human nutrition. Although they have long been used as dietary integrators in dairy cows, the effects of CLA isomers on bovine immune cells remain yet mostly undisclosed. The present study aimed to cover this gap and investigate the in vitro effects of CLA on inflammatory functions, including chemotaxis, phagocytosis, killing capability and extracellular respiratory burst of purified bovine monocytes ($CD14^+$). The apoptosis rate of monocytes was addressed as well. Once assessed the effects of different concentrations

(10, 50, 100 and 500 μM) of the two9, tin CLA isomers, namely the *cis-9,trans-11* (c9,t11) and the *trans-10,cis-12* (t10,c12), the experiments were carried out using a concentration of 50 μM of the CLA isomers, both individually and in a mixture (50:50). The immunomodulatory activity of linoleic acid, an essential FA, and stearic acid, a saturated FA, was also investigated. Only the 50:50 CLA mixture was able to reduce monocytes apoptosis and to increase the extracellular respiratory burst during experimental pro-inflammatory conditions, as assessed by measuring reactive oxygen species (ROS) production. CLA and linoleic acid had no effects on chemotaxis, phagocytosis and killing. Remarkably, treatment of monocytes with stearic acid sensibly reduced their chemotactic capability. In conclusion, the present results demonstrated that CLA isomers do have immunomodulatory effects on some bovine monocytes' functions, confirming that the mixture of the two CLA isomers is more effective than the CLA isomers individually.

Keywords: conjugated linoleic acid, monocyte, innate immunity, dairy cow

INTRODUCTION

Conjugated linoleic acid (**CLA**) is a group of naturally occurring positional and geometrical isomers of the essential omega-6 fatty acid (**FA)** linoleic acid, featuring conjugated double bonds in either cis or trans configuration (Bhattacharya et al., 2006). CLA is synthesized as an intermediate product during the biohydrogenation of linoleic acid by *Butyrivibrio fibrosolvens* rumen bacteria (Churruca et al., 2009) or through the endogenous conversion of trans-vaccenic acid by desaturase-9 in the mammary gland, first demonstrated by Bauman's team (Griinari et al., 2000).

Among the 28 CLA isomers reported so far, the *cis-9,trans-11* (**c9,t11***)* and the *trans-10,cis-12* (**t10,c12**) are the most abundant and the ones with relevant biological activities (Pariza et al., 2001; Viladomiu et al., 2016). CLA is formed in ruminants, in particular the c9, t11 isomer that is the most predominant isomer in milk fat (80-90%). The c9,t11 isomer concentration in multiparous late-lactating Holstein-Friesian cows' milk fat is 0.71 g/100g of total FA, and it sensibly increases (up to 2.5 folds) after feeding with fresh pasture (Kay et al., 2005). In other studies, the mean concentration of c9,t11 isomer in bovine milk varied between 5.04 to 11.28 mg/g of fat in animals fed with different diets, showing that CLA milk content is markedly influenced by the composition of the animal's diet (Bauman et al., 1999; Fritsche et al., 1999). The two isomers differ in their biological effects, and further different activities were demonstrated when combining them in equal amounts

(Pariza et al., 2001). Dietary supplementation of dairy cows with CLA is a frequent and relevant nutrition strategy, as it has been observed in in vivo studies a reduction in milk fat and glucose production, allowing a more efficient whole-body energy utilization, and enhanced milk yield (Selberg et al., 2004; Dänicke et al., 2012; Galamb et al., 2017). Beside the effects on metabolism, supplementing CLA as well as other Polyunsaturated Fatty Acids (**PUFA**) in dairy ruminants, has been identified as a potential strategy to mitigate the effects of the pro-inflammatory status associated to the oxidative stress related to metabolic and endocrine changes around calving in dairy cows (Sordillo, 2016). Although CLA isomers have been extensively used for improving milk quality and alleviating the magnitude of negative energy balance, the information about their impact on cow's immunity is limited. In vitro studies carried out on a model of mammary gland epithelial cells (**BME-UV1**) demonstrated that CLA can modulate inflammation and respiratory burst (Basiricò et al., 2015, 2017; Dipasquale et al., 2018). The effects of CLA on the activity of immune cells have been investigated on bovine peripheral blood mononuclear cells (**PBMC**) and resulted in contradictory results. Supplementing dams with a commercial CLA preparation (Lutrell Pure™, BASF-SE) in preceding lactation period exerted effects on the ex vivo stimulation ability of bovine PBMC (Dänicke et al., 2012). In an in vitro study assessing the effect of c9,t11 and t10,c12-CLA isomers, the inhibition of bovine isolated PBMC mitogen-activated proliferation was detected together with a marginal effect of c9,t11 isomer on cells' cytokine expression pattern (Renner et al., 2013). The apparent inconsistency of these results may be related to the fact that most of the studies were not carried out with comparable experimental designs and on an isolated population, but on PBMC, which included both lymphocytes and monocytes. Monocytes are myeloid cells derived from bone marrow. They play a pivotal role in immune defence against infections and injuries and are involved in almost all the phases of the immune reactions (Hussen and Schuberth, 2017). Given their pivotal role, the present study aimed to investigate the in vitro effect of c9, t11 and t10,c12-CLA isomers, both individually and using a mixture (50:50) of the two isomers, on bovine sorted monocyte population. To examine if and how these CLA isomers could play a role in modulating monocytes' inflammatory activities, their impact on chemotaxis, respiratory burst, phagocytosis, killing capability, and apoptosis were evaluated. The effects of other unsaturated, such as linoleic acid, and saturated FA, such as stearic acid, were assessed as well.

MATERIALS AND METHODS

Materials

Ficoll-Paque PLUS (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden), EDTA 2mM, red blood cell lysis buffer, sterile-filtered Dulbecco's Phosphate Buffered Saline without calcium and magnesium (**DPBS**) (Sigma, St. Louis, USA) were used for bovine PBMC isolation. For monocytes purification, CD14 MicroBeads, LS Columns and Pre-Separation filters 30mm (Miltenyi-Biotech, Bergisch Gladbach, Germany) and Bovine Serum Albumin (**BSA**; Sigma, St.Louis, USA) were used. After isolation, cells were resuspended in complete medium, comprising RPMI 1640 Medium with 25 Mm Hepes and L-Glutamine supplemented with 1% of Non-essential Amino Acid Solution 100X and 1% Penicillin Streptomycin Solution 100X (Euroclone, Milano, Italy), and 10% Fetal Bovine Serum (**FBS**; Sigma, St. Louis, USA). Sterile 96 wells plate MICROTEST (Becton Dickinson and Company, Franklin Lake, USA), 384 well black plates, 24-well Transwell migration plates (Costar, Corning, USA) and cryogenic vials (Sigma, St. Louis, USA) were routinely used for cell culture. The FA 9(E),11(Z)-Octadecadienoic acid and 10(E),12(Z)-Octadecadienoic acid (c9,t11 and t10,c12-CLA, respectively) (Matreya LLC, State College, USA); linoleic and stearic acid from (Sigma, St. Louis, USA) were used for cells' treatment.

Zymosan A from *Saccharomyces cerevisiae,* and Cytochrome C from equine heart, phorbol myristate acetate (**PMA**) (Sigma, St. Louis, USA), fluorescein-labelled *Escherichia coli* bioparticles K-12 strain (Invitrogen, Oregon, USA) and *Escherichia coli* American Type Culture Collection (ATCC) 25922 (strain Seattle 1946; LCG Standards) were used for chemotaxis, reactive oxygen species (**ROS**) production, phagocytosis and killing capability assays, respectively.

Purification of monocytes from blood

Peripheral blood from 33 pluriparous late lactating healthy Holstein-Friesian cows was collected during routine slaughtering procedures at a local slaughterhouse in sterile flasks containing 1.8mg K_2EDTA as anticoagulant per ml of blood. Monocytes ($CD14^+$ cells) were isolated through Ficoll 1.077 g/ml density gradient centrifugation, as previously described (Dilda et al., 2012), with few modifications. Briefly, blood was first centrifuged at 1260g, for 30 min at 4°C and the buffy coat (PBMC ring) was collected. PBMC were then diluted 1:5 in sterile cold PBS without Ca^{2+} and Mg^{2+}, containing 2mM EDTA,

layered on Ficoll and centrifuged without breaks at 1700g for 30 min at 4°C. PBMC were recovered at the interface, washed with PBS without Ca^{2+} and Mg^{2+} and treated with Red Blood Cell Lysis Buffer for red blood cells elimination. Two subsequent centrifugations (500g for 7 min with cold PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA) were carried out to remove platelets. The $CD14^+$ monocytes purification was carried out using magnetic-activated cell sorting technique (**MACS**). Isolated PBMC were incubated with anti-human CD14 microbeads for 15 min at 4°C and $CD14^+$ cells were isolated from an MD column (LS) according to the manufacturer's instructions. The homogeneity of the sorted cells (> 98%) was determined using an automatic cell counter (Sysmex). The working concentration of monocytes was then adjusted with complete medium.

Unsaturated and Saturated FA preparation

Stock solutions of unsaturated and saturated FA were prepared. The two CLA isomers (c9,t11and t10,c12-CLA), linoleic acid and stearic acid were reconstituted in ethanol at a concentration of 357 mM for CLA isomers and linoleic acid and of 70.3 mM for stearic acid. Stock solutions were stored at -20 °C and fresh dilutions with the complete medium were prepared when needed. The amounts of CLA isomers used for preliminary studies ranged from 0 to 500 μM. Additionally, the mixture of both isomers in a 50:50 proportion, linoleic and stearic acid at 50 μM were also prepared. During the first part of the study, the working concentration of CLA isomers was determined by testing the different concentration of individual c9,t11 and t10,c12-CLA isomers on monocytes' apoptosis. Once determined the CLA working solution, this was tested on monocyte immune-related functions, including chemotaxis, phagocytosis, respiratory burst, killing and apoptosis.

Apoptosis assay

Apoptosis assay was performed in triplicate on 50 $\times 10^3$ sorted monocytes seeded in 384 well black plates. The experiment was carried out on cells purified from 8 animals. Firstly, to determine CLA working concentration the cells were incubated overnight at 39 °C in the humified atmosphere 5% CO_2 with increasing concentrations of c9,t11 and t10,c12-CLA isomers (10, 50, 100 and 500 μM) or with 0.1% ethanol as control (vehicle), being the concentration of ethanol found in 500 μM of CLA isomers. At this concentration of ethanol, no effects on viability on bovine PBMC were observed, as assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetra- zolium bromide (**MTT**)-based assay (data not shown). Once determined the CLA working concentration (50 μM), the effects of other

unsaturated and saturated FA on monocytes' apoptosis were measured by incubating the cells with 50 μM of each CLA isomer, the 50:50 mixture of the two isomers, linoleic acid, stearic acid and ethanol (vehicle) as the control, with the same concentration of ethanol found in the 50 μM FA solutions (0.014%). The apoptosis rate was measured after overnight incubation by using the Apo-ONE® Homogeneous Caspase-3/7 kit (Promega, Madison, WI, USA). The caspase-3/7 reagent was added to each well and the fluorescence intensity was measured using a fluorescence plate reader Fluoroscan Ascent at 485/538 nm (absorbance/emission), every 30 minutes up to 4 hours, as previously described in bovine monocytes (Ceciliani et al., 2007).

Chemotaxis assay

Monocytes chemotaxis towards zymosan activated serum (**ZAS**) was measured as previously reported (Lecchi et al., 2008; McClelland et al., 2010) with some minor modifications. The experiment was carried out on cells purified from 8 animals. Monocytes were first pretreated overnight, in absence of chemoattractant, with 50 μM of each CLA isomer, the 50:50 mixture of the two isomers, linoleic acid and stearic acid or 0.014% ethanol as the control in 24-well Transwell migration plates, equipped with a 5 μm pore size membrane. A total of $1x10^5$ monocytes (100 μl final volume) were added in triplicates in the upper chamber, while FA and migration medium (RMPI-1640 with 1% of FBS) were added in both chambers at a final volume of 750μl. Cells were incubated overnight at 39°C in humified atmosphere 5% CO_2. After pretreating the cells, the chemotaxis was measured by adding 3mg/ml of the chemoattractant ZAS to the lower chamber, in the presence of newly added FA (50 μM) or ethanol (vehicle) as control, and again incubated for 2 h at 39°C in humified atmosphere 5% CO_2. For the negative control, cells were incubated with the vehicle without ZAS. Finally, upper chambers were removed, non-migrated cells on the upper part of the membrane were gently eliminated using a swab moistured with PBS, and migrated cells stained with Diff-Quick (Sigma, St Louis, USA) and counted in ten different fields, using light microscopy.

Determination of respiratory burst by measuring ROS production under normal and pro-inflammatory conditions

The production of extracellular superoxide anion (O_2^-) was determined by the cytochrome C reduction method as previously described (Lecchi et al., 2016). The experiment was carried out on cells purified from 8 animals. A total of $1x10^5$ monocytes (50μl) were seeded

in complete RPMI-1640 without phenol red in duplicates in 96-well sterile plates. Cells were then incubated overnight at 39°C in a humidified atmosphere of 5% CO_2, with 50 µM (50 µl) of each CLA isomers individually, the 50:50 mixture, linoleic acid and stearic acid or 0.014% ethanol (vehicle) as control at a final volume of 100 µl. At the end of the incubation time, 10 µl of cytochrome C and 90 µl of HBSS or only 100 µl of HBSS as negative control were added to each well to make up a final volume of 200 µl. Finally, to mimic possible pro-inflammatory conditions the second set of experiments was performed by adding PMA (2.5 µg/ml final concentration). The absorbance was measured every 30 min for 4 h at 550 nm with LabSystems Multiskan plate reader Spectrophotometer.

Phagocytosis assay

Monocytes' phagocytic activity was determined as previously described (Lecchi et al., 2011). The experiment was carried out on cells purified from 9 animals. First, $6x10^8$ fluorescein-labelled *Escherichia coli* bioparticles (K-12 strain) were opsonized, by incubating them with 20% of autologous serum for 30 min at 37°C. The suspension was then centrifuged at 800 g for 15 min and suspended in HBSS. Opsonized bacteria were stored at -20°C upon use. A 100 µl suspension containing $3x10^5$ monocytes was seeded in duplicates in 96-well sterile plates with 50 µM (50 µl) of each CLA isomers individually, the 50:50 mixture, linoleic acid and stearic acid or 0.014% ethanol (vehicle) as the control. The medium was then added to reach a final volume of 200 µl. Afterwards, the cells were incubated overnight at 39°C in a humidified atmosphere of 5% CO_2. Cells were then washed with sterile HBSS and 45 opsonized fluorescein-labelled *E. coli* bioparticles (100 µl) per cell were added. Monocytes were then incubated again for 2 h at 39°C in a humidified atmosphere of 5% CO_2. Cells were washed twice with HBSS and 0.4% of trypan blue was added to quench the fluorescence from non-internalized bacteria. Finally, cells were again washed with HBSS and the fluorescence intensity of fluorescein-labelled *E. coli* bioparticles was measured using a Fluoroscan Ascent FL (Thermo Scientific) at 485/538 nm (absorbance/emission).

Killing Capability assay

The intracellular bacteria-killing capability of monocytes was evaluated according to (Lecchi et al., 2013). The experiment was carried out on cells purified from 7 animals. Briefly, *E. coli* American Type Culture Collection (ATCC) 25922 (LCG Standards) was opsonized with 20% bovine serum at 37 °C for 30 min. The bacteria were washed twice

by centrifuging at 1500 g for 10 min and suspended in sterile HBSS. A total of $3x10^5$ bovine monocytes (100 µl) were suspended in complete medium-containing cryogenic vials (300 µl final volume) (Sigma, St. Louis, USA) and treated overnight with 50 µM (50 µl) of each CLA isomers individually, the 50:50 mixture of them, linoleic acid and stearic acid or 0.014% ethanol as vehicle at 39°C in a humidified atmosphere of 5% CO2. Cells were then washed and incubated with $1x10^7$ of opsonized live *E. coli* and incubated for 1 h at 39°C in a humidified atmosphere of 5% CO_2. Monocytes were centrifuged at 110g for 5 min to remove unbound bacteria and treated further for 1 h with 100 µg/ml of Gentamicin to kill any remaining extracellular bacteria. Gentamicin was eliminated by washing with HBSS and centrifuging the cells at 110g for 5 min. Finally, cells were lysed using 0.5% Triton X-100 (Sigma, St. Louis, USA) for 10 min and the surviving *E. coli* were counted on MacConkey agar plates. Results are expressed in colony forming units (**CFU**).

Statistical analysis

Statistical analyses were performed in GraphPad Prism 8.0.2, San Diego, California USA. For the data normality assessment, the Shapiro Wilk test was applied. Repeated measures one-way ANOVA and Tukey's multiple comparison test were used to evaluate ROS production, under normal and pro-inflammatory conditions, in normally distributed data. Repeated measures Friedman test and Dunn's multiple comparison test were used for apoptosis, chemotaxis, phagocytosis and killing capability, in not normal distributed samples. Statistical differences were accepted at $P \leq 0.05$.

RESULTS

Effect of c9,t11and t10,c12-CLA isomers on bovine monocyte apoptosis

To determine c9,t11and t10,c12-CLA isomers optimal concentration, a preliminary study on monocytes was carried out by incubating overnight the cells with increasing concentrations of CLA isomers (10, 50, 100 and 500 µM) or with the vehicle (ethanol), and their apoptosis rate was assessed (Supplemental Figure S1; **https://doi.org/10.3168/jds.2020-18659**). No effects were observed with any of the CLA isomers at increasing concentrations on the sorted population. The following experiments were then carried out using a concentration of 50 µM.

In the second part of the study, the differential effects of additional unsaturated (linoleic acid and the mixture 50:50 of both CLA isomers) and saturated FA (stearic acid) on

monocytes apoptosis were compared. After exposing the cells with the FA overnight, apoptosis (Figure 1) was found to be reduced (P = 0.013) only by the 50:50 mixture of CLA isomers when compared with the vehicle (ethanol).

Effect of unsaturated and saturated FA on bovine monocyte chemotaxis

In this part of the study, the capability of the unsaturated and saturated FA of modulating monocyte chemotactic activity was measured by using transwell migration plates. The chemotactic activity of monocytes was activated using ZAS, after exposing purified cells to 50 μM of the FA or the vehicle (ethanol) overnight. The results are presented in Figure 2. Co-incubation with CLA and LA did not modulate monocyte chemotaxis. On the contrary, stearic acid induced an evident decrease in the chemotactic ability of monocytes in a statistically significant way (P = 0.032) when compared with the control (vehicle), the 50:50 mixture of CLA (P = 0.049) and the t10,c12-CLA (P = 0.013); and (P < 0.001) with c9,t1-CLA.

Effect of unsaturated and saturated FA on bovine monocyte ROS production

Monocytes' production of extracellular superoxide anion at both normal and under inflammatory conditions was evaluated through the Cytochrome C reduction method. Cells were treated overnight with the FA and then the ROS production was measured every 30 min for 4 h (Supplemental Figure S2; **https://doi.org/10.3168/jds.2020-18659**). As the highest ROS levels were observed in both experimental conditions at 60 min after the addition of Cytochrome C, the effects of the different FA on the cells' superoxide anion production were only further evaluated and presented at this time point (Figure 3). Cells under normal conditions did not show any difference in ROS production (Figure 3A) as compared to control. On the contrary, an increase with the 50:50 mixture of CLA isomers was detected (P = 0.002), after inducing a pro-inflammatory challenge with PMA (Figure 3B), when compared to the control and (P = 0.003) with c9,t11-CLA and linoleic acid.

Effect of unsaturated and saturated FA on bovine monocyte phagocytosis and killing capability of E. coli

The last set of experiments was aimed to study whether the co-incubation of isolated bovine monocytes with unsaturated and saturated FA affects their phagocytic and killing capability, as determined by fluorescein-labelled *E. coli* bioparticles internalization assay and intracellular *E.coli* killing assay, respectively. The capacity of monocytes to

phagocyte (Figure 4A) and kill live *E. coli* (Figure 4B) when treated overnight with saturated and unsaturated FA was not affected when compared to the vehicle.

DISCUSSION

In this study, we reported the effects of c9,t11 and t10,c12-CLA isomers, separately and as 50:50 mixture, as well of linoleic acid, an essential FA, and stearic acid, a saturated FA, on several immunoregulatory functions of bovine monocyte ($CD14^+$), including apoptosis, chemotaxis, phagocytosis, respiratory burst and killing capability. Our main finding was that the 50:50 mixture of the c9,t11 and t10,c12-CLA isomers, when used at a concentration of 50 μM, reduced the apoptosis rate of monocytes. Co-incubating cells with the 50:50 mixture of the two isomers also increased the respiratory burst, as determined by an increase of the production of ROS, but only in an experimental pro-inflammatory environment. On the contrary, CLA does not affect any of the other monocyte immunoregulatory functions herein assessed. Remarkably, we found that stearic acid was capable of a statistically significant reduction of chemotaxis.

Conjugated linoleic acid isomers have been routinely used as a feed supplement for dairy cows due to their beneficial in vivo effects. A decrease in milk fat synthesis and its consequent improvement in energy balance, increase in milk production, improved reproductive performance and reduction of metabolic-related diseases were reported (Perfield et al., 2007; de Veth et al., 2009; Basiricò et al., 2017; Csillik et al., 2017). However, their effects on bovine immunity have been scarcely addressed.

Monocytes provide a suitable in vitro model to assess the impact CLA exerts on bovine immunity. Monocytes are circulating blood leucocytes playing a major role in the host immune defence against invading pathogens (Chávez-galán et al., 2015). They fulfil their defensive roles by migrating into inflamed tissues, producing pro-inflammatory cytokines and ROS, and eventually phagocytosing and killing engulfed pathogens. The adequate display of their functions is critical for an effective immune response.

The rate of apoptosis is regarded as a way to control the activity of blood monocytes, by either increasing or reducing their presence and activity in the inflammatory environment. Therefore, apoptosis is regarded as an integral feature of the immune system (Feig and Peter, 2007). Under this premise, this study tested whether CLA isomers affect apoptosis in sorted monocytes, demonstrating that 50:50 mixture of the two CLA isomers can reduce apoptosis. The impact of CLA on apoptosis has been widely studied on cancer cell models

in humans, and the results converge toward a pro-apoptotic and antiproliferative effect (Ochoa et al., 2004; Wang et al., 2008). Studies carried out on bovine cellular models resulted in apparently contradictory results. In bovine mammary cells (MAC-T cell line), co-incubating with 35μM concentrations of both CLA isomers promoted an increase of apoptosis rate (Keating et al., 2008), while in another study the c9,t11-CLA isomer (60 μM) was able to reduce the caspase-3 activity, thus decreasing apoptosis rate, in bovine aortic endothelial cells (Lai et al., 2005), suggesting the hypothesis that the effects of CLA are cell-specific.

For all the following experiments, the working concentration of CLA was set at 50 μ*M*. This concentration of CLA was selected following preliminary studies, that were carried out by co-incubating the monocytes with different concentrations of CLA isomers, demonstrating that there were no differences in modulating apoptosis rate using concentrations ranging from 10 μM to 500 μM. The use of a concentration of 50 μM was set to compare the present results with others from previous studies, that used the same CLA concentration (Basiricò et al., 2015, 2017; Dipasquale et al., 2018). Indeed, the concentration of 50 μM is also close to physiological levels found in human sera (10-70 μM) (Basiricò et al., 2015). Moreover, positive effects when using (50 μM) of CLA such as an improved redox status of bovine mammary cells (Basiricò et al., 2015) or atheroprotective properties in human monocytes have been reported (McClelland et al., 2010).

To further investigate that the effects reported were specific for CLA isomers and not related to an unspecific effect in response to treatment with FA, we compared the differential effects of linoleic acid as omega-6 PUFA control and stearic acid as saturated acid, which is also the main FA found in some commercially available CLA supplements, on bovine monocytes' apoptosis. We also incorporated a 50:50 mixture of both CLA isomers, as most of the animal studies reporting CLA benefits and commercially available CLA supplements use a mixture of these two isomers in roughly equal amounts (Song et al., 2005; Renner et al., 2012). The CLA mixture was the only treatment that caused a significant reduction in monocytes apoptosis when compared with the vehicle (ethanol), an effect not observed with the individual CLA isomers.

In the following set of experiments, the capability of CLA isomers to modulate chemotaxis was studied. We did not observe any difference in monocytes' migration toward ZAS when treated with CLA isomers and its mixture. These results differ from those previously reported in human monocytes, that demonstrated that CLA could modulate

monocytes/macrophages' chemotaxis by PPARγ activation and COX-2 inhibition, suggesting atheroprotective properties (McClelland et al., 2010). Intriguingly, a statistically significant reduction of chemotaxis was found when monocytes were co-incubated with stearic acid. These results are in contrast with what previously reported in human monocytes, where stearic acid was found to have a pro-inflammatory activity (Anderson et al., 2012).

In the final set of experiments, further defensive performance of monocytes in the inflammatory focus such as phagocytosis, killing and respiratory burst were measured. Treating isolated monocytes with CLA has no impact on the phagocytic and killing capability of bovine monocytes. These findings are different as compared to the reported effects of CLA on other species, including dogs and pigs, and other cellular targets like polymorphonuclear cells (**PMN**), where the t10,c12-CLA isomer increased the phagocytosis process, either indirectly (Kang et al., 2007), or directly (Kang et al., 2009). No differences in the production of extracellular superoxide anion under normal conditions were also found. On the contrary, treating the cells with PMA, to mimic inflammatory conditions, upregulated ROS production in monocytes treated with the CLA mixture. These results are in agreement with those reported in human macrophages, where both c9,t11 and t10,c12-CLA isomers upregulated ROS synthesis, through a PPARγ dependent mechanism (Stachowska et al., 2008). Remarkably, a similar effect of increasing ROS production was recently demonstrated in BME-UV1 cells (Dipasquale et al., 2018). Moreover, t10,c12-CLA isomer has already shown to increase oxidative stress in human in vivo studies (Risérus et al., 2002) and in canine PMN (Kang and Yang, 2008; Kang et al., 2009).

CONCLUSIONS

CLA has shown to present a wide range of beneficial properties for cows' health, production and welfare. However, its effects on immune cells' responses and functionality have been scarcely addressed so far. This study demonstrates that CLA exerts an anti-apoptotic activity, and can increase ROS production in an inflammatory in vitro model, suggesting that CLA may have relevant roles in modulating some in vitro monocyte immune functions. The present study provides the evidence that the effects of each CLA isomer are different and a combination of the c9,t11with t10,c12-CLA isomers induce synergic effects on at least two important monocyte immune functions, namely apoptosis and inflammatory induced respiratory burst. Besides its isomer-dependent activity, CLA

effects are also strictly related to their cellular targets, as the effects observed on mammary gland and endothelial cell lines are different compared to those on immune-related cells such as monocytes. Several aspects of the potential immunomodulatory effects of omega-6 FA are still elusive, particularly the molecular basis of the different mechanisms of action of CLA. Elucidation of these mechanisms would improve our understanding of the actions of CLA in experimental in vivo systems and determine its practical biological significance, supporting a more targeted utilisation of CLA in dairy animal nutrition. Given the wide use of stearic acid in dairy animal nutrition, its immunomodulatory effect on reducing chemotaxis deserves to be further explored.

CONFLICT OF INTEREST STATEMENT

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

ACKNOWLEDGEMENTS

This work was supported with Grant agreement nº: 765423 – MANNA (Project that has received funding from the European Union's Horizon 2020 research and innovation programme H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate). We also acknowledge the support of Prof. Tiziana Brevini and her team of the Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare the Università degli Studi di Milano, Italy, for her valuable support in the implementation of alternative cell culture systems. The present study was designed with the valuable support and suggestions of prof Umberto Bernabucci (Department of agricultural and forest sciences, University of Viterbo, Italy), Arnulf Tröscher (BASF CHEMOVATOR, Germany) and Harald Hammon (Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany).

REFERENCES

Anderson, E.K., A.A. Hill, and A.H. Hasty. 2012. Stearic acid accumulation in macrophages induces toll-like receptor 4/2-independent inflammation leading to endoplasmic reticulum stress-mediated apoptosis.. Arterioscler. Thromb. Vasc. Biol. 32:1687–95. doi:10.1161/ATVBAHA.112.250142.

Basiricò, L., P. Morera, D. Dipasquale, A. Tröscher, and U. Bernabucci. 2017. Comparison between conjugated linoleic acid and essential fatty acids in preventing oxidative stress in

bovine mammary epithelial cells. J. Dairy Sci. 100:2299–2309. doi:10.3168/jds.2016-11729.

Basiricò, L., P. Morera, D. Dipasquale, A. Tröscher, A. Serra, M. Mele, and U. Bernabucci. 2015. Conjugated linoleic acid isomers strongly improve the redox status of bovine mammary epithelial cells (BME-UV1). J. Dairy Sci. 98:7071–7082.

Bauman, D.E., L.H. Baumgard, B.A. Corl, and d J.M. Griinari. 1999. Biosynthesis of conjugated linoleic acid in ruminants. Pages 1–14 in Proc. Am. Soc. Anim. Sci.

Bhattacharya, A., J. Banu, M. Rahman, J. Causey, and G. Fernandes. 2006. Biological effects of conjugated linoleic acids in health and disease. J. Nutr. Biochem. 17:789–810. doi:10.1016/j.jnutbio.2006.02.009.

Ceciliani, F., V. Pocacqua, A. Miranda-Ribera, V. Bronzo, C. Lecchi, and P. Sartorelli. 2007. α1-Acid glycoprotein modulates apoptosis in bovine monocytes. Vet. Immunol. Immunopathol. 116:145–152. doi:10.1016/j.vetimm.2007.01.006.

Chávez-galán, L., M.L. Olleros, D. Vesin, I. Garcia, and R.A. Harris. 2015. Much more than M1 and M2 macrophages , there are also CD169 + and TCR + macrophages 6:1–15. doi:10.3389/fimmu.2015.00263.

Churruca, I., A. Fernández-Quintela, and M.P. Portillo. 2009. Conjugated linoleic acid isomers: Differences in metabolism and biological effects. BioFactors 35:105–111. doi:10.1002/biof.13.

Csillik, Z., V. Faigl, M. Keresztes, E. Galamb, H.M. Hammon, A. Tröscher, H. Fébel, M. Kulcsár, F. Husvéth, G. Huszenicza, and W.R. Butler. 2017. Effect of pre- and postpartum supplementation with lipid-encapsulated conjugated linoleic acid on reproductive performance and the growth hormone–insulin-like growth factor-I axis in multiparous high-producing dairy cows. J. Dairy Sci. 100:5888–5898. doi:https://doi.org/10.3168/jds.2016-12124.

Dänicke, S., J. Kowalczyk, L. Renner, J. Pappritz, U. Meyer, R. Kramer, E.M. Weber, S. Döll, J. Rehage, and G. Jahreis. 2012. Effects of conjugated linoleic acids fed to dairy cows during early gestation on hematological, immunological, and metabolic characteristics of cows and their calves. J. Dairy Sci. 95:3938–3953. doi:10.3168/jds.2011-4879.

Dilda, F., G. Gioia, L. Pisani, L. Restelli, C. Lecchi, F. Albonico, V. Bronzo, M. Mortarino, and F. Ceciliani. 2012. Escherichia coli lipopolysaccharides and Staphylococcus aureus enterotoxin B differentially modulate inflammatory microRNAs in bovine monocytes.. Vet. J. 192:514–6. doi:10.1016/j.tvjl.2011.08.018.

Dipasquale, D., L. Basiricò, P. Morera, R. Primi, A. Tröscher, and U. Bernabucci. 2018. Anti-

inflammatory effects of conjugated linoleic acid isomers and essential fatty acids in bovine mammary epithelial cells. Animal 12:2108–2114. doi:10.1017/S1751731117003676.

Feig, C., and M.E. Peter. 2007. How apoptosis got the immune system in shape.. Eur. J. Immunol. 37 Suppl 1:S61-70. doi:10.1002/eji.200737462.

Fritsche, J., R. Rickert, H. Steinhart, M.P. Yurawecz, M.M. Mossoba, N. Sehat, J.A.G. Roach, J.K.G. Kramer, and Y. Ku. 1999. Conjugated linoleic acid (CLA) isomers: formation, analysis, amounts in foods, and dietary intake. Lipid / Fett 101:272–276. doi:10.1002/(SICI)1521-4133(199908)101:8<272::AID-LIPI272>3.0.CO;2-W.

Galamb, E., V. Faigl, M. Keresztes, Z. Csillik, A. Tröscher, P. Elek, M. Kulcsár, G. Huszenicza, H. Fébel, and F. Husvéth. 2017. Effect of pre- and post-partum supplementation with lipid-encapsulated conjugated linoleic acid on milk yield and metabolic status in multiparous high-producing dairy cows. J. Anim. Physiol. Anim. Nutr. (Berl). 101:1026–1035. doi:10.1111/jpn.12544.

Griinari, J.M., B.A. Corl, S.H. Lacy, P.Y. Chouinard, K.V. V Nurmela, and D.E. Bauman. 2000. Conjugated Linoleic Acid Is Synthesized Endogenously in Lactating Dairy Cows by Δ9-Desaturase. J. Nutr. 130:2285–2291. doi:10.1093/jn/130.9.2285.

Hussen, J., and H.-J. Schuberth. 2017. Heterogeneity of Bovine Peripheral Blood Monocytes.. Front. Immunol. 8:1875. doi:10.3389/fimmu.2017.01875.

Jiang, J., L. Bjoerck, R. Fondén, and M. Emanuelson. 1996. Occurrence of Conjugated Cis-9, Trans-11-Octadecadienoic Acid in Bovine Milk: Effects of Feed and Dietary Regimen. J. Dairy Sci. 79:438–445. doi:https://doi.org/10.3168/jds.S0022-0302(96)76383-X.

Kang, J.-H., and M.-P. Yang. 2008. In vitro evaluation of the effect of trans-10, cis-12 conjugated linoleic acid on phagocytosis by canine peripheral blood polymorphonuclear neutrophilic leukocytes exposed to methylprednisolone sodium succinate. Am. J. Vet. Res. 69:494–500.

Kang, J.H., G.S. Lee, E.B. Jeung, and M.P. Yang. 2007. Trans-10, cis-12-conjugated linoleic acid increases phagocytosis of porcine peripheral blood polymorphonuclear cells in vitro. Br. J. Nutr. 97:117–125. doi:10.1017/S0007114507280584.

Kang, J.H., G.S. Lee, E.B. Jeung, and M.P. Yang. 2009. Trans-10, cis-12 conjugated linoleic acid modulates phagocytic responses of canine peripheral blood polymorphonuclear neutrophilic leukocytes exposed to Clostridium difficile toxin B. Vet. Immunol. Immunopathol. 130:178–186. doi:10.1016/j.vetimm.2009.02.005.

Kay, J.K., J.R. Roche, E.S. Kolver, N.A. Thomson, and L.H. Baumgard. 2005. A comparison

between feeding systems (pasture and TMR) and the effect of vitamin E supplementation on plasma and milk fatty acid profiles in dairy cows.. J. Dairy Res. 72:322–32. doi:10.1017/S0022029905000944.

Keating, A.F., F.Q. Zhao, K.A. Finucane, D.R. Glimm, and J.J. Kennelly. 2008. Effect of conjugated linoleic acid on bovine mammary cell growth, apoptosis and stearoyl Co-A desaturase gene expression. Domest. Anim. Endocrinol. 34:284–292. doi:10.1016/j.domaniend.2007.08.005.

Lai, K.L., A.P. Torres-Duarte, and J.Y. Vanderhoek. 2005. 9-trans, 11-trans-CLA: Antiproliferative and proapoptotic effects on bovine endothelial cells. Lipids 40:1107–1116. doi:10.1007/s11745-005-1474-1.

Lecchi, C., F. Ceciliani, S. Bernasconi, F. Franciosi, V. Bronzo, and P. Sartorelli. 2008. Bovine alpha-1 acid glycoprotein can reduce the chemotaxis of bovine monocytes and modulate CD18 expression. Vet. Res. 39. doi:10.1051/vetres:2008027.

Lecchi, C., G. Invernizzi, A. Agazzi, M. Ferroni, L.F. Pisani, G. Savoini, and F. Ceciliani. 2011. In vitro modulation of caprine monocyte immune functions by ω-3 polyunsaturated fatty acids. Vet. J. 189:353–355. doi:10.1016/j.tvjl.2010.09.001.

Lecchi, C., N. Rota, A. Vitali, F. Ceciliani, and N. Lacetera. 2016. In vitro assessment of the effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in bovine polymorphonuclear cells. Vet. Immunol. Immunopathol. 182:89–94. doi:10.1016/j.vetimm.2016.10.007.

Lecchi, C., A. Scarafoni, V. Bronzo, P.A. Martino, A. Cavallini, P. Sartorelli, and F. Ceciliani. 2013. α1-Acid glycoprotein modulates phagocytosis and killing of Escherichia coli by bovine polymorphonuclear leucocytes and monocytes. Vet. J. 196:47–51. doi:10.1016/j.tvjl.2012.07.022.

McClelland, S., C. Cox, R. O'Connor, M. de Gaetano, C. McCarthy, L. Cryan, D. Fitzgerald, and O. Belton. 2010. Conjugated linoleic acid suppresses the migratory and inflammatory phenotype of the monocyte/macrophage cell. Atherosclerosis 211:96–102. doi:10.1016/j.atherosclerosis.2010.02.003.

Ochoa, J.J., A.J. Farquharson, I. Grant, L.E. Moffat, S.D. Heys, and K.W.J. Wahle. 2004. Conjugated linoleic acids (CLAs) decrease prostate cancer cell proliferation: Different molecular mechanisms for cis-9, trans-11, and trans-10 cis-12 isomers. Carcinogenesis 25:1185–1191. doi:10.1093/carcin/bgh116.

Pariza, M.W., Y. Park, and M.E. Cook. 2001. The biologically active isomers of conjugated linoleic acid. Prog. Lipid Res. 40:283–298. doi:10.1016/S0163-7827(01)00008-X.

Perfield, J.W., A.L. Lock, J.M. Griinari, A. Sæbø, P. Delmonte, D.A. Dwyer, and D.E. Bauman. 2007. Trans-9, cis-11 conjugated linoleic acid reduces milk fat synthesis in lactating dairy cows. J. Dairy Sci. 90:2211–2218. doi:10.3168/jds.2006-745.

Renner, L., S. Kersten, A. Duevel, H.J. Schuberth, and S. Dänicke. 2013. Effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid, linoleic acid, phytanic acid and the combination of various fatty acids on proliferation and cytokine expression of bovine peripheral blood mononuclear cells. Nutrients 5:2667–2683. doi:10.3390/nu5072667.

Renner, L., J. Pappritz, R. Kramer, S. Kersten, G. Jahreis, and S. Dänicke. 2012. Fatty acid profile and proliferation of bovine blood mononuclear cells after conjugated linoleic acid supplementation. Lipids Health Dis. 11:1–7. doi:10.1186/1476-511X-11-63.

Risérus, U., S. Basu, S. Jovinge, G.N. Fredrikson, J. Ärnlöv, and B. Vessby. 2002. Supplementation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein: A potential link to fatty acid-induced insulin resistance. Circulation 106:1925–1929. doi:10.1161/01.CIR.0000033589.15413.48.

Selberg, K.T., A.C. Lowe, C.R. Staples, N.D. Luchini, and L. Badinga. 2004. Production and metabolic responses of periparturient Holstein cows to dietary conjugated linoleic acid and trans-octadecenoic acids.. J. Dairy Sci. 87:158–68. doi:10.3168/jds.S0022-0302(04)73153-7.

Song, H.-J., I. Grant, D. Rotondo, I. Mohede, N. Sattar, S.D. Heys, and K.W.J. Wahle. 2005. Effect of CLA supplementation on immune function in young healthy volunteers. Eur. J. Clin. Nutr. 59:508–517. doi:10.1038/sj.ejcn.1602102.

Sordillo, L.M. 2016. Nutritional strategies to optimize dairy cattle immunity. J. Dairy Sci. 99:4967–4982. doi:10.3168/jds.2015-10354.

Stachowska, E., M. Baśkiewicz-Masiuk, V. Dziedziejko, I. Gutowska, I. Baranowska-Bosiacka, M. Marchlewicz, B. Dołegowska, B. Wiszniewska, B. Machaliński, and D. Chlubek. 2008. Conjugated linoleic acid increases intracellular ROS synthesis and oxygenation of arachidonic acid in macrophages. Nutrition 24:187–199. doi:10.1016/j.nut.2007.10.018.

de Veth, M.J., D.E. Bauman, W. Koch, G.E. Mann, A.M. Pfeiffer, and W.R. Butler. 2009. Efficacy of conjugated linoleic acid for improving reproduction: A multi-study analysis in early-lactation dairy cows. J. Dairy Sci. 92:2662–2669. doi:10.3168/jds.2008-1845.

Viladomiu, M., R. Hontecillas, and J. Bassaganya-Riera. 2016. Modulation of inflammation and immunity by dietary conjugated linoleic acid. Eur. J. Pharmacol. 785:87–95. doi:10.1016/j.ejphar.2015.03.095.

Wang, L.S., Y.W. Huang, S. Liu, P. Yan, and Y.C. Lin. 2008. Conjugated linoleic acid induces apoptosis through estrogen receptor alpha in human breast tissue. BMC Cancer 8:1–14. doi:10.1186/1471-2407-8-208.

FIGURES

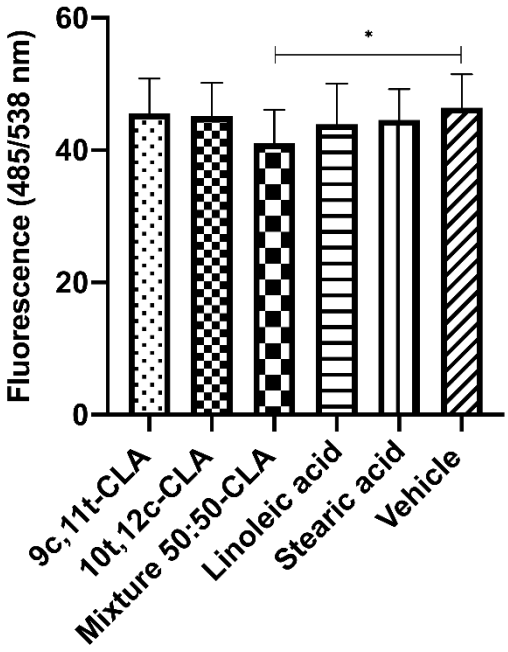


Figure 1. In vitro effect of saturated and unsaturated FA on bovine monocytes apoptosis. Caspase-3/7 enzymatic activity of bovine monocytes after overnight incubation with FA or vehicle (0.014% ethanol). Data are means ± SEM of eight independent experiments. Significance was declared for $P < 0.05$ (*).

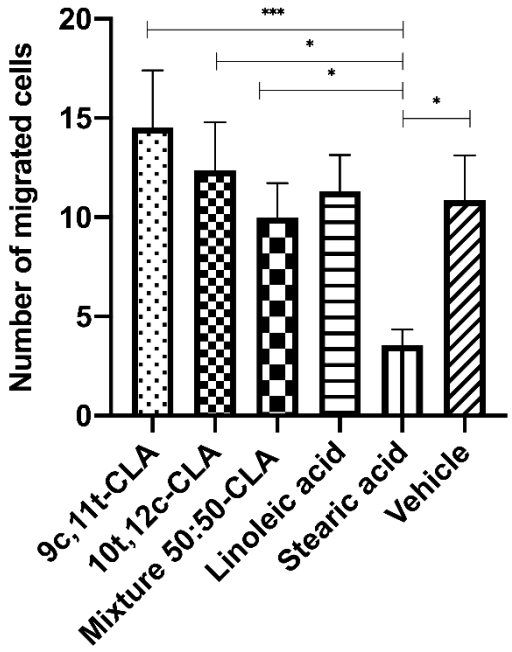


Figure 2. Differential effects of saturated and unsaturated FA on bovine monocytes chemotaxis. Cells were treated with 0.014% of ethanol as vehicle control. Data are means ± SEM of eight independent experiments. Significance was declared for $P < 0.05$ (*) and $P < 0.001$ (***).

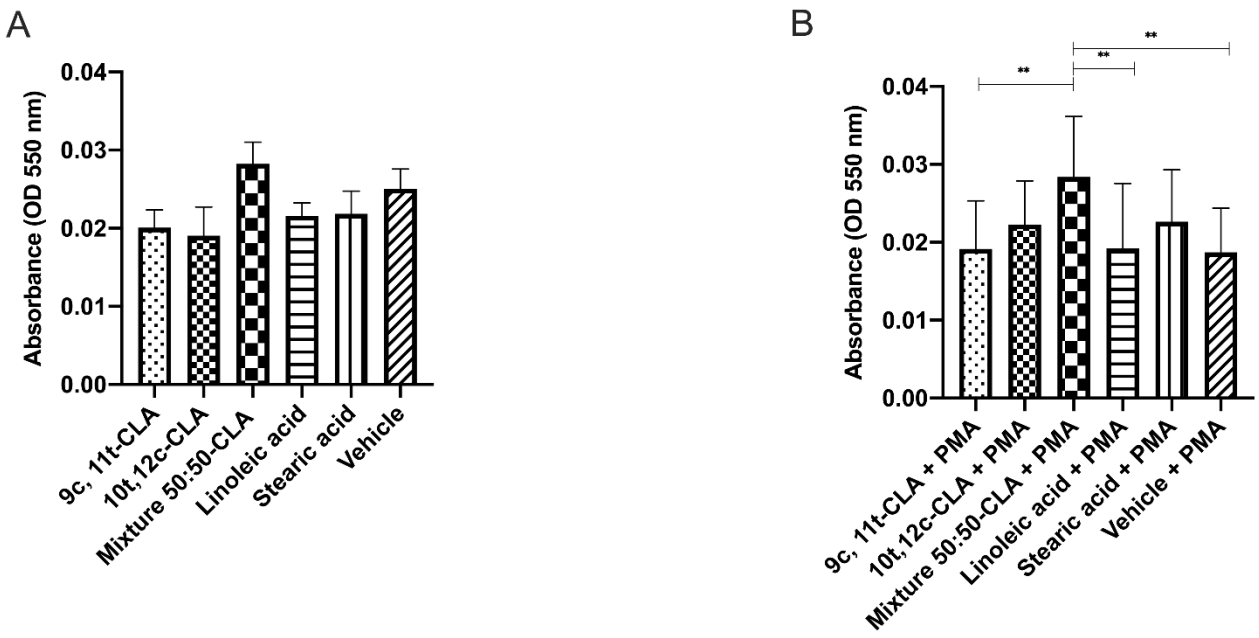


Figure 3. In vitro effect of saturated and unsaturated FA on bovine monocytes extracellular superoxide anion generation, at 60 min after the addition of Cytochrome C, under (A) normal conditions or (B) pro-inflammatory conditions (phorbol myristate acetate (PMA) stimulation). Cells treated with 0.014% of ethanol (vehicle) were considered as control. Data are means ± SEM of eight independent experiments. Significance was declared for $P < 0.01$ (**).

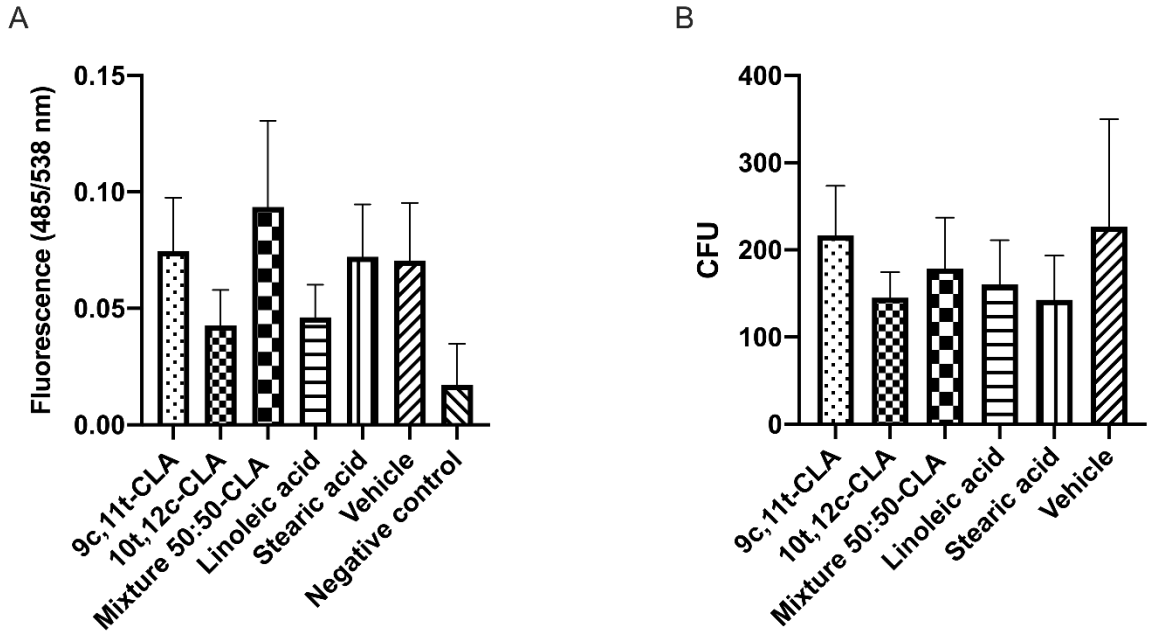


Figure 4. (A) Phagocytosis of fluorescein-labelled *Escherichia coli* bioparticles and (B) killing capability of live *Escherichia coli* by bovine monocytes after overnight exposure with FA. The results are expressed as fluorescence intensity (OD 485-538 nm) and as colony forming units (CFU), respectively. Cells treated with 0.014% of ethanol (vehicle) were considered as control. Data are means ± SEM of nine and seven independent experiments, respectively.

Manuscript draft 2: Porcine milk exosomes modulate the immune functions of CD14+ monocytes *in vitro*

Porcine milk exosomes modulate the immune functions of CD14$^+$ monocytes in vitro

G. Ávila[1], D. De Leonardis[1†], J. Filipe[1†], R. Furioso Ferreira[2,3], A. Agazzi[1], H. Sauerwein[2], M. Comi[4], V. Mrljak[3], C. Lecchi[1], F. Ceciliani[1]*

[1] Department of Veterinary Medicine and Animal Sciences, Università Degli Studi di Milano, Via dell'Università 6, 26900 Lodi, Italy

[2] Institute of Animal Science, Physiology & Hygiene Unit, University of Bonn, 53115 Bonn, Germany

[3] Faculty of Veterinary Medicine, University of Zagreb, 55 Heinzelova, Zagreb, Croatia

[4] Department of Human Science and Quality of Life Promotion, Università Telematica San Raffaele, Via di Val Cannuta 247, 00166 Roma, Italy

[†]These authors have contributed equally to this work

*** Corresponding author:**
Gabriela Ávila Morales
gabriela.avila@unimi.it

Department of Veterinary Medicine, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy
Tel: 0039 02 50334531

ABSTRACT

Exosomes are a subtype of extracellular vesicles with an endosomal origin mediating near and long-distance intercellular communication events. They are secreted by all cell types and carry nucleic acids, proteins, lipids, and metabolites, which can be transferred to recipient cells, altering their biological response. Exosomes are present in different body fluids such as blood, saliva and milk, where they can exert immunomodulatory functions after discharging their cargo. It has been demonstrated that porcine milk exosomes can accumulate in the small intestine that is rich in immune cells like macrophages. No information is available on the immunomodulatory ability of porcine milk exosomes on porcine immune cells, like monocytes, which are known precursors of gut macrophages. This study covers this gap aiming to (1) assess the in vitro uptake of milk exosomes by porcine monocytes (CD14+) and (2) evaluate the in vitro impact of porcine milk exosomes on porcine monocytes immune functions. Milk exosomes were purified from sows' milk by ultracentrifugation coupled with size exclusion chromatography (SEC). After removal of lipopolysaccharides (LPS) to allow their in vitro utilization, exosomes were characterized by Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), and Western Blotting to detect a specific exosomal marker. Monocytes were isolated from pigs' blood, using the magnetic-activated cell sorting technique (MACS). The internalization of PKH26-labeled porcine milk exosomes by monocytes was examined using fluorescence microscopy. To exclude potential cytotoxic effects, porcine monocytes were incubated with increasing exosome concentrations, and apoptosis and viability were measured. Lastly, the ability of milk exosomes to modulate the cells' immune activities was evaluated by measuring monocytes' phagocytosis, the capacity of killing bacteria, chemotaxis, and reactive oxygen species (ROS) production. Exosomes did not cause any cytotoxic effects on porcine monocytes, as their apoptosis and viability remained unchanged. Instead, the exosomes decreased monocytes' chemotaxis and phagocytosis and increased ROS production under resting (non pro-inflammatory) and pro-inflammatory conditions. No effects on killing capacity were observed. Altogether, this study provides insights into the role that milk exosomes might play in pigs' immunity, by demonstrating that milk exosomes can be internalized by porcine monocytes in vitro and exert immunomodulatory effects on inflammatory functions.

Keywords: porcine milk exosomes, exosome uptake, monocyte, phagocytosis, chemotaxis.

INTRODUCTION

Exosomes are nano-sized extracellular vesicles (30 - 160 nm) with an endosome-derived limiting membrane that mediate intercellular communication in both physiological and pathological conditions (Becker et al., 2016; Kalluri and LeBleu, 2020). They are produced by all cell types, including immune cells, through the inward budding of the endosomal membrane, which mature into multivesicular bodies **(MVB)** that when fusing with the plasma membrane release the exosomes through exocytosis (Wen et al., 2017).

As mediators of cell-to-cell communication, exosomes are secreted from donor cells to modulate short and long-distance signalling events by carrying and transferring their cargo, which can include proteins, both transmembrane and cytosolic proteins, lipids, DNA, RNA (mRNA, miRNA, non-coding RNA) and metabolites (Kalluri and LeBleu, 2020). The exosomes' cargo can then be transferred to recipient cells, altering their function (Raposo and Stoorvogel, 2013; Chow et al., 2014). Several studies report that immune cell-derived exosomes can indeed functionally transfer miRNA after their fusion with the acceptor immune cells and modulate their gene expression (Mittelbrunn et al., 2011; Montecalvo et al., 2012).

Over the past two decades, accumulating evidence has reinforced the hypothesis that exosomes can induce, amplify and/or modulate both innate and adaptive immune responses, including natural killer cells activation, macrophage differentiation and monocyte chemotaxis induction (Dalvi et al., 2017), antigen presentation, T cell activation and differentiation (Raposo et al., 1996). Immunosuppressive and anti-inflammatory roles of exosomes have also been described (Chaput and Théry, 2011). Indeed, mesenchymal stromal cells-derived exosomes exert immunomodulatory properties in human peripheral blood mononuclear cells **(PBMC)** and T cells, by increasing cell apoptosis, inducing the differentiation of T helper type 1 **(Th1)** into T helper type 2 **(Th2)**, suppressing the secretion of the pro-inflammatory cytokines tumor necrosis factor alpha (**TNF-α**) and IL-1β, and increasing the production of the anti-inflammatory cytokine transforming growth factor beta **(TGF-β)** (Chen et al., 2016b).

Exosomes are present in different body fluids such as blood, saliva, urine, semen, cerebrospinal fluid, bile, and milk (Admyre et al., 2007; Raposo and Stoorvogel, 2013). Milk exosomes are part of the complex mechanism of transmission of immunity from the mother to the offspring, by transferring immunoregulatory molecules, such as miRNA, they may play an essential role in the

development of the newborn immune system and growth (Zhou et al., 2012). Indeed, human milk exosomes resist digestion in vitro and are internalized by intestinal cells, affecting their gene expression (Lonnerdal et al., 2015). Similarly, bovine milk exosomes are internalized by human and rat intestinal cells where they release their miRNA cargo (Wolf et al., 2015).

Immune cells like human macrophages are also capable of taking up in vitro breast milk exosomes, containing functional miRNA (Lässer et al., 2011). Inter-species exosome internalization has been also observed with bovine milk exosomes, as they can enter human (Izumi et al., 2015) and murine macrophages, and splenocytes in vitro (Arntz et al., 2015). Both, bovine and porcine milk exosomes, including their cargo, can also accumulate in piglets and mice peripheral tissues rich in immune cells such as the liver, spleen, lung and the small intestine after suckling or oral administration (Manca et al., 2018). Therefore, after reaching the intestinal tract, milk exosomes could modulate the animals' immunity, as shown in vitro (Ascanius et al., 2021). As further supported by proteomic analysis, bovine milk exosome proteins are mainly involved in immunological pathways such as Fc- gamma receptor-mediated phagocytosis, antigen processing and presentation, lymphocyte receptor signalling, and NK cell-mediated cytotoxicity (Reinhardt et al., 2012).

Porcine milk exosomes have been shown to promote porcine intestinal epithelial cells proliferation in vitro and *in vivo* in mice (Chen et al., 2016a), but their effects on porcine immunity have been so far unexplored. Therefore, this study aimed to investigate the in vitro uptake of porcine milk exosomes by porcine CD14+ monocytes and whether porcine milk exosomes may influence the immune functions of monocytes, including viability, apoptosis, chemotaxis, oxidative burst, phagocytosis, and killing capacity.

MATERIALS AND METHODS

Purification and Characterization of Milk Porcine Exosomes

Purification of Milk Exosomes from Sows. Milk from multiparous healthy sows (Teaching and Research Farm Frankenforst, University of Bonn) was collected during natural milk ejection and exosomes were purified through differential ultracentrifugation coupled with size exclusion

chromatography **(SEC)**, as previously reported for porcine milk exosomes with some minor modifications (Ferreira et al., 2021). Briefly, sows' skimmed milk (7.5 mL) was centrifuged at 10,000 g for 30 min at 4 °C to remove the remaining fat, cellular debris and microvesicles. The supernatant was diluted with double-filtered (0.22 μM) sterile PBS to reach a final volume of 12.5 mL and then it was transferred to Ultra-clear quick seal ultracentrifuge tubes (Beckman Coulter, Indianapolis, CA, USA) and centrifuged at 100,000 g for 1 h at 4 °C, using a fixed rotor (Beckman Coulter TY65 fixed angle rotor, Pasadena, CA, USA). The exosome pellet was carefully collected on top of the casein pellet without disturbing it and deposited in 2 mL tubes. The collected exosomes (2 mL) were thoroughly mixed with the pipette and further purified through SEC, using the qEVOriginal columns from Izon, with a 35 nm-350 nm recovery range (Izon Science, Oxford, UK), following the manufacturer's instructions. After the void volume (3 mL), 4 fractions of 500 μL each were collected. The fractions 2 and 3 that are expected to contain the exosomes were pooled and depleted from lipopolysaccharides **(LPS)** using the ToxinEraser Endotoxin Removal Kit (GenScript, Piscataway, NJ, USA) and following the manufacturer's instructions. The purified LPS-depleted exosomes were stored at -80 °C until use.

Nanoparticle Tracking Analysis (NTA). The Nanoparticle Tracking Analysis **(NTA)** was conducted using a Nanosight NTA 3.3 (Amesbury, United Kingdom) instrument as per the manufacturer's instructions. The milk exosomes were diluted in double-filtered PBS (1:50), loaded into the chip and the particles were visualized and analyzed with the NTA 3.3 Dev Build 3.3.301 software. For the analysis, the instrument was set up to operate at 22 °C, with a syringe pump speed of 30 arbitrary units (AU) and for each sample, 5 videos of 60 sec each were recorded. Results (mean of 5 measurements) are expressed as exosome size (nm) and concentration (particles/mL).

Transmission Electron Microscopy (TEM). Milk exosomes (2.5 μL) were applied to glow-discharged carbon-coated formvar copper grids, negatively stained with 2% uranyl acetate, air-dried for 10 min and observed in a FEI Talos 120 kV transmission electron microscope (FEI Company, Netherlands). Images of exosomes were acquired by a 4 k × 4 K Ceta CMOS camera.

Identification of Exosome Marker Proteins by Western blotting. The milk exosome protein concentration was first determined with the Pierce Bicinchoninic acid **(BCA)** protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's instructions. Exosomal proteins (2 µg) were loaded on sodium dodecyl sulphate-polyacrylamide gel electrophoresis **(SDS-PAGE)** gel and Western blotted on nitrocellulose membrane, using Trans-Blot Turbo Midi 0.2 µm Nitrocellulose Transfer Packs (Bio-Rad Laboratories, Hercules, CA, USA), and the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). The membranes were blocked for 1 h with ROTI®Block 1X (Carl Roth, GmbH Co.KG, Schoemperlen, Germany) and incubated with the primary antibody rabbit anti-human TSG-101 (1:2000) (ab225877, Abcam, Cambridge, UK) for 2 h at room temperature, and then with the secondary antibody polyclonal anti-rabbit peroxidase (1:3000) (Vector Laboratory, Inc.30, Burlingame, CA, USA) for 1 h at room temperature. The immunodetection of the reactive bands was performed using the Immobilion Western chemiluminescent HRP substrate (Millipore Corporation, Billerica MA, WA, USA).

Characterization of Porcine Milk Exosomes' Immunomodulatory Effects on Porcine Monocytes

Purification of Porcine Monocytes. Peripheral blood (100 mL) from twenty 60-100 kg healthy pigs (TOPIGS) was collected during routine slaughtering procedures in sterile flasks containing 0.2% of EDTA (Sigma-Aldrich, St. Louis, MO, USA) as an anticoagulant. PBMC were isolated first through Ficoll density gradient centrifugation, as previously described for bovine blood (Ceciliani et al., 2007). Briefly, blood was first centrifuged at 1260 *g* for 30 min at 18 °C to obtain the buffy coat. The buffy coat was diluted 1:5 in cold sterile Dulbecco's PBS without Ca^{2+} and Mg^{2+} + 2 mM EDTA (Sigma-Aldrich) and carefully layered onto 3 mL of Ficoll-Paque Plus (1.077g/mL) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Cells were then centrifuged at 1700 *g* (without brakes) for 30 min at 4 °C to obtain the PBMC ring. PBMC were collected at the interface, washed twice with cold sterile PBS without Ca^{2+} and Mg^{2+} + 2 mM EDTA, centrifuged at 500 *g* for 7 min at 4 °C and incubated with Red Blood Cell Lysis (Roche Diagnostics GmbH, Mannheim, Germany) buffer for 3 min at room temperature to remove the red blood cells. Three consequent washes with cold sterile PBS+ 2 mM EDTA were performed to remove contaminant platelets. CD14+ monocytes were further purified from PBMC through magnetic-activated cell

sorting technique **(MACS)**, using CD14 MicroBeads, LS (large size) columns and 30 mm pre-separation filters (Miltenyi-Biotech, Bergisch Gladbach, Germany), as previously described for bovine samples (Ávila et al., 2020), and following the manufacturer's instructions. Monocytes were counted using an automatic cell counter (TC20™, BioRad), and cells were resuspended in complete medium Roswell Park Memorial Institute 1640 medium **(RPMI)** with L-glutamine+ 25 mM HEPES + 1% P/S + 1% non-essential amino acids and 10% exosome-depleted Fetal Bovine Serum **(FBS)**, purchased from Sigma-Aldrich.

Exosome Uptake Assay. To evaluate if porcine milk exosomes could be internalized by porcine monocytes, monocytes of four healthy animals were treated with LPS-depleted milk exosomes and visualized using fluorescence microscopy. First, 10^8 LPS-depleted exosomes (ratio of 200 exosomes/cell) were labelled with the PKH26 Red Fluorescent Cell Linker Mini (Sigma-Aldrich), following the manufacturer's instructions with minor modifications. Briefly, LPS-depleted milk exosomes or PBS (negative control) were mixed with 250 µL of Diluent C and then rapidly added to a PKH26 dye solution in diluent C (0.04×10^{-6} final concentration), which was prepared immediately before staining. The exosomes were incubated with periodic mixing for 5 minutes in dark, and then 10% of exosome depleted serum was added to stop the staining and to allow the binding of excess dye. The excess of unincorporated dye was further removed with the Exosome Spin Columns (MW3000) (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions. Before cell seeding, the sterile 4-well Nunc Lab-Tek II Chamber Slides w/Cover RS Glass Slide (Thermo Fisher Scientific, Waltham, MA, USA) were treated with 100 µL of Poly-D-lysine (50 µg/mL) (Sigma-Aldrich) for 2 h, to enhance the cells' adherence, and washed with pyrogen-free water. Then, 5×10^5 monocytes, purified from 4 animals, were seeded and co-incubated with 10^8 PKH26-labeled exosomes (150 µL) or PBS as a negative control for 22 h at 39 °C in a humidified atmosphere and 5% CO_2. After the incubation, cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature and the nuclei were stained with Hoechst 33342 (Sigma-Aldrich) (1 µg/mL) for 15 min. Finally, a drop of Invitrogen™ProLong™ Diamond Antifade Mountant (Thermo Fisher Scientific) was added to the slides and the cells were visualized using a fluorescence microscope (Eclipse E600; Nikon). The images were then analyzed with the ImageJ/Fiji software. The percentage of PKH26 exosome positive cells was determined by calculating the ratio between the cells positive for the red fluorescent dye and the total number of

cells observed in each field, multiplied by 100. Results are expressed as the mean percentage of three different fields.

Viability Assay. The viability assay was performed to assess the potential cytotoxicity of LPS-depleted milk exosomes on porcine monocytes by using the Cell proliferation kit I (MTT) (Roche Diagnostics) as already reported for bovine monocytes (Ávila et al., 2020). A total amount of 1 x 10^5 cells (25 µL) per well, purified from 5 animals, was seeded in duplicate in sterile 96-well plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA). The plates were incubated for 12 h and 22 h at 39 °C in a humidified atmosphere and 5% CO_2 with increasing numbers (10^3, 10^5, 10^7, and 10^8) of LPS-depleted milk exosomes (25 µL) or with the medium as control (no exosomes). To measure the cells' viability, 10 µL of the MTT labeling reagent were added to each well and incubated at 39 °C for 4 h, following the manufacturer's instructions. The formazan crystals were solubilized by adding 100 µL of solubilizing buffer and incubating the plates overnight at 39 °C. The absorbance was read at 550 nm with a LabSystem Multiskan plate reader Spectrophotometer (LabX, Midland, Canada).

Apoptosis Assay.To evaluate whether milk exosomes could affect porcine monocytes' apoptosis, the enzymatic activity of Caspase-3/7 was measured. Briefly, 5 x 10^4 cells (12.5 µL) from 5 animals were seeded in duplicate in sterile 384-well black plates (Corning Inc., Kennebunk, ME, USA), as previously described for bovine samples (Ceciliani et al., 2007). The cells were incubated for 12 h and 22 h at 39 °C in a humidified atmosphere and 5% CO_2 with increasing numbers (10^3, 10^5,10^7, and 10^8) of LPS-depleted porcine milk exosomes (12.5 µL) or the medium as control (no exosomes). The apoptosis assay was carried out using the Apo-ONE® reagent Homogeneous Caspase-3/7 kit (Promega, Madison, WI, USA), following the manufacturer's instructions. The fluorescence of 485/538 nm (absorbance/emission) was measured with a fluorescence plate reader Fluoroscan Ascent (Thermo Fisher Scientific) every 30 min for 2 h.

Chemotaxis Assay.The monocytes' chemotaxis towards zymosan activated serum **(ZAS)** was measured as previously reported, with some minor changes (Ávila et al., 2020). Briefly, ZAS was prepared by activating FBS-exosome depleted serum (Sigma-Aldrich) with 15 mg/ml of Zymosan

A from *Saccharomyces cerevisiae* (Sigma-Aldrich) for 60 minutes at 39 °C, and incubated for 30 minutes at 56 °C to destroy complement components, as previously described for bovine monocytes (Lecchi et al., 2008). Then, 50 microliters of monocytes (1 x 10^5) from 5 animals were seeded in duplicate in the upper chamber of a sterile 24-well Transwell migration plates (Corning Inc.), equipped with an 8 μm pore size membrane. Cells were then pretreated with 2 x 10^7 LPS-depleted milk exosomes (50 μL in the upper chamber and 650 μL in the lower chamber), in a ratio of 200 exosomes/cell and the absence of a chemoattractant, for 22 h at 39 °C and 5% CO_2. After the incubation, ZAS (3 mg/mL) was added in the lower chamber, and the cells were incubated in the presence of newly added milk exosomes or migration medium (RMPI-1640 with 1% of exosome-depleted FBS) as control (no exosomes) again for 2 h at 39 °C and 5% CO_2. Finally, non-migrated cells in the upper chambers of the membrane were gently removed with a swab moistened with PBS, and migrated cells were stained with Diff-Quick (Sigma-Aldrich) and counted in 10 different fields, using light microscopy (inverted microscope).

Phagocytosis Assay. The phagocytosis assay was carried out by measuring the fluorescence of fluorescein-labelled *Escherichia coli (E. coli)* K-12 strain bioparticles (Invitrogen) as previously performed (Lecchi et al., 2016). Opsonization of fluorescein-labelled *E. coli* bioparticles (K-12 strain) was performed by incubating 80 μL of bacteria suspension (5 x 10^6 *E.coli*/μL) with 20% of exosome-depleted FBS (20 μL) for 30 min at 39 °C. The suspension was centrifuged at 800 x *g* for 15 min and suspended in PBS. A total of 3 x 10^5 monocytes (100 μL) from 7 animals were seeded in duplicate in 96-well plates and treated with 100 μL of LPS-depleted milk exosomes (ratio of 200 exosomes/cell) or with the medium as control (no exosomes). Cells were then incubated at 39 °C and 5% CO_2 for 22 h. The cells were washed with PBS and fluorescein-labeled *E. coli* bioparticles with a ratio of 45 particles/cell were added and co-incubated for 2 h at 39 °C. Cells were washed with PBS to remove non-internalized bioparticles and incubated with 50 μL trypan blue 0.4% for 1 min at room temperature to remove and quench the fluorescence of the non-internalized bacteria, respectively. After removal of the trypan blue, the fluorescence (485/538 nm) was measured using the microplate reader Fluoroscan Ascent (Thermo Fisher Scientific).

Killing Capacity Assay. The intracellular bacterial killing capacity was determined according to as reported previously (Lecchi et al., 2013). The *E. coli* American Type Culture Collection

(ATCC) 25922 (strain Seattle 1946; LCG Standards Ltd., Teddington, UK) were opsonized with 20% exosome depleted FBS (20 µL), incubated at 37 °C for 30 min. The bacteria were washed twice by centrifugation at 1500 *g* for 10 min at 4 °C and suspended with PBS. A total of 3 x 10^5 monocytes (100 µL) from 5 animals was seeded in duplicate in cryogenic vials. Cells were then treated with 6 x 10^7 LPS-depleted milk exosomes (100 µL), in a ratio of 200 exosomes/cell, or with the medium as control (no exosomes) and were incubated for 22 h at 39 °C and 5% CO_2. After the incubation, cells were incubated for 1 h at 39 °C and 5% CO_2 with 1 x 10^7 opsonized live *E. coli*. The unbound bacteria were removed by centrifugation and by further treating the cells with 100 µg/mL of gentamicin for 1 h. Gentamicin was eliminated by washing the cells with PBS and centrifuging at 110 x *g* for 5 min. Finally, cells were lysed with 0.5% Triton x-100 (Sigma-Aldrich) on ice for 10 min, and after overnight incubation at 37 °C, the colonies forming units **(CFU)** of surviving bacteria were counted on MacConkey agar plates. Results were then expressed as CFU/mL.

Reactive Oxygen Species (ROS) Production Assay. The production of extracellular superoxide anions **(O_2-)** was determined with the cytochrome C reduction assay, as previously described (Lecchi et al., 2016). A total of 1 x 10^5 monocytes (50 µL) from 6 animals was seeded in complete medium without phenol red in triplicate in 96-well sterile plates and co-incubated for 22 h at 39 °C + 5% CO_2 with 2 x 10^7 exosomes (50 µL) in a ratio of 200 exosomes/cell. After the incubation period, the production of O_2- was measured under resting (non- pro-inflammatory) and pro-inflammatory conditions when challenged with phorbol myristate acetate **(PMA)**. In the normal conditions, 10 µL of Cytochrome C from the equine heart (1 mM), (Sigma-Aldrich) were added; while to mimic a pro-inflammatory challenge 10 µL of Cytochrome C and 2 µL PMA (2.5 µg/mL final concentration; Sigma-Aldrich) were added. Medium without phenol red was added to all wells to have a final volume of 200 µL. The absorbance was measured every 30 min for 4 h at 550 nm with a LabSystem Multiskan plate reader spectrophotometer (LabX).

Statistical Analyses. Statistical analyses were performed in GraphPad Prism 8.0.2. Data normality was assessed with the Shapiro Wilk test. Repeated measures one-way ANOVA and Tukey's multiple comparison tests were used for normally distributed samples from viability and apoptosis at 22 h assays, while a Friedman test and Dunn's multiple comparisons tests were applied for

apoptosis at 12 h. Paired t-tests were used for phagocytosis, killing capacity, chemotaxis, ROS production and exosome uptake assays. Statistical differences were accepted at $P \leq 0.05$.

RESULTS

Purification and Characterization of Milk Porcine Exosomes

To examine and determine the size and concentration of the isolated LPS-depleted milk exosomes NTA was performed. The size of the purified porcine milk exosomes was 155 nm ± 8.9 nm (SEM) and the concentration was 2.55 x 10^{10} particles/mL (Figure 1A).

The structural and morphological features of porcine LPS-depleted milk exosomes were assessed by TEM negative staining. The exosomes presented a round or cup shape (Figure 1B) and were enclosed by a lipid bilayer membrane (Figure 1C).

The protein content of the LPS-depleted milk exosomes was quantified with the BCA protein quantification kit, and a total concentration of 147 μg/mL was obtained (data not shown). The presence of the exosome marker TSG-101 in milk exosomes, before and after LPS depletion was confirmed by Western blot analysis (Figure 1D). The detected band for this cytosolic protein was observed at the reported molecular weight (44 kDa).

Characterization of Exosomes' Immunomodulatory Effects on Porcine Monocytes

***Porcine Milk Exosomes were Internalized by Porcine Monocytes* in vitro.** To evaluate whether porcine milk exosomes can be taken up by porcine monocytes, the cells were co-cultured with 10^8 PKH26-labeled milk exosomes (LPS-depleted) for 22 h and then visualized using fluorescence microscopy. No fluorescence was observed in the PKH26-labeled PBS (negative control) treated cells (Figure 2A). Porcine PKH26-labeled milk exosomes (in red) were internalized by the monocytes (blue) in vitro and they were mainly located in the cells' cytoplasm, surrounding the nucleus (Figure 2B, C). These results were further confirmed when determining the percentage of PKH26-positive cells, which was higher ($P = 0.03$) in the exosome group than in the negative control (Figure 2D).

Porcine Milk Exosomes did not Affect Porcine Monocytes' Spontaneous Apoptosis and Viability. To determine if the milk exosomes could exert cytotoxic effects on porcine monocytes, cells were incubated with increasing numbers (10^3, 10^5, 10^7, and 10^8) of exosomes or with complete medium only as control (no exosomes) for 12 and 22 h and their apoptosis and viability were measured. Increasing concentrations of milk exosomes showed no cytotoxic effects on porcine monocytes during the time, as neither their spontaneous apoptosis nor viability at 12 h (Figure 3A, C) and 22 h (Figure 3B, D); were affected.

Porcine Milk Exosomes Modulate Porcine Monocytes' Chemotaxis. The chemotactic activity was measured after activating the cells either treated with porcine milk exosomes or medium (positive control) with the chemoattractant, ZAS. Porcine milk exosomes did modulate monocyte chemotaxis ($P = 0.05$), as a decrease in the number of migrated cells was observed when compared with the positive control without exosomes (Figure 4).

Porcine Milk Exosomes Decreased Porcine Monocytes' Phagocytic Capacity, but not their Killing Capacity. The capacity of monocytes to phagocyte (Figure 5A) *E.coli* bioparticles when treated with milk exosomes (10^7) was decreased ($P = 0.02$) compared to the control. However, no effects were observed in their capacity to kill live *E. coli* (Figure 5B).

Porcine Milk Exosomes Increased Porcine Monocytes' ROS Production. The monocytes' production of extracellular O_2- under both, resting and pro-inflammatory conditions, was also evaluated. Cells co-cultured with porcine LPS-depleted milk exosomes under resting conditions showed an increase in their ROS production at 60 minutes ($P = 0.04$) (Figure 6A) as compared to control. Co-incubation with milk exosomes also affected the ROS production of monocytes after inducing a proinflammatory challenge with PMA; in detail, an increase after 90 minutes ($P = 0.03$) and 120 minutes ($P < 0.01$) was detected (Figure 6B) when compared to the control.

DISCUSSION

Exosomes are important mediators of intercellular communication. They fulfil this critical role by transferring their cargo of regulatory molecules (nucleic acids, proteins, lipids and metabolites) to recipient cells, altering their biological response, including the balancing of the immune response (Kalluri and LeBleu, 2020).

The contribution of milk exosomes to intercellular communication and regulation of cellular processes remains still undisclosed. To the best of our knowledge, the present study reports for the first time the in vitro uptake of sows' milk exosomes by porcine monocytes (CD14+), and the ability of these exosomes in modulating monocytes immune activities, including chemotaxis, phagocytosis and ROS production. Our main findings were that porcine milk exosomes are internalized by porcine monocytes and that they modulated in vitro the cells' chemotaxis, phagocytosis, and ROS production, the latter under both, resting and pro-inflammatory conditions.

In the first part of the study, LPS-depleted porcine milk exosomes were isolated and characterized using NTA, TEM and Western blotting. The NTA results are consistent with the size range previously reported for exosomes (~ 40 - 160 nm) (Kalluri and LeBleu, 2020), and with the average size (152 nm) of porcine milk exosomes from different lactation stages (Ferreira et al., 2021). However, particles with sizes larger than 160 nm and up to 819 nm were also detected in our NTA results, suggesting that other extracellular vesicles **(EVs)** - known to have sizes ranging from 50 nm to 1 μm – were also isolated. The co-isolation of different subpopulations of EVs has been previously reported (Bebelman et al., 2018; Mathieu et al., 2019), revealing the technical challenges and limitations that we still face with the currently available isolation methods to obtain pure exosome isolates. The concentration of the porcine milk exosomes determined by the NTA was also consistent with the concentrations observed in previous studies in bovine (1.4×10^{11} particles/ml) and human (8.0×10^{10} particles/ml) (Vaswani et al., 2019), and porcine milk (2.4×10^{11} particles/mL) (Ferreira et al., 2021).

The TEM micrographs showed the typical rounded and/or cup-shaped morphology of exosomes - the latter being related to the drying process for imaging analysis (Raposo and Stoorvogel, 2013) - and the lipid bilayer membrane surrounding them, in agreement with the already reported structure for porcine milk exosomes (Ferreira et al., 2021). Immunoblotting results further confirmed the presence of the widely recognized exosome marker protein, TSG-101 (tumour

susceptibility gene 101), a cytosolic protein (44 kDa) involved in the origin and biogenesis process of exosomes (Kalluri and LeBleu, 2020). This protein has routinely been used for defining bovine milk exosomes (Samuel et al., 2017; Vaswani et al., 2019) and, more recently, for porcine milk exosomes as well (Ferreira et al., 2021).

The second part of the study it was evaluated the in vitro immunomodulatory activity of milk exosomes on porcine monocytes functions. In a first step, we examined if milk exosomes are taken up by monocytes. Our results revealed that indeed these nanovesicles can be internalized by the cells and that they were located mainly in their cytoplasm near the nucleus, as seen with fluorescence microscopy. These results agree with those of human (Lässer et al., 2011) and bovine milk exosomes (Izumi et al., 2015), which can be also uptaken by human and murine macrophages (Arntz et al., 2015). In a second step, to rule out any potential cytotoxic effects of porcine milk exosomes, and subsequently, determine their subtoxic working concentration, we evaluated the apoptosis and viability of porcine monocytes. Milk exosomes did not affect the monocytes spontaneous apoptosis and viability during the time. These results are consistent with previous studies on human PBMC, where exosomes derived from mesenchymal stromal cells and breast milk did not affect the viability of the cells (Admyre et al., 2006; Khare et al., 2018). Moreover, when con-incubated with other cellular models, such as the porcine intestinal epithelial cells IPEC-J2, milk exosomes have not shown detrimental effects in vitro on the viability when measured with the MTT assay, enhancing their proliferation (Chen et al., 2016a). However, our results differ from the anti-apoptotic and protective effects that porcine milk exosomes have produced on LPS-treated intestinal epithelial cells, by attenuating their apoptosis and enhancing their viability (Xie et al., 2019), suggesting that exosomes do have pleiotropic functions that may depend on the type of cell that produces the exosomes, of the cargo they carry and transfer, and of the recipient cells.

For all the following experiments, a total of 10^7 exosomes, with a ratio of 200 exosomes/cell, and an incubation time of 22 h were selected, as no cytotoxic effects were observed in our preliminary experiments. This concentration was based on a preliminary study (Hong et al., 2017) that investigated the immunomodulatory effects after the internalizing of the exosomes on rhesus macaques PBMC and CD4+ T cells, using a similar range of 10^6 - 10^8 exosomes. Moreover, we decided to select the closest exosome concentration to that found physiologically in porcine milk that could still be used in the in vitro assays.

Porcine milk exosomes decreased monocyte chemotaxis by reducing their migration towards ZAS. To the best of the authors' knowledge, this is the first study reporting the milk exosomes activity on immune cells. Our study confirms the results reported in other models, such as the effects of human exosomes from atherogenic macrophages that inhibited both human and mouse macrophages migration by transferring miRNA, in particular, the miR-146a (Nguyen et al., 2018). Moreover, proteomic analysis of milk exosomes also confirmed the presence of exosome proteins involved in the migration and cell movement, as pathways such as the chemokine signalling and leukocyte transendothelial migration were found to be enriched (Reinhardt et al., 2012). It must be said that the effects of exosomes on chemotaxis likely depend on the source of the exosomes, and the type of the delivered cargo since other contradictory effects of exosomes on chemotaxis have been previously reported (Li et al., 2020).

The monocytes' phagocytic capacity was decreased by porcine milk exosomes but did not affect their killing capacity. These results could be explained by the immunosuppressive effects already attributed to milk exosomes (Ahn et al., 2021) and their miRNA cargo (Melnik et al., 2021) in downregulating murine macrophages phagocytosis via the suppression of nuclear factor-κB (NF-κB) (Zhu et al., 2014). Proteomic studies of bovine milk exosomes also demonstrated that exosomal proteins were involved in Fcγ receptor-mediated phagocytosis, suggesting that milk exosomes can modulate this inflammatory function (Reinhardt et al., 2012). However, the current information on the effects of milk exosomes on phagocytosis is limited, and contradictory, as other studies reported an effect of upregulating phagocytosis (Singhto et al., 2018).

Our findings show that porcine milk exosomes increased the production of extracellular superoxide anion under both resting and pro-inflammatory conditions after the PMA challenge, suggesting that they might play an important role in enhancing the monocytes' response. Our results agree with those previously reported in other models, where different sources of exosomes and cellular targets were used, and that demonstrated that eosinophil-derived exosomes increased the ROS production in patients with asthma (Cañas et al., 2017), while macrophage-derived exosomes induced the production of ROS in injured neurons (Hervera et al., 2018). The molecules necessary for ROS production like NADPH oxidase (Janiszewski et al., 2004) and cytochrome P450 (Kumar et al., 2017) were also found in platelet-derived exosomes and plasma exosomes, respectively. However, recent works have instead pointed towards an antioxidant and protective role of exosomes against oxidative stress (Bodega et al., 2019). For example, bovine milk

exosomes inhibit ROS production and increase activities of the antioxidant enzymes SOD and GPX (Wang et al., 2021), and suppress murine macrophages ROS production under hypoxic conditions (Matic, 2019).

CONCLUSIONS

The results of this study demonstrated that LPS-depleted porcine milk exosomes modulate some immune functions of porcine monocytes in vitro. Specifically, milk exosomes decreased monocytes' phagocytosis and chemotaxis, while increasing their ROS production under resting and pro-inflammatory conditions, suggesting they can exert pleiotropic functions on the cells, as both immunosuppressive and/ or immune-enhancing effects were observed. To the best of our knowledge, we also demonstrated for the first time that milk exosomes are taken up by monocytes in vitro, which could potentially explain the way exosomes exert their immunomodulation. However, our study does not provide evidence on the exact molecular mechanisms underlying such effects. Their elucidation using OMIC technologies like transcriptomics and proteomics would help us to better understand the biological significance in experimental in vivo systems. Finally, our results also suggest a potentially critical role of porcine milk exosomes in the sow-to-pigletttransmission of regulatory molecules and consequently immunomodulation. Hence, it would be also of great interest to perform further in vitro experiments, using antibodies to block ligand/receptor interactions or chemical inhibitors of phagocytosis and/or endocytosis to further elucidate the underlying molecular mechanisms of exosome uptake.

ETHICS STATEMENT

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The procedures for the blood collection were carried out during routine slaughtering procedures. Milk for isolating exosomes was collected at the Teaching and Research Farm Frankenforst of the University of Bonn. This farm holds a permit according to the statuary provisions of the European and German animal welfare law (Art 11, Para 1, Clause 1a TierschG) for breeding and keeping farm animals (cattle, sheep, pigs, chicken, and quail). The milk samples

were non-invasively obtained during the naturally occurring milk-let down reflex when the piglets were suckled, without using oxytocin injections.

ACKNOWLEDGMENTS

This work was supported with the European Union's Horizon 2020 research and innovation program H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate) [Grant agreement nº: 765423, 2017] – MANNA. We also acknowledge the support of Valentina Lodde of the Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare at the Università Degli Studi di Milano, Italy; and Carlotta Catozzi of the Microbiota and Antitumor Immunity group at the Istituto Europeo di Oncologia, Italy, for their valuable support in the microscopic evaluation and digital imaging acquisition in the exosome uptake experiment. We also thank Samantha Milanesi from the Laboratorio di Biologia dei Leucociti from the Istituto Clinico Humanitas, Italy, for performing the NTA analysis; and Andrea Raimondi from the ALEMBIC, Experimental Imaging Center at the IRCCS San Raffaele Scientific Institute for the TEM analysis. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Admyre, C., S.M. Johansson, S. Paulie, and S. Gabrielsson. 2006. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. Eur. J. Immunol. 36:1772–1781. doi:10.1002/eji.200535615.

Admyre, C., S.M. Johansson, K.R. Qazi, J.-J. Filén, R. Lahesmaa, M. Norman, E.P.A. Neve, A. Scheynius, and S. Gabrielsson. 2007. Exosomes with Immune Modulatory Features Are Present in Human Breast Milk. J. Immunol. 179:1969–1978. doi:10.4049/jimmunol.179.3.1969.

Ahn, G., Y.-H. Kim, and J.-Y. Ahn. 2021. Multifaceted effects of milk-exosomes (Mi-Exo) as a modulator of scar-free wound healing. Nanoscale Adv. 3:528–537. doi:10.1039/D0NA00665C.

Arntz, O.J., B.C.H. Pieters, M.C. Oliveira, M.G.A. Broeren, M.B. Bennink, M. de Vries, P.L.E.M. van Lent, M.I. Koenders, W.B. van den Berg, P.M. van der Kraan, and F.A.J. van de Loo. 2015. Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models. Mol. Nutr. Food Res. 59:1701–1712. doi:https://doi.org/10.1002/mnfr.201500222.

Ascanius, S.R., M.S. Hansen, M.S. Ostenfeld, and J.T. Rasmussen. 2021. Milk-Derived Extracellular Vesicles Suppress Inflammatory Cytokine Expression and Nuclear Factor-κB Activation in Lipopolysaccharide-Stimulated Macrophages. Dairy 2:165–178.

Ávila, G., C. Catozzi, D. Pravettoni, G. Sala, P. Martino, G. Meroni, C. Lecchi, and F. Ceciliani. 2020. In vitro effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes. J. Dairy Sci. 103:8554–8563. doi:10.3168/jds.2020-18659.

Bebelman, M.P., M.J. Smit, D.M. Pegtel, and S.R. Baglio. 2018. Biogenesis and function of extracellular vesicles in cancer. Pharmacol. Ther. 188:1–11. doi:https://doi.org/10.1016/j.pharmthera.2018.02.013.

Becker, A., B.K. Thakur, J.M. Weiss, H.S. Kim, H. Peinado, and D. Lyden. 2016. Extracellular

Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. Cancer Cell 30:836–848. doi:10.1016/j.ccell.2016.10.009.

Bodega, G., M. Alique, L. Puebla, J. Carracedo, and R.M. Ramírez. 2019. Microvesicles: ROS scavengers and ROS producers. J. Extracell. vesicles 8:1626654. doi:10.1080/20013078.2019.1626654.

Cañas, J.A., B. Sastre, C. Mazzeo, M. Fernández-Nieto, J.M. Rodrigo-Muñoz, A. González-Guerra, M. Izquierdo, P. Barranco, S. Quirce, J. Sastre, and V. del Pozo. 2017. Exosomes from eosinophils autoregulate and promote eosinophil functions. J. Leukoc. Biol. 101:1191–1199. doi:10.1189/jlb.3AB0516-233RR.

Ceciliani, F., V. Pocacqua, A. Miranda-Ribera, V. Bronzo, C. Lecchi, and P. Sartorelli. 2007. α1-Acid glycoprotein modulates apoptosis in bovine monocytes. Vet. Immunol. Immunopathol. 116:145–152. doi:10.1016/j.vetimm.2007.01.006.

Chaput, N., and C. Théry. 2011. Exosomes: Immune properties and potential clinical implementations. Semin. Immunopathol. 33:419–440. doi:10.1007/s00281-010-0233-9.

Chen, T., M.Y. Xie, J.J. Sun, R.S. Ye, X. Cheng, R.P. Sun, L.M. Wei, M. Li, D.L. Lin, Q.Y. Jiang, Q.Y. Xi, and Y.L. Zhang. 2016a. Porcine milk-derived exosomes promote proliferation of intestinal epithelial cells. Sci. Rep. 6:1–12. doi:10.1038/srep33862.

Chen, W., Y. Huang, J. Han, L. Yu, Y. Li, Z. Lu, H. Li, Z. Liu, C. Shi, F. Duan, and Y. Xiao. 2016b. Immunomodulatory effects of mesenchymal stromal cells-derived exosome. Immunol. Res. 64:831–840. doi:10.1007/s12026-016-8798-6.

Chow, A., W. Zhou, L. Liu, M.Y. Fong, J. Champer, D. Van Haute, A.R. Chin, X. Ren, B.G. Gugiu, Z. Meng, W. Huang, V. Ngo, M. Kortylewski, and S.E. Wang. 2014. Macrophage immunomodulation by breast cancer-derived exosomes requires Toll-like receptor 2-mediated activation of NF-κ B. Sci. Rep. 4. doi:10.1038/srep05750.

Dalvi, P., B. Sun, N. Tang, and L. Pulliam. 2017. Immune activated monocyte exosomes alter microRNAs in brain endothelial cells and initiate an inflammatory response through the TLR4/MyD88 pathway. Sci. Rep. 7:1–12. doi:10.1038/s41598-017-10449-0.

Ferreira, R.F., T. Blees, F. Shakeri, A. Buness, M. Sylvester, G. Savoini, A. Agazzi, V. Mrljak, and H. Sauerwein. 2021. Comparative proteome profiling in exosomes derived from porcine colostrum versus mature milk reveals distinct functional proteomes. J. Proteomics 104338. doi:https://doi.org/10.1016/j.jprot.2021.104338.

Hervera, A., F. De Virgiliis, I. Palmisano, L. Zhou, E. Tantardini, G. Kong, T. Hutson, M.C. Danzi, R.B.-T. Perry, C.X.C. Santos, A.N. Kapustin, R.A. Fleck, J.A. Del Río, T. Carroll, V. Lemmon, J.L. Bixby, A.M. Shah, M. Fainzilber, and S. Di Giovanni. 2018. Reactive oxygen species regulate axonal regeneration through the release of exosomal NADPH oxidase 2 complexes into injured axons. Nat. Cell Biol. 20:307–319. doi:10.1038/s41556-018-0039-x.

Hong, X., B. Schouest, and H. Xu. 2017. Effects of exosome on the activation of CD4+ T cells in rhesus macaques: a potential application for HIV latency reactivation. Sci. Rep. 7:15611. doi:10.1038/s41598-017-15961-x.

Izumi, H., M. Tsuda, Y. Sato, N. Kosaka, T. Ochiya, H. Iwamoto, K. Namba, and Y. Takeda. 2015. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. J. Dairy Sci. 98:2920–2933. doi:10.3168/jds.2014-9076.

Janiszewski, M., A.O. do Carmo, M.A. Pedro, E. Silva, E. Knobel, and F.R.M. Laurindo. 2004. Platelet-derived exosomes of septic individuals possess proapoptotic NAD(P)H oxidase activity: A novel vascular redox pathway*. Crit. Care Med. 32. doi:10.1097/01.CCM.0000114829.17746.19

Kalluri, R., and V.S. LeBleu. 2020. The biology, function, and biomedical applications of exosomes. Science 367: eaau6977. doi: 10.1126/science.aau6977.

Khare, D., R. Or, I. Resnick, C. Barkatz, O. Almogi-Hazan, and B. Avni. 2018. Mesenchymal Stromal Cell-Derived Exosomes Affect mRNA Expression and Function of B-Lymphocytes . Front. Immunol. 9:3053. https://doi.org/10.3389/fimmu.2018.03053

Kumar, S., N. Sinha, K.A. Gerth, M.A. Rahman, M.M. Yallapu, and N.M. Midde. 2017. Specific packaging and circulation of cytochromes P450, especially 2E1 isozyme, in human plasma exosomes and their implications in cellular communications. Biochem. Biophys. Res.

Commun. 491:675–680. doi:https://doi.org/10.1016/j.bbrc.2017.07.145.

Lässer, C., V. Seyed Alikhani, K. Ekström, M. Eldh, P. Torregrosa Paredes, A. Bossios, M. Sjöstrand, S. Gabrielsson, J. Lötvall, and H. Valadi. 2011. Human saliva, plasma and breast milk exosomes contain RNA: Uptake by macrophages. J. Transl. Med. 9:9. doi:10.1186/1479-5876-9-9.

Lecchi, C., F. Ceciliani, S. Bernasconi, F. Franciosi, V. Bronzo, and P. Sartorelli. 2008. Bovine alpha-1 acid glycoprotein can reduce the chemotaxis of bovine monocytes and modulate CD18 expression. Vet. Res. 39. doi:10.1051/vetres:2008027.

Lecchi, C., N. Rota, A. Vitali, F. Ceciliani, and N. Lacetera. 2016. In vitro assessment of the effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in bovine polymorphonuclear cells. Vet. Immunol. Immunopathol. 182:89–94. doi:10.1016/j.vetimm.2016.10.007.

Lecchi, C., A. Scarafoni, V. Bronzo, P.A. Martino, A. Cavallini, P. Sartorelli, and F. Ceciliani. 2013. α1-Acid glycoprotein modulates phagocytosis and killing of Escherichia coli by bovine polymorphonuclear leucocytes and monocytes. Vet. J. 196:47–51. doi:10.1016/j.tvjl.2012.07.022.

Li, X., R. Liu, Y. Wang, W. Zhu, D. Zhao, X. Wang, H. Yang, E.C. Gurley, W. Chen, P.B. Hylemon, and H. Zhou. 2020. Cholangiocyte-Derived Exosomal lncRNA H19 Promotes Macrophage Activation and Hepatic Inflammation under Cholestatic Conditions. Cells 9. doi:10.3390/cells9010190.

Lonnerdal, B., X. Du, Y. Liao, and J. Li. 2015. Human milk exosomes resist digestion in vitro and are internalized by human intestinal cells. FASEB J. 29:121.3. doi:https://doi.org/10.1096/fasebj.29.1_supplement.121.3.

Manca, S., B. Upadhyaya, E. Mutai, A.T. Desaulniers, R.A. Cederberg, B.R. White, and J. Zempleni. 2018. Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. Sci. Rep. 8:1–11. doi:10.1038/s41598-018-29780-1.

Mathieu, M., L. Martin-Jaular, G. Lavieu, and C. Théry. 2019. Specificities of secretion and uptake

of exosomes and other extracellular vesicles for cell-to-cell communication. Nat. Cell Biol. 21:9–17. doi:10.1038/s41556-018-0250-9.

Matic, S. 2019. Stability of bovine milk exosomes and their effect on proliferation of RAW 264 . 7 cells. MS Thesis. TRACE: Tennessee Research and Creative Exchange, University of Tennessee, Knoxville.

Melnik, B.C., W. Stremmel, R. Weiskirchen, S.M. John, and G. Schmitz. 2021. Exosome-Derived MicroRNAs of Human Milk and Their Effects on Infant Health and Development. Biomolecules 11:851. doi:10.3390/biom11060851.

Mittelbrunn, M., C. Gutiérrez-Vázquez, C. Villarroya-Beltri, S. González, F. Sánchez-Cabo, M.Á. González, A. Bernad, and F. Sánchez-Madrid. 2011. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat. Commun. 2:282. doi:10.1038/ncomms1285.

Montecalvo, A., A.T. Larregina, W.J. Shufesky, D. Beer Stolz, M.L.G. Sullivan, J.M. Karlsson, C.J. Baty, G.A. Gibson, G. Erdos, Z. Wang, J. Milosevic, O.A. Tkacheva, S.J. Divito, R. Jordan, J. Lyons-Weiler, S.C. Watkins, and A.E. Morelli. 2012. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 119:756–766. doi:10.1182/blood-2011-02-338004.

Nguyen, M.-A., D. Karunakaran, M. Geoffrion, H.S. Cheng, K. Tandoc, L. Perisic Matic, U. Hedin, L. Maegdefessel, J.E. Fish, and K.J. Rayner. 2018. Extracellular Vesicles Secreted by Atherogenic Macrophages Transfer MicroRNA to Inhibit Cell Migration. Arterioscler. Thromb. Vasc. Biol. 38:49–63. doi:10.1161/ATVBAHA.117.309795.

Raposo, G., H.W. Nijman, W. Stoorvogel, R. Liejendekker, C. V Harding, C.J. Melief, and H.J. Geuze. 1996. B lymphocytes secrete antigen-presenting vesicles. J. Exp. Med. 183:1161–1172. doi:10.1084/jem.183.3.1161.

Raposo, G., and W. Stoorvogel. 2013. Extracellular vesicles: Exosomes, microvesicles, and friends. J. Cell Biol. 200:373–383. doi:10.1083/jcb.201211138.

Reinhardt, T.A., J.D. Lippolis, B.J. Nonnecke, and R.E. Sacco. 2012. Bovine milk exosome

proteome. J. Proteomics 75:1486–1492. doi:https://doi.org/10.1016/j.jprot.2011.11.017.

Samuel, M., D. Chisanga, M. Liem, S. Keerthikumar, S. Anand, C.-S. Ang, C.G. Adda, E. Versteegen, M. Jois, and S. Mathivanan. 2017. Bovine milk-derived exosomes from colostrum are enriched with proteins implicated in immune response and growth. Sci. Rep. 7:5933. doi:10.1038/s41598-017-06288-8.

Singhto, N., R. Kanlaya, A. Nilnumkhum, and V. Thongboonkerd. 2018. Roles of Macrophage Exosomes in Immune Response to Calcium Oxalate Monohydrate Crystals. Front. Immunol. 9:316. doi:10.3389/fimmu.2018.00316.

Vaswani, K., M.D. Mitchell, O.J. Holland, Y. Qin Koh, R.J. Hill, T. Harb, P.S.W. Davies, and H. Peiris. 2019. A Method for the Isolation of Exosomes from Human and Bovine Milk. J. Nutr. Metab. 2019:5764740. doi:10.1155/2019/5764740.

Wang, L., Z. Shi, X. Wang, S. Mu, X. Xu, L. Shen, and P. Li. 2021. Protective effects of bovine milk exosomes against oxidative stress in IEC-6 cells. Eur. J. Nutr. 60:317–327. doi:10.1007/s00394-020-02242-z.

Wen, C., R.C. Seeger, M. Fabbri, L. Wang, A.S. Wayne, and A.Y. Jong. 2017. Biological roles and potential applications of immune cell-derived extracellular vesicles. J. Extracell. Vesicles 6:1–19. doi:10.1080/20013078.2017.1400370.

Wolf, T., S.R. Baier, and J. Zempleni. 2015. The Intestinal Transport of Bovine Milk Exosomes Is Mediated by Endocytosis in Human Colon Carcinoma Caco-2 Cells and Rat Small Intestinal IEC-6 Cells. J. Nutr. 145:2201–2206. doi:10.3945/jn.115.218586.

Xie, M.-Y., L.-J. Hou, J.-J. Sun, B. Zeng, Q.-Y. Xi, J.-Y. Luo, T. Chen, and Y.-L. Zhang. 2019. Porcine Milk Exosome MiRNAs Attenuate LPS-Induced Apoptosis through Inhibiting TLR4/NF-κB and p53 Pathways in Intestinal Epithelial Cells. J. Agric. Food Chem. 67:9477–9491. doi:10.1021/acs.jafc.9b02925.

Zhou, Q., M. Li, X. Wang, Q. Li, T. Wang, Q. Zhu, X. Zhou, X. Wang, X. Gao, and X. Li. 2012. Immune-related microRNAs are abundant in breast milk exosomes. Int. J. Biol. Sci. 8:118–123. doi:10.7150/ijbs.8.118.

Zhu, F., W. Yue, and Y. Wang. 2014. The nuclear factor kappa B (NF-κB) activation is required for phagocytosis of staphylococcus aureus by RAW 264.7 cells. Exp. Cell Res. 327:256–263. doi:https://doi.org/10.1016/j.yexcr.2014.04.018.

FIGURES

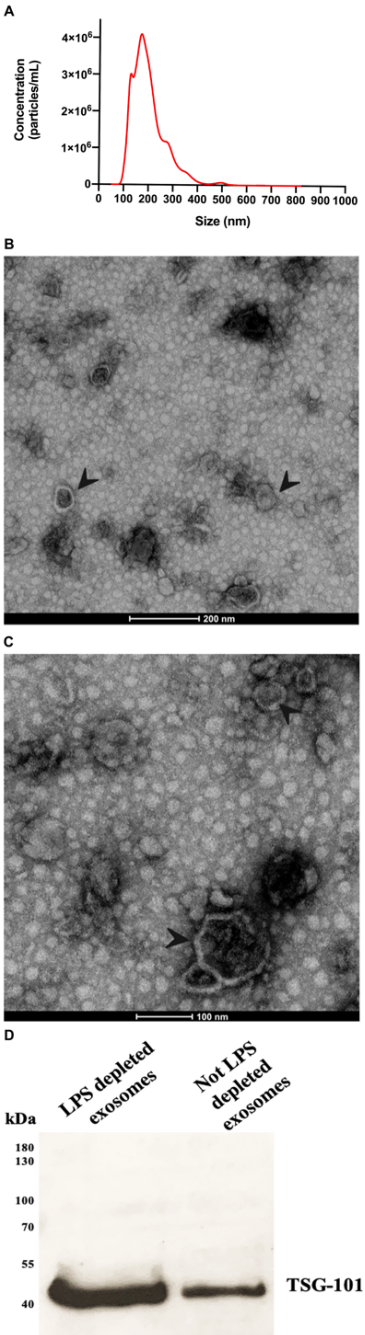


Figure 1. Characterization of LPS-depleted porcine milk exosomes purified by ultracentrifugation coupled with Size Exclusion Chromatography (SEC). (A) Nanoparticle Tracking Analysis (NTA) of LPS-depleted porcine milk exosomes. The plot of NTA analysis shows the size (nm) in the x-

axis and the concentration (particles/mL) in the y-axis. The size of the exosomes was 156 ± 8.9 nm. Transmission Electron Microscopy (TEM) micrographs showed that porcine milk exosomes have a characteristic (B) round and/or cup shape and are (C) limited by a lipid bilayer, indicated by the black arrows. Scale bars: 100 and 200 nm, respectively. (D) Representative western blotting analysis of the exosome marker TSG-101 in porcine milk exosomes. TSG-101 (44 kDa) was detected in both LPS-depleted (first lane) and non-LPS depleted porcine milk exosomes, confirming the isolation of milk exosomes.

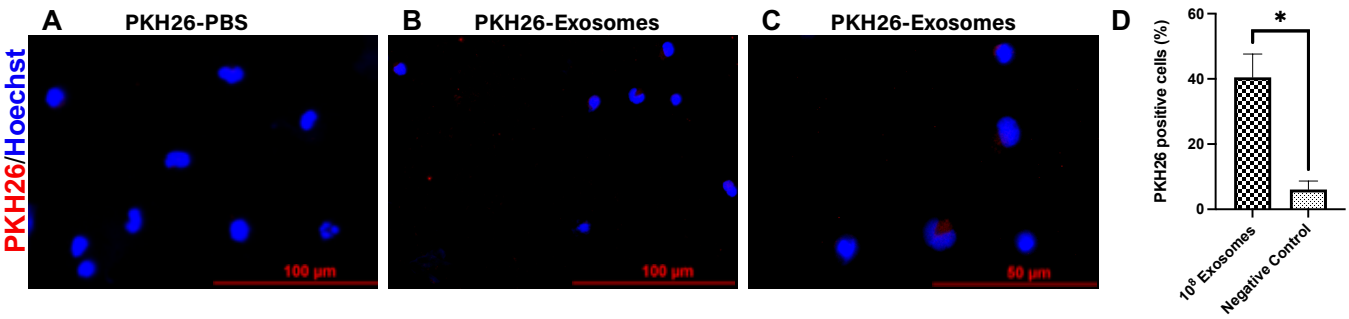


Figure 2. Uptake of LPS-depleted porcine milk exosomes by porcine monocytes in vitro. Cells were incubated with 10^8 of PKH26-labeled milk exosomes or PKH26-PBS (negative control) for 22 h, their nuclei stained with Hoechst and examined by fluorescence microscopy: red, PKH26-labeled exosomes; blue, nuclei. Fluorescence microscopy image of cells treated with (A) PKH26-PBS; (B, C) PKH26-labeled milk exosomes [(C) shows a higher magnification of (B)]; (D) Percentage of PKH26 positive cells that internalize PKH26-labeled exosomes. Data are means ± SEM of four experiments. Significance was declared at $P \leq 0.05$ (*). Scale bars: 100 and 50 μm, respectively.

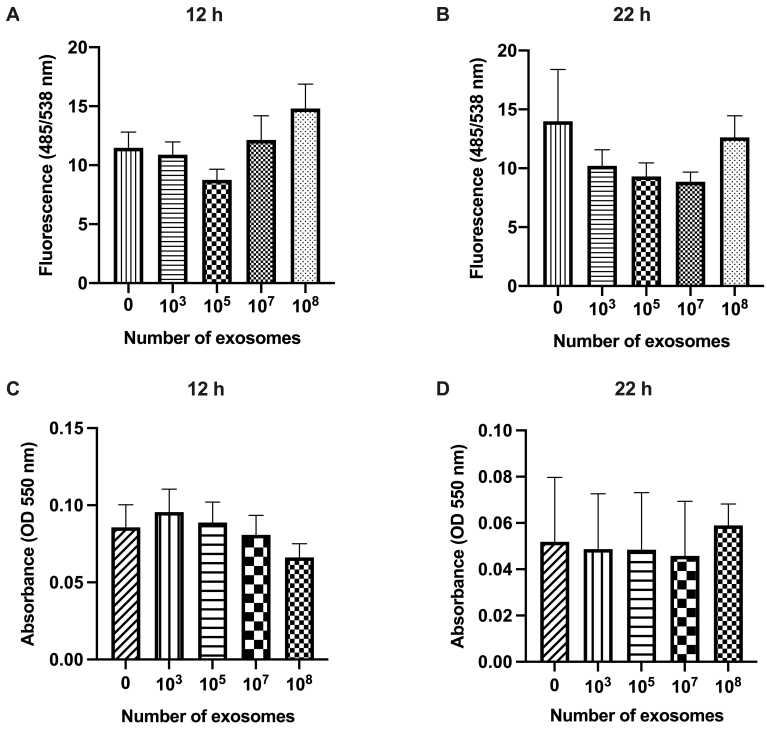


Figure 3. In vitro effect of LPS-depleted milk porcine exosomes on porcine monocytes' apoptosis and viability. Cells were treated with increasing numbers (10^3,10^5,10^7 and 10^8) of porcine milk exosomes or only medium as control (no exosomes), and apoptosis at (A) 12 h and (B) 22 h was measured. Cells' viability at (C) 12 h and (D) 22 h was also examined. Caspase-3/7 enzymatic activity and 2,5-diphenyl tetrazolium bromide (MTT) reduction by metabolically active cells were measured for apoptosis and viability, respectively. The results are expressed as fluorescence intensity (485/538 nm) for apoptosis and absorbance (OD 550 nm) for viability. Data are means ± SEM of five experiments. OD = optical density.

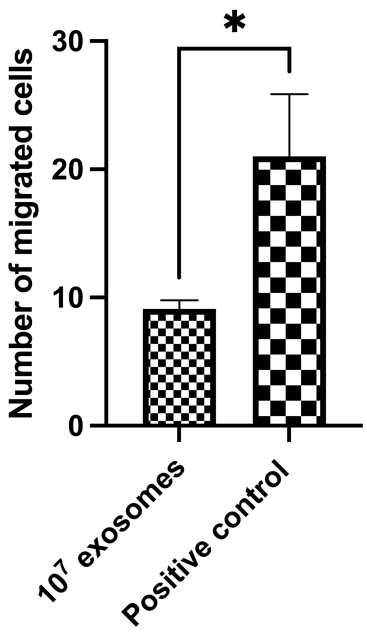


Figure 4. In vitro effects of LPS-depleted porcine milk exosomes on porcine monocytes chemotaxis. Milk exosomes (10^7) and medium (positive control) pre-treated cells were both activated with the chemoattractant Zymosan Activated Serum (ZAS) in the presence or absence of milk exosomes for 2 h. Data are means ± SEM of five experiments. Significance was declared for $P \leq 0.05$ (*).

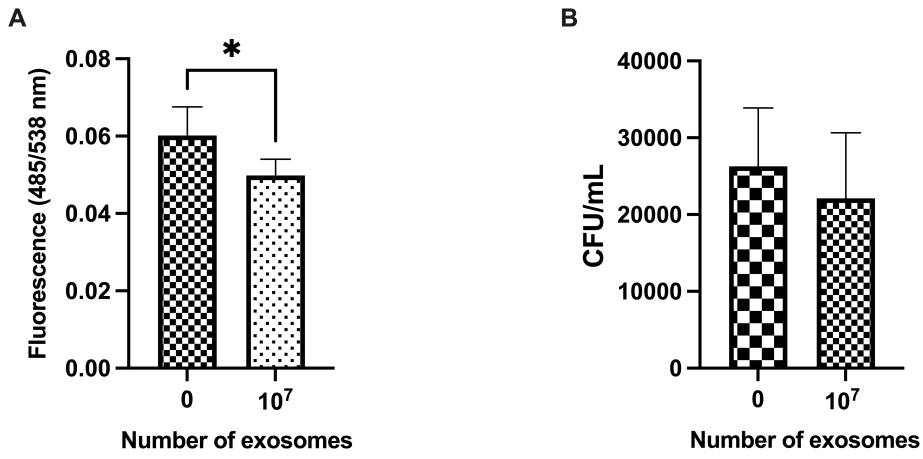


Figure 5. (A) Phagocytosis of fluorescein-labelled *Escherichia coli* bioparticles and (B) killing capacity of live *E. coli* by porcine monocytes after 22 h incubation with LPS-depleted porcine milk exosomes (10^7). Cells treated with only medium (no exosomes) were considered as control. The results are expressed as fluorescence intensity (485/538 nm) and as colony-forming units/mL (CFU/mL), respectively. Data are means ± SEM of seven and five experiments for phagocytosis and killing capacity, respectively. Significance was declared for $P \leq 0.05$ (*).

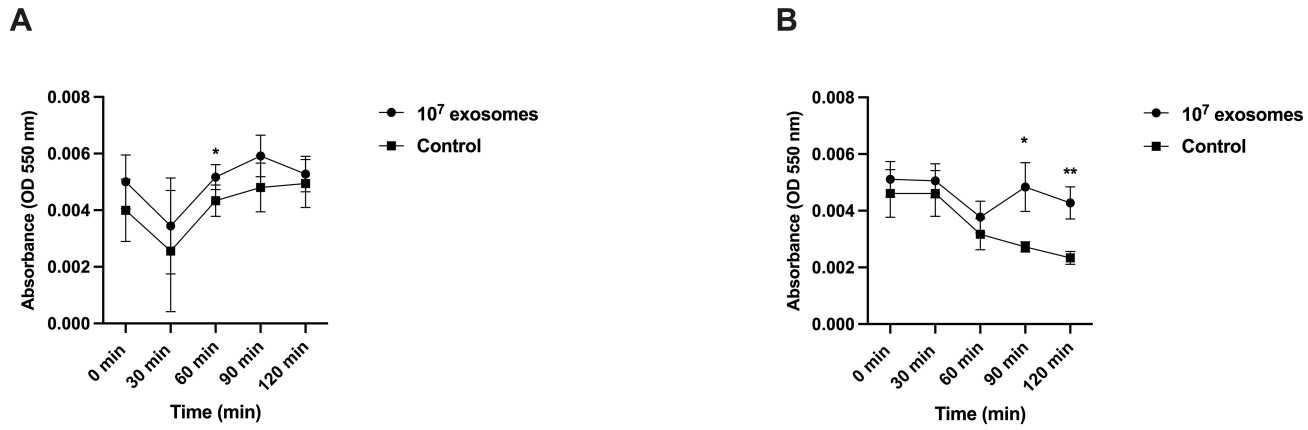


Figure 6. In vitro effects of LPS-depleted porcine milk exosomes on porcine monocytes' extracellular superoxide anion generation (O_2^-) after the addition of cytochrome C, under (A) resting conditions or (B) proinflammatory conditions [phorbol myristate acetate (PMA) challenge]. Cells treated with only medium (no exosomes) were considered as control. The results are expressed as absorbance values (OD 550 nm). Data are means ± SEM of six experiments. Significance was declared at $P \leq 0.05$ (*) and $P \leq 0.01$ (**). OD = optical density.

Manuscript draft 3: Immunomodulatory effects of long-chain n-3 polyunsaturated fatty acids (PUFA) on porcine monocytes (CD14+) immune response *in vitro*

Immunomodulatory effects of long-chain n-3 polyunsaturated fatty acids (PUFA) on porcine monocytes (CD14+) immune response *in vitro*

G. Ávila[a], S. Di Mauro[a†], J. Filipe[a†], Alessandro Agazzi[a], Marcello Comi[b], Cristina Lecchi[a], Fabrizio Ceciliani[a]*

[a]*Department of Veterinary Medicine and Animal Sciences, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy*
[b]*Department of Human Science and Quality of Life Promotion, Università Telematica San Raffaele, Via di Val Cannuta 247, 00166 Roma, Italy*

*Corresponding author: Gabriela Ávila Morales
† These authors have contributed equally to this work
E-mail address: gabriela.avila@unimi.it
Department of Veterinary Medicine, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy
Tel: 0039 02 50334531

ABSTRACT

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are omega-3 long-chain polyunsaturated fatty acids (n3-PUFAs) found mostly in fish oil. They have been commonly used as dietary integrators in human and animal nutrition, modulating the immune system, mostly by exerting anti-inflammatory activities as demonstrated by *in vivo* and *in vitro* studies. The precise mechanisms of action at the background of EPA and DHA immunomodulatory activity are still not fully elucidated. Moreover, no information on their effects on porcine monocytes immune response is available yet. To cover this gap, the study aimed to evaluate DHA and EPA's *in vit*ro impact on porcine monocytes (CD14+) defensive functions. Briefly, monocytes were isolated from the blood of twenty-six healthy pigs, using a magnetic-activated cell sorting technique (MACS). Monocytes were first treated with increasing concentrations of DHA and EPA (25, 50, 100 and 200 µM) and apoptosis and viability were measured to assess potential cytotoxic effects. Once determined EPA and DHA subtoxic working concentrations (25, 50 and 100 µM), their effects on

chemotaxis, phagocytosis and total, intracellular and extracellular reactive oxygen species (ROS) production were evaluated. DHA and EPA only decreased porcine monocytes viability at the highest concentration (200 μM), but not their apoptosis. DHA (100 μM) decreased the cells' chemotaxis, while EPA (25 μM) increased their intracellular ROS production after 60 minutes under non-inflammatory or resting conditions and at 90 minutes under pro-inflammatory conditions (PMA challenge). EPA (50 μM) decreased monocytes' intracellular ROS levels only under resting conditions at 30 minutes. No effects were observed on porcine monocytes phagocytic capacity. In conclusion, this study demonstrates that DHA and EPA can exert differential *in vitro* immunomodulatory effects in pigs, by dampening monocytes chemotaxis and potentiating their oxidative burst, respectively.

***Keywords**:* PUFA, pig, monocyte, chemotaxis, ROS production, anti-inflammatory

Abbreviations

PUFA, polyunsaturated fatty acids; MACS, magnetic-activated cell sorting; FA, fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; GPR120, G protein-coupled receptor 120; PPARγ, peroxisome proliferator-activated receptor γ; NFκB, nuclear factor κB; SPM, specialized pro-resolving lipid mediators; MTT, 3-(4,5- dimethyl thiazol -2-yl)-2,5-diphenyl tetrazolium bromide; ROS; reactive oxygen species; ZAS, zymosan activated serum; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HRP, horseradish peroxidase; DPI, diphenyleneiodonium chloride; O_2^-, superoxide anion; H_2O_2, hydrogen peroxide.

1. Introduction

Omega-3 polyunsaturated fatty acids (n3-PUFAs) are fatty acids (FA) that naturally contain more than one double bond in their structure (De Caterina, 2011). They are divided into: 1) short chain n3-PUFAs: α-linolenic acid (ALA), and 2) the longer-chain n3-PUFAs: eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) (Calder, 2013). Long-chain n3-PUFAs are mostly abundant in, seaweeds and other fish oil supplements, being the diet their main source (Calder, 2015). EPA and DHA have been long used as dietary fatty acids in both human and animal nutrition, as they have been demonstrated to have beneficial properties,

improving their antioxidant defense mechanisms, growth, fertility, health and immunity (Calder, 2007; Fu et al., 2021; Lee et al., 2019).

As structural and functional components of the immune cells' membrane phospholipids, EPA and DHA are known to mainly alter immune cell functions by changing the cells' membrane FA composition after their incorporation, thus affecting the membrane's fluidity, lipid raft formation, cell signaling and consequent gene expression (Gutiérrez et al., 2019). Their immunomodulatory and anti-inflammatory actions can be also attributed to other mechanisms such as: inhibition of arachidonic acid (ARA) metabolism, direct interaction with the cell surface and intracellular receptors, G protein-coupled receptor 120 (GPR120) and the anti-inflammatory peroxisome proliferator-activated receptor γ (PPARγ), respectively. The inhibition of ARA metabolism and direct modulation of PPARγ signaling pathway have been associated with reduced production of pro-inflammatory eicosanoids and expression inflammatory genes (Al-Khalaifah, 2020; Calder, 2017). Moreover, EPA and DHA are known substrates for anti-inflammatory and specialized pro-resolving lipid mediators (SPM) such as resolvins, protectins, lipoxins and meresins that mediate the resolution (turn off) of the inflammation (Chiang and Serhan, 2020). The defensive actions of immune cells can be affected by EPA and DHA *in vitro* and *in vivo, including* the polarization of murine macrophages towards an anti-inflammatory M2 phenotype (Chang et al., 2015), the immunomodulation of goat monocyte and neutrophil phagocytosis and ROS production (Lecchi et al., 2011; Pisani et al., 2009). Finally, the increase of EPA and DHA in human diets also reduced monocytes and neutrophils chemotaxis *ex vivo* (Schmidt et al., 1992).

In pigs, the effects of n-3 PUFA coming from fish oil have been shown to influence their gut health and immunity, being of special importance for transition periods such as postweaning, where the piglets are more susceptible to inflammation (Lauridsen, 2020). A low dietary ω6:ω3 ratio (4:1) during gestation and lactation has beneficial effects for weaning survival rate, weight gain, and ω3 enrichment in colostrum and milk (Nguyen et al., 2020). Additionally, EPA and DHA caused *in vitro* cytoprotective and proliferative effects on porcine enterocytes (IPEC-J2) submitted to different biological and chemical stresses (LPS and H_2O_2) (Sundaram et al., 2020). These findings confirmed the potential of EPA and DHA in maintaining pigs' gut health, integrity and immunity, especially under pro-inflammatory conditions. Lastly, dietary fish oil supplementation in weaned piglets also modulated the inflammatory responses of porcine alveolar macrophages *ex vivo*, with a negative correlation between the n-3 PUFA content and PGE_2, TNF-α and IL-8 concentrations,

which lead to a decreased production of pro-inflammatory cytokines and eicosanoids (Møller and Lauridsen, 2006).

Even though previous studies have already demonstrated the immunomodulatory effects of EPA and DHA on pigs, they have mainly focused on assessing their effects on the production of inflammatory cytokines, eicosanoids, and gut health and integrity. In addition, the exact mechanisms underlying the direct effects of EPA and DHA on porcine immune cells' response have not been yet fully elucidated, and to the best of our knowledge, no studies of their *in vitro* impact on porcine monocytes - the main effectors immune cells - are available. Therefore, in this study, we aimed at covering this gap by investigating the *in vitro* effects that DHA and EPA may exert on porcine monocytes defensive functions, including chemotaxis, phagocytosis and oxidative burst. Their effects on the cells' viability and apoptosis were also evaluated.

2. Materials and methods

2.1. Materials

Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), EDTA 0.5 M, red blood cell lysis buffer, and sterile-filtered Dulbecco's PBS without calcium and magnesium (Sigma-Aldrich, St. Louis, MO, USA) were used for porcine PBMC isolation. For monocytes purification CD14 MicroBeads, Large Size (LS) columns, 30-mm pre-separation filters (Miltenyi-Biotech, Bergisch Gladbach, Germany) and 0.5% BSA (Sigma-Aldrich) were used. Once isolated, the cells were resuspended in complete medium RPMI 1640 with 25mM HEPES and L-glutamine complemented with 1% nonessential amino acid solution 100× and 1% penicillin-streptomycin solution 100×, and 10% FBS (Sigma-Aldrich). DHA (cis-4,7,10,13,16,19-Docosahexaenoic acid sodium salt) and EPA (cis-5,8,11,14,17-Eicosapentaenoic acid sodium salt) used for stimulating the cells were purchased from Sigma-Aldrich. Zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich), BioParticles® *E. coli* Fluorescent Particles (Invitrogen) were used for chemotaxis and phagocytosis assay, respectively. For the ROS production assays, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), luminol, 4-Aminophthalhydrazide (isoluminol), horseradish peroxidase (HRP), diphenyleneiodonium chloride (DPI) and PMA were purchased from Sigma-Aldrich.

2.2.PUFA preparation

DHA (14.3 mM) and EPA (15.4 mM) stock solutions were prepared by adding 1 mL of endotoxin-free water (Sigma-Aldrich). The stock solutions were then filtered with 0.22 µm filters (Millipore Corporation, Billerica MA, WA, USA), aliquoted and stored in the dark at -80 °C until use. Immediately before their use, EPA and DHA working dilutions were prepared with a complete medium. Increasing concentrations of DHA and EPA (0, 25, 50, 100 and 200 µM) were first used for determining their working concentration, as previously described for caprine monocytes (Lecchi et al., 2011). The subtoxic concentrations of 25, 50 and 100 µM were selected to perform the further experiments.

2.3. Purification of porcine monocytes (CD14+) from blood

Peripheral blood (100 mL) from twenty-six 60-100 kg healthy pigs (TOPIGS) was collected during routine slaughtering procedures in sterile flasks containing 0.2% of EDTA as an anticoagulant. PBMC were isolated first through Ficoll density gradient centrifugation, as described for bovine blood with some minor modifications (Ceciliani et al., 2007). Briefly, blood was first centrifuged at 1260 g for 30 min at 18 °C to collect the buffy coat. The collected buffy coat was diluted 1:5 in cold sterile-filtered PBS without Ca^{2+} and Mg^{2+} + 2 mM EDTA and carefully layered onto 3 mL of Ficoll-Paque Plus (1.077g/mL). A second centrifugation step was performed at 1700 g (without brakes) for 30 min at 4 °C to obtain the PBMC ring. The PBMC were collected at the interface, washed with cold sterile PBS without Ca^{2+} and Mg^{2+} + 2 mM EDTA and centrifuged at 500 g for 7 min at 4 °C. To remove the red blood cells, Red Blood Cell Lysis buffer was added to the monocytes and then incubated for 3 min at room temperature. Washes with cold sterile PBS + 2 mM EDTA were performed to remove contaminant platelets. Monocytes (CD14+) were further purified from PBMC through magnetic-activated cell sorting technique (MACS), as previously described for bovine samples (Ávila et al., 2020), following the manufacturer's instructions. Monocytes were counted and their viability assessed through Trypan blue exclusion (>90%), using an automatic cell counter (TC20^{TM}, BioRad). Finally, monocytes were resuspended in a complete medium at the desired concentration for each assay.

2.4.Viability assay

To assess potential cytotoxic effects of DHA and EPA and to select the subtoxic working concentrations, the cell proliferation kit I (MTT) (Roche Diagnostics GmbH, Mannheim,

Germany) was used, following the manufacturer's instructions and as previously described with bovine monocytes (Catozzi et al., 2020). Briefly, monocytes ($1x10^5$) from 7 different animals were seeded in duplicates in clear sterile 96-well plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and incubated with increasing concentrations of DHA and EPA (25, 50, 100 and 200 μM) or with only medium (0 μM) as a control for 22 h at 39 °C + 5% CO_2. After the incubation time, the MTT labelling reagent (10 μL) was added and the cells were incubated for 4 hours at 39°C + 5% CO_2. To solubilize the produced formazan crystals, 100 μL of solubilization buffer was added and the plates were incubated overnight at 39 °C + 5% CO_2. The absorbance was measured with a LabSystems Multiskan plate reader spectrophotometer (LabX, Midland, Canada) at 550 nm.

2.5.Apoptosis assay

To evaluate the effects of DHA and EPA on porcine monocytes apoptosis, the enzymatic activity of Caspase-3/7 was measured, as previously described for bovine monocytes with minor modifications (Ceciliani et al., 2007). Briefly, $5x10^4$ monocytes (12.5 μL) from 6 animals were seeded in duplicates in sterile black 384-well plates (Corning Inc., Costar, Kennebunk, ME, USA) and incubated with increasing concentrations (25, 50, 100 and 200 μM) of EPA and DHA (12.5 μL) or with only medium (0 μM) as a control for 22 hours at 39 °C + 5% CO_2. After the incubation period, the cells' apoptosis was measured using the Apo-ONE® Homogeneous Caspase-3/7 kit (Promega, Madison, WI, USA), following the manufacturer's instructions. The fluorescence was then measured every 30 minutes for 2 hours with the Fluoroscan Ascent (Thermo Fisher Scientific, Vantaa, Finland) at 485/538 nm (absorbance/emission).

2.6.Chemotaxis assay

Monocytes chemotaxis towards ZAS was measured as previously reported (Ávila et al., 2020; Lecchi et al., 2008), with some minor changes. Firstly, $1x10^5$ monocytes (50 μL) from 6 animals were seeded in duplicates onto the semi-permeable membrane (8 μm pore size) of the upper chamber of a sterile 24-well Transwell migration plate (Corning Inc., Costar). The cells were then pre-treated, in the absence of ZAS, with 100 μM of DHA and EPA (50 μL in the upper chamber and 650 μL in the lower chamber) or only migration medium (RMPI-1640 with 1% of heat-inactivated FBS) as a control for 22 hours at 39 °C + 5% CO_2. After the incubation period, the

medium was removed from both chambers and again 100 μM of EPA and DHA or migration medium were added to both chambers. To measure the cells' chemotaxis, 3 mg/mL of ZAS were added only to the lower chamber of the plate in both, the PUFA and medium treated cells (positive control), and cells were incubated further for 2 hours at 39 °C + 5% CO_2. Cells incubated only with migration medium, but without ZAS were considered as the negative control. Finally, the non-migrated cells were removed from the upper membrane, and those that migrated were stained using Diff Quick Staining (Sigma-Aldrich) and counted using an inverted microscope at 40x. ZAS was prepared using porcine serum, as previously described for bovine monocytes (Lecchi et al., 2008).

2.7.Phagocytosis assay

Monocytes' phagocytic activity was determined as previously described (Lecchi et al., 2011). Briefly, a total of $3x10^5$ monocytes (100 μl) from 7 different animals were seeded in duplicates in sterile 96-well plates (Becton Dickinson and Company). They were incubated with increasing concentrations (25, 50 and 100 μM) of EPA and DHA (100 μl) or with only medium (0 μM) as a control for 22 hours at 39 °C and 5% CO_2. After the incubation period, fluorescein-labelled *Escherichia coli* bioparticles (K-12 strain*)* were first opsonized with 20% non-decomplemented pig serum at 39 °C for 30 minutes. Cells were then washed with sterile PBS and 100 μl of opsonized bioparticles/cell (45 bioparticles/cell) were added to the wells and cells were incubated for 2 hours at 39 °C and 5% CO_2. Non-internalized bioparticles were then removed by washing the cells with PBS and, their fluorescence was further quenched by incubating the monocytes with 50 μl of 0.4% trypan blue for 1 minute at room temperature. Trypan blue was then washed, and PBS (100 μl) was added to all the wells. Finally, the fluorescence was measured using the microplate reader Fluoroscan Ascent FL (Thermo Fisher Scientific) at 485/538 nm (absorbance/emission).

2.8. Total, intracellular and extracellular ROS production assay

The production of intracellular, total and extracellular - superoxide anion (O_2-) ROS were determined under both, resting and pro-inflammatory conditions (PMA challenge), with H_2DCFDA, luminol and isoluminol in the presence of HRP, as previously described for bovine polymorphonuclear cells (PMN) with some minor modifications (Grob et al., 2020; Rinaldi et al.,

2007; Wang et al., 2020). Briefly, a total of $1x10^5$ monocytes (100 µL) from 6 animals was seeded in complete medium without phenol red in duplicates in sterile black (Corning Inc., Costar) or white 96-well plates (Nunclon Delta Surface, Thermo Fisher Scientific) for intracellular; and total and extracellular ROS assays, respectively. Cells were then co-incubated with increasing concentrations of DHA and EPA (25, 50 and 100 µM) or medium as control (0 µM) for 22 h at 39 °C + 5% CO_2.

For the intracellular ROS evaluation, after the incubation period, cells were washed twice with PBS at room temperature to remove the FBS, as it can interfere with the activation of the fluorescent ROS indicator probe, H_2DCFDA. To load the fluorescent probe into the cells, 200 µL of H_2DCFDA (10 µM final concentration) or PBS alone (negative control) were added and cells were incubated for 20 min at 39 °C + 5% CO_2. The loading buffer was then removed and 200 µL of medium without phenol red and FBS or 200 µL of PMA (2.5 µg/mL of final concentration) were added to the cells to recreate the non-inflammatory (resting) and pro-inflammatory conditions, respectively. Fluorescence intensity was measured immediately (0 min) and then every 30 min for 2 h, using a fluorescence plate reader Fluoroscan Ascent at 485/538 nm (absorbance/emission).

For the total ROS and extracellular O_2^- production determination, 50 µL of luminol (500 µM final concentration) or isoluminol (100 µM final concentration) + 4 U/mL HRP were added to the cells to reach a final volume of 200 µL, respectively. To mimic the pro-inflammatory conditions, PMA (2.5 µg/mL final concentration; Sigma-Aldrich) was also added to the cells. The effect of the ROS inhibitor, DPI – a NADPH oxidase inhibitor - on monocytes total and extracellular ROS generation was also assessed, so monocytes treated with only medium (no PUFA) were first treated with 8 µM of DPI for 10 min at 39 °C + 5% CO_2 and then luminol and isoluminol + HRP, with or without PMA, were added as mentioned above. The chemiluminescence was then immediately measured (0 min) and then every 30 min for 2 h using a plate reader luminometer (Glomax 96 Microplate Luminometer, Promega). Data for total and extracellular ROS assays are presented as relative chemiluminescence units (RLU).

2.9.Statistical analyses

Statistical analyses were performed in GraphPad Prism 9.1.2 for Mac OS X, GraphPad Software (San Diego, California, USA). Data normality was assessed by applying the Shapiro-Wilk test.

For normally distributed data, a repeated measures one-way ANOVA followed by Tukey's multiple comparison tests was used for all the assays; while for not normally distributed data in the ROS production assays a Friedman test followed by Dunn's multiple comparisons test was applied. Specifically, for ROS production assays a repeated measures one-way ANOVA or Friedman test was applied to assess exclusively the effect of the different treatments (DHA and EPA concentrations and control) within each time point (0, 30, 60, 90 and 120 min). Statistical differences were accepted at $P \leq 0.05$.

3. Results

3.1. DHA and EPA affect porcine monocytes' viability at high concentrations

As the first step of this study, a preliminary experiment was performed to determine the subtoxic working concentrations of EPA and DHA. Only the highest concentration (200 μM) of DHA and EPA ($P = 0.01$) had cytotoxic effects on porcine monocytes, as a decrease in the cells' viability was observed when compared to the control (Fig. 1A, B). Porcine monocytes viability was also decreased at 200 μM of DHA when compared to 100 μM DHA, 50 μM DHA, and 25 μM DHA ($P = 0.01$); and 200 μM of EPA when compared to EPA 100 μM EPA, 50 μM EPA ($P = 0.01$), and 25 μM EPA ($P = 0.02$).

Despite a decrease in the cell viability was observed at 200 μM of DHA and EPA, no effects on the apoptosis of porcine monocytes were observed when treated with the increasing concentrations of DHA (Fig. 1C) nor EPA (Fig. 1D).

3.2.DHA modulates porcine monocytes' chemotaxis

Porcine monocytes' chemotactic activity towards the chemoattractant, ZAS, was measured after pre-treating the cells with either 100 μM of DHA or EPA or medium (positive control) for 22 h. DHA suppressed ($P = 0.05$) the chemotaxis of porcine monocytes, as the number of migrated cells was lower when compared to the positive control without PUFA (Fig. 2A). No effects were observed with 100 μM of EPA (Fig. 2B). Finally, as expected positive control cells also presented a higher number of migrated cells ($P = 0.01$) when compared to those not activated with ZAS (negative control), confirming the chemoattractant activity.

3.3.DHA and EPA don't have any effects on porcine monocytes' phagocytic capacity

To further study EPA and DHA's effects on porcine monocytes defensive response, porcine monocytes phagocytic capacity was measured, using the fluorescein-labelled *E. coli* bioparticles internalization assay. However, increasing concentrations (25, 50 and 100 μM) of both DHA (Fig. 3A) and EPA (Fig. 3B) did not modulate porcine monocytes phagocytosis when compared to the control (0 μM).

3.4.EPA increases porcine monocytes' intracellular ROS production

No effects with DHA on the cells' intracellular ROS production were observed under resting conditions (non-inflammatory) nor pro-inflammatory conditions (Fig. 4A, B) within each timepoint. On the contrary, cells treated with 25 μM of EPA showed an increase ($P = 0.05$) in their intracellular ROS production at 60, 90 and 120 minutes, under resting conditions, as compared to control (Fig. 4C), while 50 μM of EPA caused a decrease ($P = 0.03$) in the cells' ROS production at 30 minutes under resting conditions. Moreover, the ROS production under resting conditions was also increased with 25 μM of EPA at 60 ($P = 0.02$), 90 ($P = 0.03$) and 120 minutes ($P = 0.04$) when compared with 50 μM of EPA; and at 60 ($P = 0.03$), 90 ($P = 0.04$) and 120 minutes ($P = 0.03$), as compared to 100 μM of EPA (significance not shown in Fig. 4C).

Lastly, under pro-inflammatory conditions, only cells treated with 25 μM of EPA had an increase in their intracellular ROS production, but only at 90 minutes ($P = 0.01$), when compared to the control (Fig. 4D). Even though there is an increasing trend in the ROS production when compared to the control, no significant effects were observed with 25 μM of EPA at 120 min ($P = 0.06$).

3.5.Total and extracellular ROS Production is not affected by DHA and EPA

Firstly, under resting conditions, porcine monocytes did not produce detectable total ROS (Fig. 5A, C) when measured with the luminol chemiluminescence assay, as only negative values were obtained and similar to those observed in the cells treated with the ROS inhibitor (DPI) and the negative control (cells without luminol). In a same manner, the extracellular ROS (Fig. 6A, C), which were measured with the isoluminol + HRP chemiluminescence assay, were also not detectable during the first 60 minutes, but started to increase in the cells treated with 100 μM of DHA and 50, 25 μM of EPA and control (0 μM) at 90 and 120 min. EPA at 100 μM slightly increased the ROS production to detectable levels at 90 min, but it decreased again at 120 min. Under pro-inflammatory conditions, all cells treated with the increasing concentrations with DHA,

EPA or without PUFA (control) produced detectable total (Fig. 5B, D) and extracellular ROS (Fig. 6B, D) after being challenged with PMA, while for those treated with the negative control or DPI (ROS inhibitor) remained undetectable. Finally, none of the increasing concentrations (25, 50 and 100 μM) of DHA or EPA modulated porcine monocytes total (Fig. 5) nor extracellular ROS production (Fig. 6), neither under resting nor pro-inflammatory conditions when compared to control (0 μM).

4. Discussion

The immunomodulatory and anti-inflammatory effects of the n-3 PUFA, EPA and DHA, have been widely reported in both human and animal studies (Al-Khalaifah, 2020; Calder, 2017). However, no information on their *in vitro* effects on porcine monocytes defensive functions is available yet. We present in this study for the first time the capacity of DHA and EPA in modulating two main porcine monocytes' inflammatory functions, namely chemotaxis and intracellular ROS production. Our main findings were that the cells' chemotaxis was suppressed by DHA, and their intracellular ROS production was increased by EPA under both, resting and pro-inflammatory conditions.

In the first part of the study, to examine if EPA and DHA had any potential cytotoxic effects on porcine monocytes, and subsequently, to determine their subtoxic working concentrations, we evaluated their apoptosis and viability. Both DHA and EPA decreased porcine monocytes' viability, but only when using them at the highest concentration (200 μM). Interestingly, despite the clear reduction in the viability of the cells - determined by measuring their metabolic activity - at this concentration, the apoptosis of the cells remained unchanged. These results suggest, that EPA and DHA cytotoxic effects might not be induced through apoptosis, but possibly by other cell death mechanisms such as necrosis. Similarly, in a previous study it was observed that the n-3 PUFA ALA also caused an increase in the percentage of dead prostate cancer cells, but did not affected their apoptosis (Eser O. et al., 2013). In the same study EPA did induce the cells' apoptosis, but the percentage of dead cells still exceeded that of apoptotic cells, indicating that the cell death observed after EPA treatment was not all attributed to apoptosis. Moreover, EPA and DHA have indeed been shown to increase the necrosis in human cancer cell lines (Chiu and Wan, 1999; Finstad et al., 1994); and both apoptosis and necrosis in macrophage cell line, but necrosis in a greater extent (Martins de Lima et al., 2006). These results should be considered with caution

as they were reported in cancer and immune cell lines, which their response greatly defer to that of healthy primary cells, but no information on primary immune cells is available so far. However, our results are consistent with previous studies on caprine monocytes, where also 200 μM of both PUFA decreased the viability of the cells (Lecchi et al., 2011), and on human mononuclear cells in which concentrations higher than 150 μM caused the same effect (Jaudszus et al., 2013).

For all the following experiments, only the lower concentrations of DHA and EPA (25, 50 and 100 μM) were used, as no cytotoxic effects were observed in our and others previous experiments *in vitro* at similar concentrations (Lecchi et al., 2013, 2011; Zhao et al., 2005).

The effects of DHA and EPA were examined on three of the main defensive functions of porcine monocytes, including chemotaxis, phagocytosis and ROS production. Only DHA (100 μM) was able to reduce porcine monocytes chemotaxis towards ZAS. The ability of DHA and EPA in suppressing immune cells migration has been widely documented in other models such as in human neutrophils and monocytes (Schmidt et al., 1992), being indeed one of the key anti-inflammatory and pro-resolving effects of these long-chain n-3 PUFAs (Calder, 2017). Our study confirmed this effect in porcine monocytes. The exact mechanisms by which DHA and EPA inhibit chemotaxis are not fully clear yet, but this inhibition has been attributed to down-regulated expression of receptors for chemoattractants, reduced production of some chemoattractants (e.g. LTB_4) and down-regulated expression of adhesion molecule genes, via NFκB, PPAR-γ and GPR120 (Calder, 2015). Additionally, SPM have been shown to inhibit the transendothelial migration of human neutrophils (Serhan et al., 2000), which could be caused by the reduction in actin polymerization (Krishnamoorthy et al., 2010), and of monocytes and macrophages as reviewed elsewhere (Balta et al., 2021). Lastly, reduction in neutrophils' cell membrane fluidity after EPA incorporation, was also proposed to be one of the reasons for the suppression of the cells' chemotaxis (Sipka et al., 1996).

In our experiment the porcine monocytes' phagocytic capacity was on the contrary not affected neither by DHA nor EPA at any of the concentrations tested (25, 50 and 100 μM). These results differ from those observed in murine macrophages, and caprine monocytes and neutrophils, where an increase in their capacity to engulf zymosan and apoptotic cells (Chang et al., 2015), and *E.coli* were observed, respectively (Lecchi et al., 2011; Pisani et al., 2009). However, the current information on the effects of DHA and EPA on phagocytosis is limited, and contradictory, as other

in vivo and *in vitro* studies reported no effects at all (Al-Khalaifah, 2020; Rees et al., 2006), or a downregulating effect on phagocytosis (Sipka et al., 1996).

In the last set of experiments, we assessed the effects of increasing concentrations of DHA and EPA on porcine monocytes' overall capacity to generate ROS measuring the intracellular, extracellular and total ROS levels, under both resting and pro-inflammatory conditions (PMA-challenge). Our findings show that only EPA at the lowest concentration (25 μM) was able to increase the production of intracellular ROS under both resting and pro-inflammatory conditions at different time points. These findings suggest that EPA might play an important role in enhancing the monocytes' immune response and support the idea that PUFAs should be considered a substantial source of ROS (Schönfeld and Wojtczak, 2008). However, it should be also noted that EPA was able to decrease the cells intracellular ROS production only under resting conditions and at an intermediate concentration (50 μM) at 30 minutes. Our results agree with those previously reported in rat neutrophils, where EPA caused a greater effect, by increasing the *in vitro* production of intracellular (H_2O_2) and extracellular ROS (H_2O_2 and O_2^-) at similar concentrations (12.5 - 150 μM), under resting and pro-inflammatory conditions (Paschoal et al., 2013). Similarly, in LPS-stimulated murine macrophages EPA (10 and 100 μM) increased the total ROS production after the PMA challenge (Ambrozova et al., 2010). Nevertheless, in our study, no impact on the extracellular O_2^- nor the total ROS levels was detected, as determined with the isoluminol and luminol-dependent chemiluminescence assays, respectively: this could be mainly due to the different methods of ROS quantification used in the studies and the different cell types and activation states. The lack of significant results observed is consistent with what was found in goat monocytes, where DHA and EPA also didn't modulate their extracellular O_2^- production (Lecchi et al., 2011). On the contrary, in caprine neutrophils only DHA induced a down-regulation in extracellular O_2^- production *in vitro* (Pisani et al., 2009), suggesting that these two n-3 PUFA exert differential immunomodulatory effects that might also vary between species and cell types. Moreover, dual effects of DHA and EPA in modulating extracellular O_2^- production in the mitochondria have already been reported (Schönfeld and Wojtczak, 2008). The exact mechanisms underlying these dual effects of EPA and DHA in immune cells ROS production are still not completely clarified, but it is thought they could up-regulate ROS production via NADPH oxidase (Paschoal et al., 2013), while a down-regulation could be due to a decreased transcription factor activation (Gutiérrez et al., 2019).

5. Conclusions

In conclusion, the results of this study demonstrate for the first time that EPA and DHA modulate different immune functions of porcine monocytes *in vitro*. Specifically, DHA decreased porcine monocytes' chemotaxis, while EPA mainly increased their intracellular ROS production under resting (non-inflammatory) and pro-inflammatory conditions (PMA challenge). This suggests that long-chain n-3 PUFA can exert anti-inflammatory, inflammation resolving, and/or immune-enhancing effects in pigs. Therefore, our results further support the hypothesis that EPA and DHA do not act only as unspecific immune repressors, as they could boost the immune response of the animals, especially when at risk of infections and still be able to counteract inflammation through anti-inflammatory and pro-resolving mechanisms. This immunomodulatory potential makes the supplementation of EPA and DHA in pigs' diets a valuable option to be included in nutrition strategies to improve the animals' overall health and immune status. However, our study does not provide evidence on the exact molecular mechanisms underlying such effects, and their elucidation using a system biology approach, including transcriptomic and proteomic analyses, could help us to better understand the biological significance in *in vivo* systems.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper's content.

Ethics statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The procedures for the blood collection were carried out during routine slaughtering procedures.

Acknowledgements

We acknowledge the support of Nicoletta Rizzi of the Direzione Servizi per la Ricerca, Centro Zootecnico Sperimentale at the Università Degli Studi di Milano, Italy, for her valuable support in the set-up of the luminol and isoluminol chemiluminescence assays for the ROS production measurement. We also thank Alba Martín González of the Departament of Nutrition and

Production of Herbivores at the Instituto de Ganadería de Montaña, CSIC-Universidad de León, Spain, for her help in the *in vitro* ROS production assays.

Funding

This study was supported by the European Union's Horizon 2020 research and innovation programme H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate) [Grant agreement nº: 765423, 2017] – MANNA.

References

Al-Khalaifah, H., 2020. Modulatory Effect of Dietary Polyunsaturated Fatty Acids on Immunity, Represented by Phagocytic Activity. Front. Vet. Sci. 7, 569939. https://doi.org/10.3389/fvets.2020.569939

Ambrozova, G., Pekarova, M., Lojek, A., 2010. Effect of polyunsaturated fatty acids on the reactive oxygen and nitrogen species production by raw 264.7 macrophages. Eur. J. Nutr. 49, 133–139. https://doi.org/10.1007/s00394-009-0057-3

Ávila, G., Catozzi, C., Pravettoni, D., Sala, G., Martino, P., Meroni, G., Lecchi, C., Ceciliani, F., 2020. In vitro effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes. J. Dairy Sci. 103, 8554–8563. https://doi.org/10.3168/jds.2020-18659

Balta, M.G., Papathanasiou, E., Christopoulos, P.F., 2021. Specialized Pro-Resolving Mediators as Potential Regulators of Inflammatory Macrophage Responses in COVID-19 . Front. Immunol. .

Calder, P.C., 2017. Omega-3 fatty acids and inflammatory processes: from molecules to man. Biochem. Soc. Trans. 45, 1105–1115. https://doi.org/10.1042/BST20160474

Calder, P.C., 2015. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1851, 469–484. https://doi.org/https://doi.org/10.1016/j.bbalip.2014.08.010

Calder, P.C., 2013. Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? Br. J. Clin. Pharmacol. 75, 645–662. https://doi.org/10.1111/j.1365-2125.2012.04374.x

Calder, P.C., 2007. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot. Essent. Fat. Acids 77, 327–335. https://doi.org/10.1016/j.plefa.2007.10.015

Catozzi, C., Ávila, G., Zamarian, V., Pravettoni, D., Sala, G., Ceciliani, F., Lacetera, N., Lecchi, C., 2020. In-vitro effect of heat stress on bovine monocytes lifespan and polarization. Immunobiology 225, 151888. https://doi.org/https://doi.org/10.1016/j.imbio.2019.11.023

Ceciliani, F., Pocacqua, V., Miranda-Ribera, A., Bronzo, V., Lecchi, C., Sartorelli, P., 2007. α1-Acid glycoprotein modulates apoptosis in bovine monocytes. Vet. Immunol. Immunopathol. 116, 145–152. https://doi.org/10.1016/j.vetimm.2007.01.006

Chang, H.Y., Lee, H.-N., Kim, W., Surh, Y.-J., 2015. Docosahexaenoic acid induces M2 macrophage polarization through peroxisome proliferator-activated receptor γ activation. Life Sci. 120, 39–47. https://doi.org/https://doi.org/10.1016/j.lfs.2014.10.014

Chiang, N., Serhan, C.N., 2020. Specialized pro-resolving mediator network: an update on production and actions. Essays Biochem. 64, 443–462. https://doi.org/10.1042/EBC20200018

Chiu, L.C.M., Wan, J.M.F., 1999. Induction of apoptosis in HL-60 cells by eicosapentaenoic acid (EPA) is associated with downregulation of bcl-2 expression. Cancer Lett. 145, 17–27. https://doi.org/https://doi.org/10.1016/S0304-3835(99)00224-4

De Caterina, R., 2011. n–3 Fatty Acids in Cardiovascular Disease. N. Engl. J. Med. 364, 2439–2450. https://doi.org/10.1056/NEJMra1008153

Eser O., P., Vanden Heuvel P., J., Araujo, J., Thompson T., J., 2013. Marine- and plant-derived ω-3 fatty acids differentially regulate prostate cancer cell proliferation. Mol Clin Oncol 1, 444–452. https://doi.org/10.3892/mco.2013.76

Finstad, H.S., Kolset, S.O., Holme, J.A., Wiger, R., Farrants, A.-K.Ö., Blomhoff, R., Drevon, C.A., 1994. Effect of n-3 and n-6 Fatty Acids on Proliferation and Differentiation of Promyelocytic Leukemic HL-60 Cells. Blood 84, 3799–3809. https://doi.org/https://doi.org/10.1182/blood.V84.11.3799.bloodjournal84113799

Fu, Y., Wang, Y., Gao, H., Li, D., Jiang, R., Ge, L., Tong, C., Xu, K., 2021. Associations among Dietary Omega-3 Polyunsaturated Fatty Acids, the Gut Microbiota, and Intestinal Immunity. Mediators Inflamm. 2021, 8879227. https://doi.org/10.1155/2021/8879227

Grob, D., Conejeros, I., Velásquez, Z.D., Preußer, C., Gärtner, U., Alarcón, P., Burgos, R.A., Hermosilla, C., Taubert, A., 2020. Trypanosoma brucei brucei Induces Polymorphonuclear Neutrophil Activation and Neutrophil Extracellular Traps Release . Front. Immunol. .

Gutiérrez, S., Svahn, S.L., Johansson, M.E., 2019. Effects of omega-3 fatty acids on immune cells.

Int. J. Mol. Sci. 20, 5028.

Jaudszus, A., Gruen, M., Watzl, B., Ness, C., Roth, A., Lochner, A., Barz, D., Gabriel, H., Rothe, M., Jahreis, G., 2013. Evaluation of suppressive and pro-resolving effects of EPA and DHA in human primary monocytes and T-helper cells[S]. J. Lipid Res. 54, 923–935. https://doi.org/https://doi.org/10.1194/jlr.P031260

Krishnamoorthy, S., Recchiuti, A., Chiang, N., Yacoubian, S., Lee, C.-H., Yang, R., Petasis, N.A., Serhan, C.N., 2010. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. Proc. Natl. Acad. Sci. 107, 1660 LP – 1665. https://doi.org/10.1073/pnas.0907342107

Lauridsen, C., 2020. Effects of dietary fatty acids on gut health and function of pigs pre- and post-weaning. J. Anim. Sci. 98, skaa086. https://doi.org/10.1093/jas/skaa086

Lecchi, C., Ceciliani, F., Bernasconi, S., Franciosi, F., Bronzo, V., Sartorelli, P., 2008. Bovine alpha-1 acid glycoprotein can reduce the chemotaxis of bovine monocytes and modulate CD18 expression. Vet. Res. 39. https://doi.org/10.1051/vetres:2008027

Lecchi, C., Invernizzi, G., Agazzi, A., Ferroni, M., Pisani, L.F., Savoini, G., Ceciliani, F., 2011. In vitro modulation of caprine monocyte immune functions by ω-3 polyunsaturated fatty acids. Vet. J. 189, 353–355. https://doi.org/10.1016/j.tvjl.2010.09.001

Lee, S.A., Whenham, N., Bedford, M.R., 2019. Review on docosahexaenoic acid in poultry and swine nutrition: Consequence of enriched animal products on performance and health characteristics. Anim. Nutr. 5, 11–21. https://doi.org/https://doi.org/10.1016/j.aninu.2018.09.001

Martins de Lima, T., Cury-Boaventura, M.F., Giannocco, G., Nunes, M.T., Curi, R., 2006. Comparative toxicity of fatty acids on a macrophage cell line (J774). Clin. Sci. 111, 307–317. https://doi.org/10.1042/CS20060064

McClelland, S., Cox, C., O'Connor, R., de Gaetano, M., McCarthy, C., Cryan, L., Fitzgerald, D., Belton, O., 2010. Conjugated linoleic acid suppresses the migratory and inflammatory phenotype of the monocyte/macrophage cell. Atherosclerosis 211, 96–102. https://doi.org/10.1016/j.atherosclerosis.2010.02.003

Møller, S., Lauridsen, C., 2006. Dietary fatty acid composition rather than vitamin E supplementation influence ex vivo cytokine and eicosanoid response of porcine alveolar macrophages. Cytokine 35, 6–12. https://doi.org/https://doi.org/10.1016/j.cyto.2006.07.001

Nguyen, T.X., Agazzi, A., Comi, M., Bontempo, V., Guido, I., Panseri, S., Sauerwein, H., Eckersall, P.D., Burchmore, R., Savoini, G., 2020. Effects of Low ω6: ω3 Ratio in Sow Diet and Seaweed Supplement in Piglet Diet on Performance, Colostrum and Milk Fatty Acid Profiles, and Oxidative Status. Animals 10, 2049.

Paschoal, V.A., Vinolo, M.A.R., Crisma, A.R., Magdalon, J., Curi, R., 2013. Eicosapentaenoic (EPA) and Docosahexaenoic (DHA) Acid Differentially Modulate Rat Neutrophil Function In Vitro. Lipids 48, 93–103. https://doi.org/10.1007/s11745-012-3726-6

Pisani, L.F., Lecchi, C., Invernizzi, G., Sartorelli, P., Savoini, G., Ceciliani, F., 2009. In vitro modulatory effect of ω-3 polyunsaturated fatty acid (EPA and DHA) on phagocytosis and ROS production of goat neutrophils. Vet. Immunol. Immunopathol. 131, 79–85. https://doi.org/10.1016/j.vetimm.2009.03.018

Rees, D., Miles, E.A., Banerjee, T., Wells, S.J., Roynette, C.E., Wahle, K.W.J., Calder, P.C., 2006. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men. Am. J. Clin. Nutr. 83, 331–342. https://doi.org/10.1093/ajcn/83.2.331

Rinaldi, M., Moroni, P., Paape, M.J., Bannerman, D.D., 2007. Evaluation of assays for the measurement of bovine neutrophil reactive oxygen species. Vet. Immunol. Immunopathol. 115, 107–25. https://doi.org/10.1016/j.vetimm.2006.09.009

Schmidt, E.B., Varming, K., Pedersen, J.O., Lervang, H.-H., Grunnet, N., Jersild, C., Dyerberg, J., 1992. Long-term supplementation with n-3 fatty acids, II: effect on neutrophil and monocyte chemotaxis. Scand. J. Clin. Lab. Invest. 52, 229–236. https://doi.org/10.3109/00365519209088790

Schönfeld, P., Wojtczak, L., 2008. Fatty acids as modulators of the cellular production of reactive oxygen species. Free Radic. Biol. Med. 45, 231–241. https://doi.org/https://doi.org/10.1016/j.freeradbiomed.2008.04.029

Serhan, C.N., Clish, C.B., Brannon, J., Colgan, S.P., Chiang, N., Gronert, K., 2000. Novel Functional Sets of Lipid-Derived Mediators with Antiinflammatory Actions Generated from Omega-3 Fatty Acids via Cyclooxygenase 2–Nonsteroidal Antiinflammatory Drugs and Transcellular Processing. J. Exp. Med. 192, 1197–1204. https://doi.org/10.1084/jem.192.8.1197

Sipka, S., Dey, I., Buda, C., Csongor, J., Szegedi, G., Farkas, T., 1996. The Mechanism of

Inhibitory Effect of Eicosapentaenoic Acid on Phagocytic Activity and Chemotaxis of Human Neutrophil Granulocytes. Clin. Immunol. Immunopathol. 79, 224–228. https://doi.org/https://doi.org/10.1006/clin.1996.0072

Sundaram, T.S., Giromini, C., Rebucci, R., Baldi, A., 2020. Omega-3 polyunsaturated fatty acids counteract inflammatory and oxidative damage of non-transformed porcine enterocytes. Animals 10, 956.

Wang, J., Liu, Z., Han, Z., Wei, Z., Zhang, Y., Wang, K., Yang, Z., 2020. Fumonisin B1 triggers the formation of bovine neutrophil extracellular traps. Toxicol. Lett. 332, 140–145. https://doi.org/https://doi.org/10.1016/j.toxlet.2020.07.006

Figures

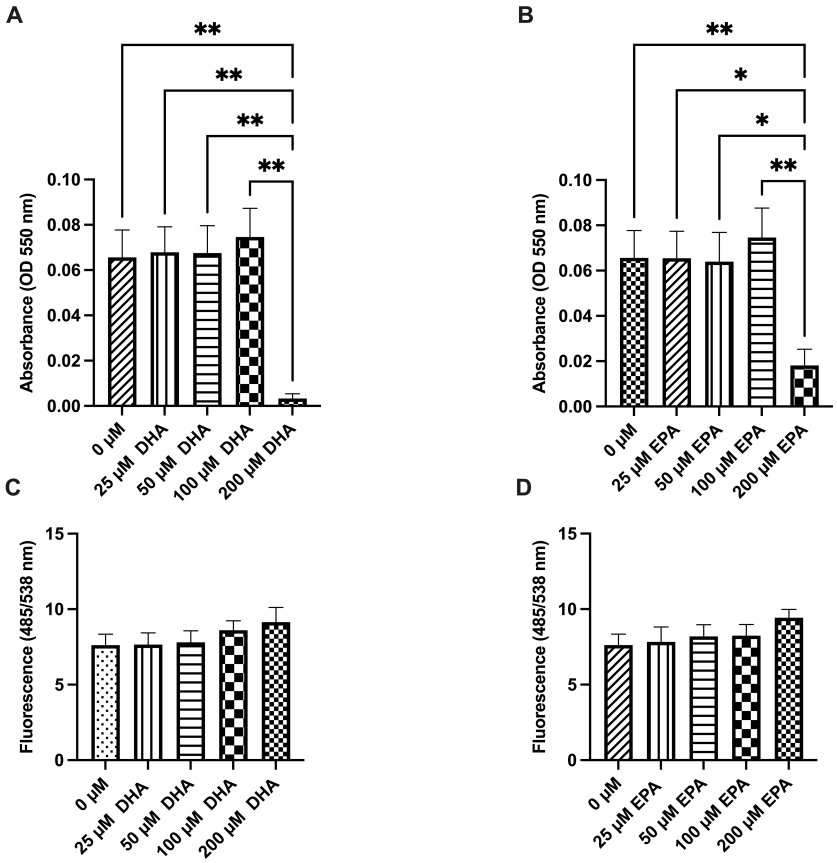


Fig. 1. *In vitro* effects of DHA and EPA on porcine monocytes' apoptosis and viability. Cells were treated with increasing concentrations (25, 50, 100 and 200 µM) of DHA and EPA or medium as control (0 µM) for 22 h. Cells' viability after DHA (A) and EPA (B) treatment was measured. The effects of DHA (C) and EPA (D) on porcine monocytes apoptosis were also examined. The 2,5-diphenyl tetrazolium bromide (MTT) reduction by metabolically active cells and the caspase-3/7 enzymatic activity were measured for viability and apoptosis, respectively. The results are expressed absorbance (OD 550 nm) for viability and fluorescence intensity (485/538 nm) for apoptosis. Data are means ± SEM of seven and six experiments for viability and apoptosis, respectively. OD = optical density. Significance was declared at P ≤0.05 (*) and P ≤ 0.01 (**).

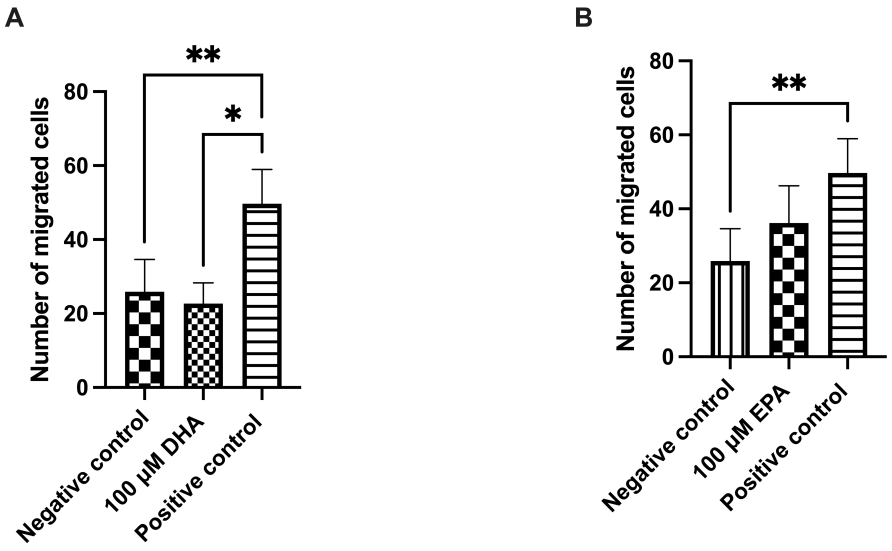


Fig. 2. *In vitro* impact of DHA and EPA on porcine monocytes chemotaxis. Cells pre-treated for 22 h with 100 µM of DHA (A) or EPA (B) and medium (positive control) were activated with the chemoattractant Zymosan Activated Serum (ZAS) in the presence of DHA and EPA for 2 h. Cells pre-treated with only medium (no PUFA) that were not activated with ZAS were considered as a negative control. Data are means ± SEM of six experiments. Significance was declared at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

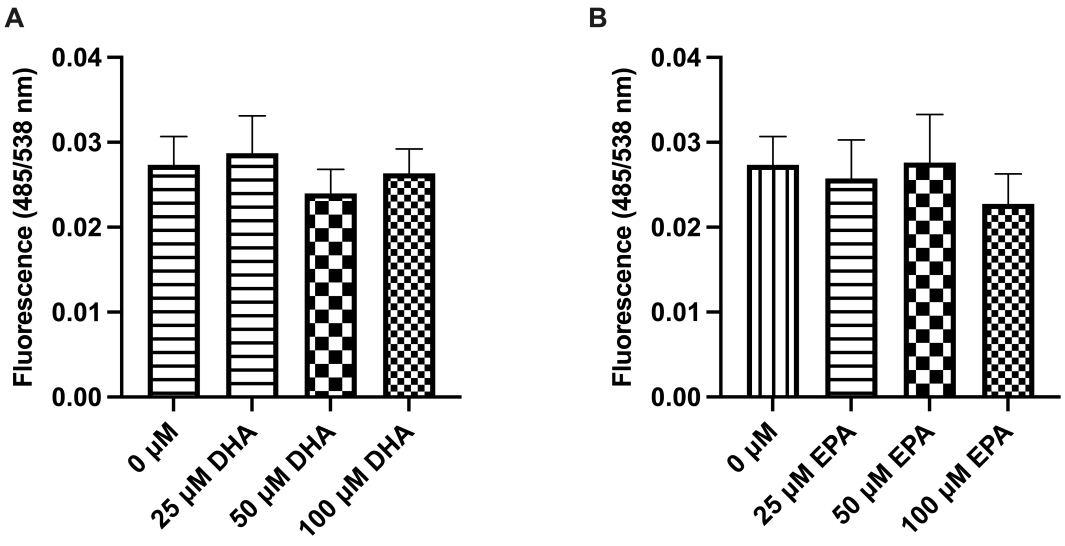


Fig. 3. Phagocytosis of fluorescein-labelled *Escherichia coli* bioparticles by porcine monocytes treated with DHA and EPA. Cells were pre-treated with increasing concentrations (25, 50 and 100 µM) of DHA (A) and EPA (B) or medium (0 µM) as a control for 22 h. The results are expressed as fluorescence intensity (485/538 nm). Data are means ± SEM of seven experiments.

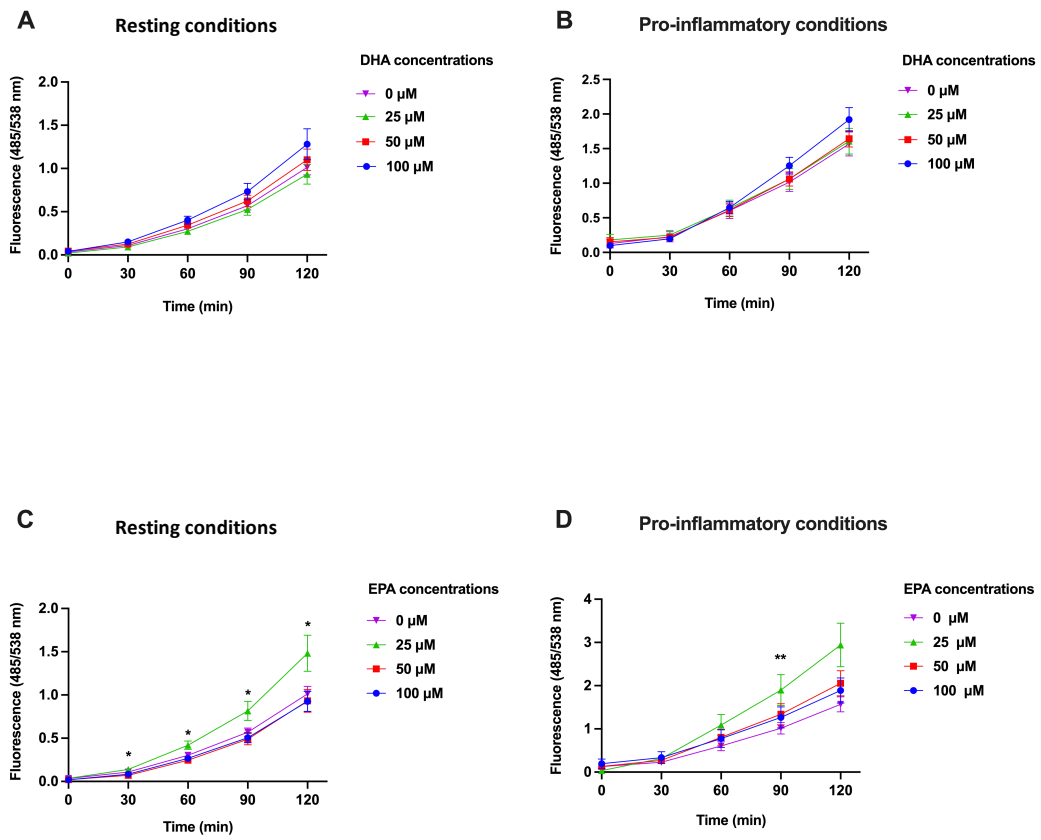


Fig. 4. *In vitro* effects of DHA and EPA on porcine monocytes' intracellular reactive oxygen species (ROS) generation. Cells were first pre-treated with increasing concentrations (25, 50 and 100 µM) of DHA and EPA or medium as control (0 µM) for 22 h. The intracellular ROS levels were then measured on the cells pre-treated with DHA (panels A and B) or EPA (panels C and D) under both resting (non pro-inflammatory) conditions or pro-inflammatory conditions (PMA challenge), using the fluorescent ROS indicator, H_2DCFDA. Fluorescence intensity (485/538 nm) was measured every 30 minutes for 2 h. Data are means ± SEM of six experiments. Significance was declared at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

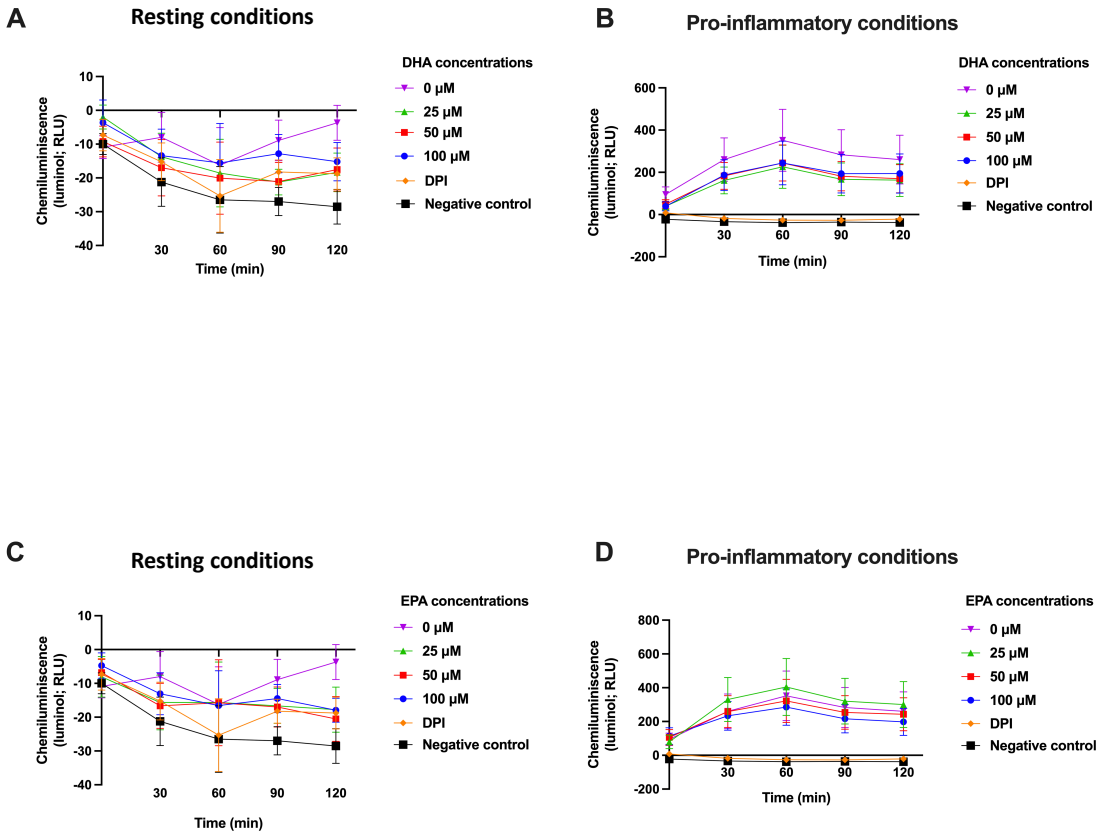


Fig. 5. Effects of DHA and EPA on *in vitro* porcine monocytes' total reactive oxygen species (ROS) generation. Cells were first pre-treated with increasing concentrations (25, 50 and 100 µM) of DHA and EPA or medium as control (0 µM) for 22 h. The total ROS levels were determined using luminol-dependent chemiluminescent assay, on the cells pre-treated with DHA (panels A and B) or EPA (panels C and D) under resting conditions (non pro-inflammatory) or pro-inflammatory conditions (PMA challenge). Chemiluminescence was measured every 30 minutes for 2 h and the results are expressed as relative light units (RLU). Data are means ± SEM of six experiments.

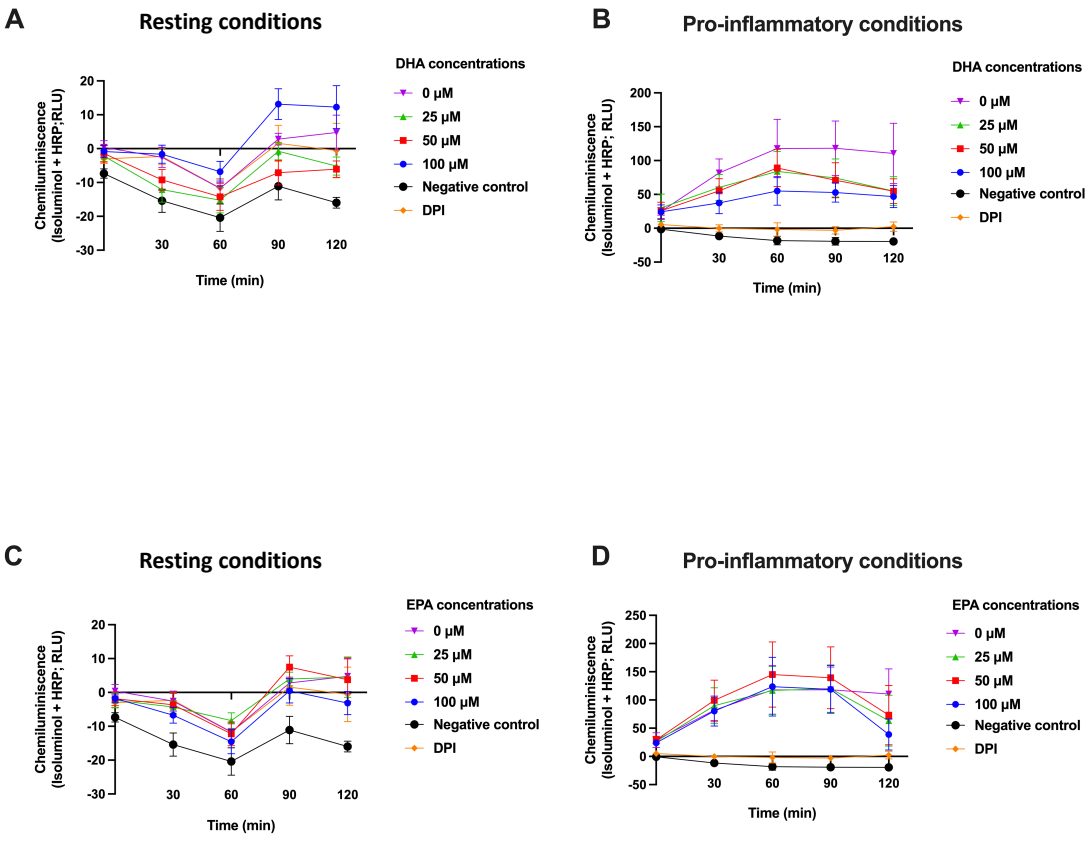


Fig. 6. Porcine monocytes' extracellular superoxide anion (O_2^-) production after DHA and EPA *in vitro* treatment. Cells were first pre-treated with increasing concentrations (25, 50 and 100 µM) of DHA and EPA or medium as control (0 µM) for 22 h. The extracellular ROS levels were then determined by adding isoluminol + horseradish peroxidase (HRP), on the cells pre-treated with DHA (panels A and B) or EPA (panels C and D) under resting conditions (non pro-inflammatory) or pro-inflammatory conditions (PMA challenge). Chemiluminescence was measured every 30 minutes for 2 h and the results are expressed as relative light units (RLU). Data are means ± SEM of six experiments.

Paper 4: Short Communication. Anti-inflammatory activity of citrus pectin on chicken monocytes' immune response

The following results have been published in Veterinary Immunology and Immunopathology (2021), https://doi.org/10.1016/j.vetimm.2021.110269

Short Communication

Anti-inflammatory activity of citrus pectin on chicken monocytes' immune response

G. Ávila[a], D. De Leonardis[a], G. Grilli[a], C. Lecchi[a], F. Ceciliani[a]*

[a]Department of Veterinary Medicine, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy

[*]Corresponding author: Gabriela Ávila Morales
E-mail address: gabriela.avila@unimi.it
Department of Veterinary Medicine, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy
Tel: 0039 02 50334531

Highlights

- CP did not affect chicken monocytes' apoptosis and viability
- CP inhibited the chemotactic activity of chicken monocytes
- CP reduced the phagocytic activity of chicken monocytes
- CP exerts *in vitro* immunomodulatory effects in poultry

ABSTRACT

Pectin is a dietary fibre composed of galacturonic acid, primarily found in the citrus fruits' cell walls. Citrus pectin (CP) has demonstrated antioxidative, anticancer, and anti-inflammatory properties in humans and animals. In broilers, CP supplementation improves energy utilization and nutrient digestibility, but limited information on its effects on chicken immunity is available so far. This study aimed to assess the *in vitro* impact of CP on chicken monocytes' immune response. Cells were purified from whole

blood of healthy chickens and incubated with increasing concentrations (0, 0.25, 0.5, 0.75, 1 mg/mL) of CP to determine CP working concentration. The effects of different CP concentrations on cells' apoptosis and viability were assessed by measuring caspase-3 and -7 and the cells' metabolic activity (MTT assay), respectively. CP had no dose-dependent effect on monocyte apoptosis and viability. Then, the effects of CP (0.5 mg/mL) on chicken monocytes' chemotaxis and phagocytosis were assessed by measuring transwell migration and fluorescein-labelled *E. coli* incorporation, respectively. CP inhibited both monocytes' chemotaxis and phagocytosis. These data demonstrate that CP exerts an immunomodulatory role in chicken monocytes, supporting its integration in nutrition strategies that might be beneficial for the animal's immunity and health.

***Keywords**:* chicken, citrus pectin, monocyte, anti-inflammatory, apoptosis, chemotaxis

Abbreviations

CP, citrus pectin; MTT, 3-(4,5- dimethyl thiazol -2-yl)-2,5-diphenyl tetrazolium bromide; DM, methyl esterification; TLR, toll-like receptor; HBSS, hank's balanced salt solution; ZAS, zymosan activated serum; MCP, modified citrus pectin.

1. Introduction

Pectin is a family of complex polysaccharides, primarily composed of repeating units of galacturonic acid, joined by α1 → 4 glycosidic linkages, creating a linear polymer (Ridley et al., 2001). Different degrees of methyl esterification (DM) of the pectin carboxyl groups can be observed, often associated with structural and functional differences of the pectin (Chen et al., 2006; Salman et al., 2008).

Pectin is found in all plants' cell walls, but it is most abundant in citrus fruits (Sahasrabudhe et al., 2018). Citrus pectin (CP) is widely used as dietary fibre in both human and animal nutrition (Langhout and Schutte, 1996; Leclere et al., 2013). Previous *in vitro* and *in vivo* studies demonstrated CP's antioxidative (Sanders et al., 2004), anti-diabetic (Liu et al., 2016), anticancer (Glinsky and Raz, 2009; Salehi et al., 2018), anti-inflammatory (Sahasrabudhe et al., 2018; Salman et al., 2008) and other immunomodulatory activities (Chen et al., 2006). *In vitro* studies performed in human

PBMC demonstrated that CP with higher esterification degrees (60 and 90%) inhibits in a dose-dependent manner the production of the proinflammatory cytokine IL-1β, while increases the secretion of anti-inflammatory cytokines IL-1ra and IL-10 (Salman et al., 2008). Low DM citrus pectin blocked immune receptors in human dendritic cells and murine macrophages by inhibiting the Toll-like receptor 1 (TLR1) and Toll-like receptor 2 (TLR2) proinflammatory pathways. The production of IL-6 and IL-10 was also reduced in human dendritic cells, and only of IL-6 in murine macrophages (Sahasrabudhe et al., 2018). Lemon pectin can also activate human THP-1 monocytic cell line, in a TLR and DM dependent manner, and exert a protective effect on the human epithelial barrier (Vogt et al., 2016). The immunomodulatory effects observed in these studies were all dependent on the DM of the citrus pectin used, in which generally the higher the DM (> 50%), the stronger the effects observed.

Although CP supplementation of broilers' diet improves the energy utilization, nutrient digestibility, increasing productive performance (Silva et al., 2013), limited information of CP effects on chicken immunity is available so far. The impact of CP on immune functions of chicken mononuclear cells have not been assessed yet either. The present investigation aims to cover this gap by evaluating the *in vitro* impact of CP (55-70% DM) on chicken monocytes' immune response, including its effects on spontaneous apoptosis, viability, chemotaxis and phagocytosis.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Methylcellulose, EDTA, NaCl (Sigma, St. Louis, USA) were used for PBMC isolation. Pectin esterified (55-70%) potassium salt from citrus fruit, cell tested, (Sigma, St. Louis, USA) was used for treating the cells.

2.1.2. Reagents

Ficoll-Paque PLUS (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden), Percoll, red blood cell lysis buffer, sterile-filtered PBS and HBSS, endotoxin-free water (Sigma, St. Louis, USA) were used for PBMC isolation. Cells were resuspended in complete medium, comprising RPMI 1640 Medium with 25 Mm HEPES and L-Glutamine (Sigma,

St. Louis, USA), supplemented with 1% of Non-essential Amino Acid Solution 100X and 1% Penicillin Streptomycin Solution 100X (Euroclone, Milano, Italy), and 10% FBS (Sigma, St. Louis, USA).

Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Milano, Italy) and Cell Proliferation Kit I (MTT) (Roche, Mannheim, Germany) were used for apoptosis and viability assays, respectively. Chemotaxis and phagocytosis were measured by using Zymosan A from *Saccharomyces cerevisiae* and fluorescein-labelled *Escherichia coli* bioparticles K-12 strain (Invitrogen, Oregon, USA), respectively.

2.2. Purification of monocytes from blood

Chicken monocytes were isolated from peripheral blood through double discontinuous gradient centrifugation, as previously described for chicken (He et al., 2007; Kogut et al., 1995), with few modifications. Briefly, fifty mL of peripheral blood from 42 days-old hybrid broilers (ROSS 308) were collected during routine slaughtering procedures at a local slaughterhouse and pooled in sterile flasks containing 0.2% EDTA per mL of blood as anticoagulant. The blood was mixed (1.5:1 v/v) with methylcellulose 1% (25 centiposes) and centrifuged at 40g without brakes for 30 min at 4°C. The supernatant was then collected, diluted (1:1) with PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA and layered over a double discontinuous Ficoll-Percoll gradient (specific gravity 1.077 g/mL of Ficoll over 1.119 g/mL of Percoll) in 15 mL conical centrifuge tubes, and centrifuged at 200g without brakes for 30 min at 4°C. The PBMC ring was collected at the Ficoll/supernatant interface, washed twice with PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA and centrifuged at 200g for 10 min at 4°C to remove the contaminating thrombocytes. For red blood cells elimination, Red Blood Cell Lysis Buffer was added to the cells for 3 min at room temperature, and cells were washed with PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA until the supernatant was clear. Finally, the PBMC were counted, and their viability assessed with trypan blue exclusion. The purification of monocytes was carried out by allowing the PBMC to adhere for 2 h at room temperature to the cell culture treated plates of each assay, as previously described (He et al., 2007). Non-adherent cells were removed, and monocytes were washed with complete warm medium. The monocytes' purity was determined by microscopic examination of Diff-Quick stained cytospin smears. Cell viability, measured with the Trypan Blue exclusion method, was higher than 95%. At the microscope examination, monocyte purity was of 70% approximately.

2.3.Citrus pectin preparation

Citrus pectin esterified (55-70%) potassium salt was reconstituted in endotoxin-free water at a concentration of 10 mg/mL. Briefly, 2 mL of endotoxin-free water were added to 20 mg of CP, and the solution was then vortexed thoroughly until it was dissolved. The CP solution was filtered with 0.22 μm filters. Aliquots from the stock solution were prepared and stored at 4°C until use. Working dilutions with complete medium were freshly prepared. The amounts of CP used for determining its working concentration were 0, 0.25, 0.5, 0.75, 1 mg/mL. Once selected the CP working solution, a concentration of 0.5 mg/mL was used to measure chemotaxis and phagocytosis.

2.4.Viability assay (MTT)

To assess the potential toxicity of CP on chicken monocytes, the cells' viability was evaluated by using the Cell Proliferation Kit I (MTT), as previously reported (Catozzi et al., 2020). The experiment was carried out on cells purified from 3 different pools, consisting of 9 animals each. A total of 1×10^5 PBMC (100 μl) were seeded in duplicate in 96-well sterile plates MICROTEST (Becton Dickinson and Company, Franklin Lake, USA) to let the monocytes adhere to the plates. After removing the non-adherent cells, monocytes were incubated 20 h with increasing CP concentrations (0.25, 0.5, 0.75 and 1 mg/mL) or with medium (control) at 41°C in humified atmosphere 5% CO_2. After the incubation period, 10 μl of MTT labelling reagent was added to each well and incubated at 41°C for 4 h. The formazan crystals were solubilized by adding 100 μl of the solubilization buffer and incubating at 41°C overnight, following the manufacturer's instructions. Finally, the absorbance was read at 550 nm with LabSystems Multiskan plate reader Spectrophotometer.

2.5.Apoptosis assay

Apoptosis assay was performed in duplicates; 5×10^4 PBMC were seeded in 384-well black plates (Costar, Corning, USA). The experiment was carried out on the adhered monocytes purified from 4 different pools of 9 animals each. To determine CP working concentration, the cells were incubated for 20 h at 41°C in a humified atmosphere of 5%

CO_2 with increasing CP concentrations (0.25, 0.5, 0.75 and 1 mg/mL) medium as control and the spontaneous apoptosis was measured. The apoptosis was measured after 20 h of incubation by using the Apo-ONE® Homogeneous Caspase-3/7 kit. The caspase-3/7 reagent was added to each well, and the fluorescence intensity was measured using a fluorescence plate reader Fluoroscan Ascent at 485/538 nm (absorbance/emission), every 30 minutes up to 2 hours and after overnight incubation, as previously described in bovine monocytes with minor modifications (Ceciliani et al., 2007).

2.6.Chemotaxis assay

Monocytes chemotaxis towards zymosan activated serum (ZAS) was measured as previously reported (Ávila et al., 2020; Lecchi et al., 2008), with minor changes. The experiment was performed on cells purified from 5 different pools, consisting of 9 animals each. Monocytes were first pretreated overnight, in the absence of chemoattractant, with 0.5 mg/mL of CP or migration medium only (RMPI-1640 with 1% of FBS) as a control (vehicle) in 24-well Transwell migration plates (Costar, Corning, USA), equipped with an 8 μm pore size membrane. First, a total number of $1x10^5$ PBMC (100 μl) were added in duplicates in the upper chamber, and monocytes were allowed to adhere for 2 h at room temperature to the upper insert membrane of the transwell plate. After incubation, non-adherent cells were removed and monocytes washed with the warm medium. Adhered monocytes were pretreated with 0.5 mg/ mL of CP or with migration medium by adding 100 μl of CP or medium to the upper chamber and 650 μl to the lower chamber, for 20 h at 41°C in a humified atmosphere of 5% CO_2. After pretreating the cells, chemotaxis was measured by adding 3 mg/mL of the chemoattractant ZAS to the lower chamber, in the presence of newly added CP (0.5 mg/mL) or migration medium (vehicle) in both chambers, and again incubated for 2 h at 41°C in a humified atmosphere of 5% CO_2. Cells incubated with migration medium but without ZAS were considered as the negative control. Upper chambers were removed, and non-migrated cells on the upper part of the membrane were eliminated using a cotton swab moistured with PBS. Finally, migrated cells were stained with Diff-Quick (Sigma, St Louis, USA) and counted in ten different fields, using light microscopy (inverted microscope). The chemoattractant ZAS was prepared as previously described in bovine monocytes (Lecchi et al., 2008), using chicken serum.

2.7.Phagocytosis assay

Monocytes' phagocytosis was determined as previously described (Lecchi et al., 2011). The experiment was carried out on cells purified from 6 different pools, consisting of 9 animals each. In a first step, $6x10^8$ fluorescein-labelled *Escherichia coli* bioparticles (K-12 strain) were opsonized with 20% of chicken serum for 30 min at 37°C. Opsonized bacteria were centrifuged at 800 g for 15 min, suspended in HBSS and stored at -20°C until use. A total of $3x10^5$ PBMC (100 µl) were seeded in duplicates in 96-well sterile plates for monocyte isolation for 2 h at room temperature. After removing non-adherent cells and washing the monocytes with warm HBSS, 100 µl of 0.5 mg/mL CP or only medium (vehicle) as the control was added to each well. The medium was added to reach a final volume of 200 µl, and cells were incubated for 20 h at 41°C in a humidified atmosphere of 5% CO_2. Cells were then washed with sterile HBSS, and a total of 45 opsonized fluorescein-labelled *E. coli* bioparticles (100 µl) per well were added. Monocytes were incubated again for 2 h at 41°C in a humidified atmosphere of 5% CO_2. Cells were washed twice with HBSS and 0.4% of trypan blue was added to quench the fluorescence from non-internalized bacteria. Cells were finally washed with HBSS and the fluorescence intensity of fluorescein-labelled *E. coli* bioparticles was measured using a Fluoroscan Ascent at 485/538 nm (absorbance/emission).

2.8.Statistical analyses

Statistical analyses were performed in GraphPad Prism 8.0.2. Data normality of apoptosis, viability and chemotaxis was assessed by applying the Shapiro Wilk test and the Kolmogorov Smirnov test for phagocytosis. For the assessment of CP's effect on the apoptosis, viability and chemotaxis, repeated measures one-way ANOVA and Tukey's multiple comparison tests were used for normally distributed samples and a one-tailed paired t-test for phagocytosis. Statistical differences were accepted at $P \leq 0.05$.

3. Results and discussion

In the present study, we reported the effects of CP on chicken monocytes' immune functions, including chemotaxis and phagocytosis. To test the impact of CP on monocytes, viability and apoptosis were also measured.

The purity of the isolated monocytes was approximately 70%, the contaminant cells being mostly thrombocytes, which is consistent with what was reported in previous studies (Ma et al., 2019; Reddy et al., 2016). Since avian thrombocytes are nucleated cells with sizes and densities similar to lymphocytes and monocytes, they are often purified together with them. Moreover, their capacity to adhere to the plastic makes their complete depletion from monocytes cultures challenging (Mudroňová et al., 2014).

Pectin has shown immunomodulatory roles by preventing immunological diseases like asthma (Zhang et al., 2016), allergies, colitis and inflammatory bowel diseases through directly regulating inflammation in several animal models (Ishisono et al., 2019; Sahasrabudhe et al., 2018). In broilers, including CP in the diet has improved the animals' growth's performance (Silva et al., 2013), but information about its immunomodulatory activity in chickens is still absent.

As the first step of this study, we measured chicken monocytes' viability and apoptosis to determine the optimal working concentration of CP and to evaluate potential CP cytotoxic effects. Cells' viability (Fig. 1A) and spontaneous apoptosis (Fig. 1B) were not affected by increasing CP concentrations. Apoptosis plays a critical role in regulating and shaping the immune system, as it maintains cellular homeostasis by eliminating the excess of immune cells (Feig and Peter, 2007). Therefore, apoptosis can be regarded as a way of controlling immune cells' activity in the inflammatory focus. Modified citrus pectin (MCP) can induce apoptosis of breast (Salehi et al., 2018), prostate (Jackson et al., 2007) and colon (Olano-Martin et al., 2003) cancer cells, without damaging normal cells even at high concentrations (600 μg/mL) (Chauhan et al., 2005). The same increasing concentrations of CP (0.25, 0.5, 0.75 and 1 mg/mL) herein tested, had also no cytotoxic effects on a macrophage murine cell line (RAW264.7) (Chen et al., 2006), suggesting that the impact of CP might be cell-specific.

In the second step of this investigation, CP's effect (0.5 mg/mL) on the chemotaxis of chicken monocytes was assessed. This concentration was used throughout the next experiments, as monocytes' viability and apoptosis remained unchanged at this concentration, and no other cytotoxic effects in immune cells, including macrophages, have been described (Fan et al., 2018). On the contrary, several immunomodulatory effects, specifically anti-inflammatory ones, have been observed (Chen et al., 2006; Salman et al., 2008).

The activation of medium treated (vehicle) cells with ZAS enhanced the chemotaxis of monocytes compared to the negative control (cells without ZAS) ($P < 0.01$). Co-

incubation with CP decreased (P = 0.03) chemotaxis rate of cells compared to ZAS activated cells treated with medium (vehicle), while no differences were observed compared to cells without ZAS (negative control) (Fig. 2). The present findings demonstrated that CP reduces the *in vitro* monocytes' chemotaxis in chicken. These results are consistent with those reported in cancer cells, which revealed that smaller sizes of MCP fractions inhibit their migration (do Prado et al., 2019). A similar inhibitory effect on the migration of a murine fibroblasts treated with 0.5 mg/mL of ginseng pectin was observed by changing the cells' morphology and organization of actin filaments, and by reducing cell adhesion and spreading to the substratum (Fan et al., 2018). Specifically, MCP inhibited cancer and endothelial cells' migration through the direct inhibition of Galectin-3, a carbohydrate-binding protein expressed by numerous cell types, including monocytes, which is involved in cell migration and phagocytosis (Nangia-Makker et al., 2002; Simon and Green, 2005). Indeed, pectins can modulate directly innate immune responses by interacting with monocytes and macrophages' pattern recognition receptors (PPR) like Galectin-3 and Toll-like receptors (TLR) (Beukema et al., 2020). The direct inhibition of Galectin-3 and TLR2, TLR1 and TLR4 by CP (Ishisono et al., 2017; Sahasrabudhe et al., 2018) could explain the suppression of some monocytes' immune responses and consequently of its anti-inflammatory effects. On the other hand, after their binding, pectins can also present TLR2/4 activating properties, and as reported previously, TLR2/4 signalling can also cause a rapid arrest of human monocytes' chemotaxis (Yi et al., 2012).

The last set of experiments was performed to explore CP's capacity in modulating chicken monocytes' phagocytosis. Monocytes were treated with CP (0.5 mg/mL) or complete medium (vehicle) as control for 20 h at 41°C, and their phagocytic capability was measured. Fig. 3 shows a reduction (P = 0.03) in the phagocytosis of CP treated monocytes when compared to the control. These results evidence that CP inhibited another important monocyte defensive function, phagocytosis. This result differs from those previously reported, showing that a high DM pectin (around 85% DM) isolated from berries increased peritoneal macrophages' phagocytosis (Wang et al., 2015). However, contrasting results of pectin's immunomodulatory effects on leukocytes are often reported, as the content of galacturonic acid and the degree of DM determine pectin's ability to decrease immune reactivity. Pectins with higher than 80% of galacturonic acid decreased the *in vivo* accumulation of murine macrophages, while increasing or no effects were seen with pectins with less than 75% of galacturonic acid

(Popov and Ovodov, 2013). On the contrary, CP with lower DM showed anti-inflammatory properties in mice *in vivo*, a feature lost as their DM increases (Popov et al., 2013).

Finally, the suppression of chicken monocytes' phagocytosis could also be explained by the inhibition of Galectin-3, which plays a significant role in macrophage phagocytosis, as a major component of phagosomes and phagocytic cups (Sano et al., 2003).

In conclusion, this is the first study to indicate that CP inhibits two main inflammatory functions of chicken monocytes', namely chemotaxis and phagocytosis, suggesting potential anti-inflammatory roles. Moreover, our results evidence that the effects of CP on apoptosis and viability are cell-specific, as the pro-apoptotic effects often observed in cancer cells were not seen on chicken monocytes. Altogether, these results reinforce the concept that CP may play an essential role in immunity, specifically in dampening inflammation. Such potential anti-inflammatory functions make CP an attractive dietary fibre for novel nutrition strategies to enhance the animals' health. Besides, these results highlight the importance to formulate balanced diets in chicken, specially considering that an excessive CP content may induce an immunosuppressive status, increasing the risk of developing opportunistic diseases. Further analyses on other inflammatory immune functions (e.g., oxidative burst, killing capability and cytokine production) and integration of system biology approaches might be highly valuable to elucidate CP biological significance.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper's content.

Acknowledgements

We acknowledge the support of Valentina Lodde and her team of the Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare at the Università degli Studi di Milano,–Italy, for her valuable support in the microscopic evaluation and digital imaging acquisition of chicken thrombocytes and monocytes; and to Rodrigo Guabiraba-Brito, from the Infection and Innate Immunity in Monogastric

Livestock Department, Centre INRAE, Val de Loire, Nouzilly, France, for his valuable support in chicken PBMC isolation and thrombocyte elimination.

Funding

This study was supported by the European Union's Horizon 2020 research and innovation programme H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate) [Grant agreement nº: 765423, 2017] – MANNA.

References

Ávila, G., Catozzi, C., Pravettoni, D., Sala, G., Martino, P., Meroni, G., Lecchi, C., Ceciliani, F., 2020. In vitro effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes. J. Dairy Sci. 103, 8554–8563. https://doi.org/10.3168/jds.2020-18659

Beukema, M., Faas, M.M., de Vos, P., 2020. The effects of different dietary fiber pectin structures on the gastrointestinal immune barrier: impact via gut microbiota and direct effects on immune cells. Exp. Mol. Med. 52, 1364–1376. https://doi.org/10.1038/s12276-020-0449-2

Catozzi, C., Ávila, G., Zamarian, V., Pravettoni, D., Sala, G., Ceciliani, F., Lacetera, N., Lecchi, C., 2020. In-vitro effect of heat stress on bovine monocytes lifespan and polarization. Immunobiology 225, 151888. https://doi.org/https://doi.org/10.1016/j.imbio.2019.11.023

Ceciliani, F., Pocacqua, V., Miranda-Ribera, A., Bronzo, V., Lecchi, C., Sartorelli, P., 2007. α1-Acid glycoprotein modulates apoptosis in bovine monocytes. Vet. Immunol. Immunopathol. 116, 145–152. https://doi.org/10.1016/j.vetimm.2007.01.006

Chauhan, D., Li, G., Podar, K., Hideshima, T., Neri, P., He, D., Mitsiades, N., Richardson, P., Chang, Y., Schindler, J., Carver, B., Anderson, K.C., 2005. A Novel Carbohydrate-Based Therapeutic GCS-100 Overcomes Bortezomib Resistance and Enhances Dexamethasone-Induced Apoptosis in Multiple Myeloma Cells. Cancer Res. 65, 8350 LP – 8358. https://doi.org/10.1158/0008-5472.CAN-05-0163

Chen, C.H., Sheu, M.T., Chen, T.F., Wang, Y.C., Hou, W.C., Liu, D.Z., Chung, T.C.,

Liang, Y.C., 2006. Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways. Biochem. Pharmacol. 72, 1001–1009. https://doi.org/10.1016/j.bcp.2006.07.001

do Prado, S.B.R., Shiga, T.M., Harazono, Y., Hogan, V.A., Raz, A., Carpita, N.C., Fabi, J.P., 2019. Migration and proliferation of cancer cells in culture are differentially affected by molecular size of modified citrus pectin. Carbohydr. Polym. 211, 141–151. https://doi.org/https://doi.org/10.1016/j.carbpol.2019.02.010

Fan, Y., Sun, L., Yang, S., He, C., Tai, G., Zhou, Y., 2018. The roles and mechanisms of homogalacturonan and rhamnogalacturonan I pectins on the inhibition of cell migration. Int. J. Biol. Macromol. 106, 207–217. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2017.08.004

Feig, C., Peter, M.E., 2007. How apoptosis got the immune system in shape. Eur. J. Immunol. 37 Suppl 1, S61-70. https://doi.org/10.1002/eji.200737462

Glinsky, V. V., Raz, A., 2009. Modified citrus pectin anti-metastatic properties: one bullet, multiple targets. Carbohydr. Res. 344, 1788–1791. https://doi.org/10.1016/j.carres.2008.08.038

He, H., Genovese, K.J., Nisbet, D.J., Kogut, M.H., 2007. Synergy of CpG oligodeoxynucleotide and double-stranded RNA (poly I:C) on nitric oxide induction in chicken peripheral blood monocytes. Mol. Immunol. 44, 3234–3242. https://doi.org/10.1016/j.molimm.2007.01.034

Ishisono, K., Mano, T., Yabe, T., Kitaguchi, K., 2019. Dietary Fiber Pectin Ameliorates Experimental Colitis in a Neutral Sugar Side Chain-Dependent Manner. Front. Immunol. 10, 2979. https://doi.org/10.3389/fimmu.2019.02979

Ishisono, K., Yabe, T., Kitaguchi, K., 2017. Citrus pectin attenuates endotoxin shock via suppression of Toll-like receptor signaling in Peyer's patch myeloid cells. J. Nutr. Biochem. 50, 38–45.

Jackson, C.L., Dreaden, T.M., Theobald, L.K., Tran, N.M., Beal, T.L., Eid, M., Gao, M.Y., Shirley, R.B., Stoffel, M.T., Kumar, M.V., Mohnen, D., 2007. Pectin induces apoptosis in human prostate cancer cells: correlation of apoptotic function with pectin structure. Glycobiology 17, 805–819. https://doi.org/10.1093/glycob/cwm054

Kogut, M.H., McGruder, E.D., Hargis, B.M., Corner, D.E., DeLoach, J.R., 1995. In vivo activation of heterophil function in chickens following injection with Salmonella enteritidis-immune lymphokines. J. Leukoc. Biol. 57, 56–62.

Langhout, D.J., Schutte, J.B., 1996. Nutritional Implications of Pectins in Chicks in Relation to Esterification and Origin of Pectins. Poult. Sci. 75, 1236–1242. https://doi.org/10.3382/ps.0751236

Lecchi, C., Ceciliani, F., Bernasconi, S., Franciosi, F., Bronzo, V., Sartorelli, P., 2008. Bovine alpha-1 acid glycoprotein can reduce the chemotaxis of bovine monocytes and modulate CD18 expression. Vet. Res. 39. https://doi.org/10.1051/vetres:2008027

Lecchi, C., Invernizzi, G., Agazzi, A., Ferroni, M., Pisani, L.F., Savoini, G., Ceciliani, F., 2011. In vitro modulation of caprine monocyte immune functions by ω-3 polyunsaturated fatty acids. Vet. J. 189, 353–355. https://doi.org/10.1016/j.tvjl.2010.09.001

Leclere, L., Cutsem, P. Van, Michiels, C., 2013. Anti-cancer activities of pH- or heat-modified pectin. Front. Pharmacol. 4 OCT, 1–8. https://doi.org/10.3389/fphar.2013.00128

Liu, Y., Dong, M., Yang, Z., Pan, S., 2016. Anti-diabetic effect of citrus pectin in diabetic rats and potential mechanism via PI3K/Akt signaling pathway. Int. J. Biol. Macromol. 89, 484–488. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2016.05.015

Ma, Y., Liang, Y., Wang, N., Cui, L., Chen, Z., Wu, H., Zhu, C., Wang, Z., Liu, S., Li, H., 2019. Avian Flavivirus Infection of Monocytes/Macrophages by Extensive Subversion of Host Antiviral Innate Immune Responses. J. Virol. 93, e00978-19. https://doi.org/10.1128/JVI.00978-19

Mudroňová, D., Kožár, M., Molnár, L., Trbolová, A., 2014. Viability and discrimination of avian peripheral blood mononuclear cells and thrombocytes intended for improvement of wound healing in birds. Acta Vet. Hung. 62, 334–339. https://doi.org/10.1556/AVet.2014.014

Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Bresalier, R., Raz, A., 2002. Inhibition of Human Cancer Cell Growth and Metastasis in Nude Mice by Oral Intake of Modified Citrus Pectin. JNCI J. Natl. Cancer Inst. 94, 1854–1862. https://doi.org/10.1093/jnci/94.24.1854

Olano-Martin, E., Rimbach, G.H., Gibson, G.R., Rastall, R.A., 2003. Pectin and pectic-oligosaccharides induce apoptosis in in vitro human colonic adenocarcinoma cells. Anticancer Res. 23, 341–346.

Popov, S. V., Ovodov, Y.S., 2013. Polypotency of the immunomodulatory effect of

pectins. Biochem. 78, 823–835. https://doi.org/10.1134/S0006297913070134

Popov, S. V, Markov, P.A., Popova, G.Y., Nikitina, I.R., Efimova, L., Ovodov, Y.S., 2013. Anti-inflammatory activity of low and high methoxylated citrus pectins. Biomed. Prev. Nutr. 3, 59–63. https://doi.org/https://doi.org/10.1016/j.bionut.2012.10.008

Reddy, V.R.A.P., Trus, I., Desmarets, L.M.B., Li, Y., Theuns, S., Nauwynck, H.J., 2016. Productive replication of nephropathogenic infectious bronchitis virus in peripheral blood monocytic cells, a strategy for viral dissemination and kidney infection in chickens. Vet. Res. 47, 70. https://doi.org/10.1186/s13567-016-0354-9

Ridley, B.L., O'Neill, M.A., Mohnen, D., 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 57, 929–967. https://doi.org/https://doi.org/10.1016/S0031-9422(01)00113-3

Sahasrabudhe, N.M., Beukema, M., Tian, L., Troost, B., Scholte, J., Bruininx, E., Bruggeman, G., van den Berg, M., Scheurink, A., Schols, H.A., Faas, M.M., de Vos, P., 2018. Dietary fiber pectin directly blocks toll-like receptor 2-1 and prevents doxorubicin-induced ileitis. Front. Immunol. 9, 1–19. https://doi.org/10.3389/fimmu.2018.00383

Salehi, F., Behboudi, H., Kavoosi, G., Ardestani, S.K., 2018. Oxidative DNA damage induced by ROS-modulating agents with the ability to target DNA: A comparison of the biological characteristics of citrus pectin and apple pectin. Sci. Rep. 8, 13902. https://doi.org/10.1038/s41598-018-32308-2

Salman, H., Bergman, M., Djaldetti, M., Orlin, J., Bessler, H., 2008. Citrus pectin affects cytokine production by human peripheral blood mononuclear cells. Biomed. Pharmacother. 62, 579–582. https://doi.org/10.1016/j.biopha.2008.07.058

Sanders, L.M., Henderson, C.E., Hong, M.Y., Barhoumi, R., Burghardt, R.C., Wang, N., Spinka, C.M., Carroll, R.J., Turner, N.D., Chapkin, R.S., Lupton, J.R., 2004. An Increase in Reactive Oxygen Species by Dietary Fish Oil Coupled with the Attenuation of Antioxidant Defenses by Dietary Pectin Enhances Rat Colonocyte Apoptosis. J. Nutr. 134, 3233–3238. https://doi.org/10.1093/jn/134.12.3233

Sano, H., Hsu, D.K., Apgar, J.R., Yu, L., Sharma, B.B., Kuwabara, I., Izui, S., Liu, F.-T., 2003. Critical role of galectin-3 in phagocytosis by macrophages. J. Clin. Invest. 112, 389–397. https://doi.org/10.1172/JCI17592

Silva, V.K., de Souza Morita, V., Boleli, I.C., 2013. Effect of pectin extracted from citrus pulp on digesta characteristics and nutrient digestibility in broilers chickens. Rev.

Bras. Zootec. 42, 575–583. https://doi.org/10.1590/S1516-35982013000800007

Simon, S.I., Green, C.E., 2005. Molecular Mechanics and Dynamics of Leukocyte Recruitment During Inflammation. Annu. Rev. Biomed. Eng. 7, 151–185. https://doi.org/10.1146/annurev.bioeng.7.060804.100423

Vogt, L.M., Sahasrabudhe, N.M., Ramasamy, U., Meyer, D., Pullens, G., Faas, M.M., Venema, K., Schols, H.A., de Vos, P., 2016. The impact of lemon pectin characteristics on TLR activation and T84 intestinal epithelial cell barrier function. J. Funct. Foods 22, 398–407. https://doi.org/https://doi.org/10.1016/j.jff.2016.02.002

Wang, H., Gao, T., Du, Y., Yang, H., Wei, L., Bi, H., Ni, W., 2015. Anticancer and immunostimulating activities of a novel homogalacturonan from Hippophae rhamnoides L. berry. Carbohydr. Polym. 131, 288–296. https://doi.org/https://doi.org/10.1016/j.carbpol.2015.06.021

Yi, L., Chandrasekaran, P., Venkatesan, S., 2012. TLR Signaling Paralyzes Monocyte Chemotaxis through Synergized Effects of p38 MAPK and Global Rap-1 Activation. PLoS One 7, e30404.

Zhang, Z., Shi, L., Pang, W., Liu, W., Li, J., Wang, H., Shi, G., 2016. Dietary Fiber Intake Regulates Intestinal Microflora and Inhibits Ovalbumin-Induced Allergic Airway Inflammation in a Mouse Model. PLoS One 11, e0147778.

Figures

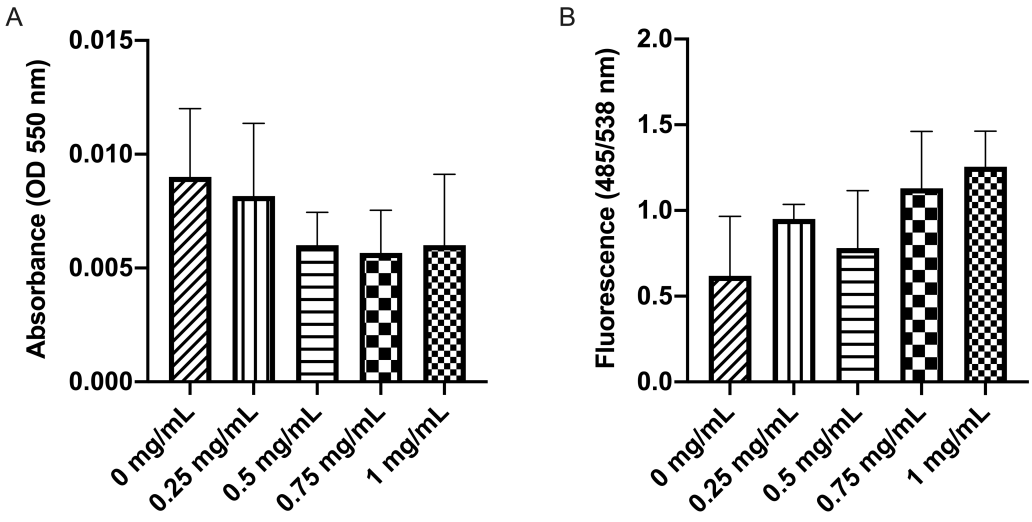


Fig. 1. *In vitro* effect of increasing concentrations (0.25, 0.5, 0.75 and 1 mg/mL) of citrus pectin (CP) on chicken monocytes' (A) viability and (B) apoptosis. 3-(4,5- dimethyl thiazol -2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction by metabolic active cells and caspase-3/7 enzymatic activity of chicken monocytes treated with citrus pectin or medium as control (0 mg/mL) were measured for viability and apoptosis, respectively. Data are means ± SEM of three and four animals, for viability and apoptosis, respectively. All experiments were carried out using duplicates for each condition.

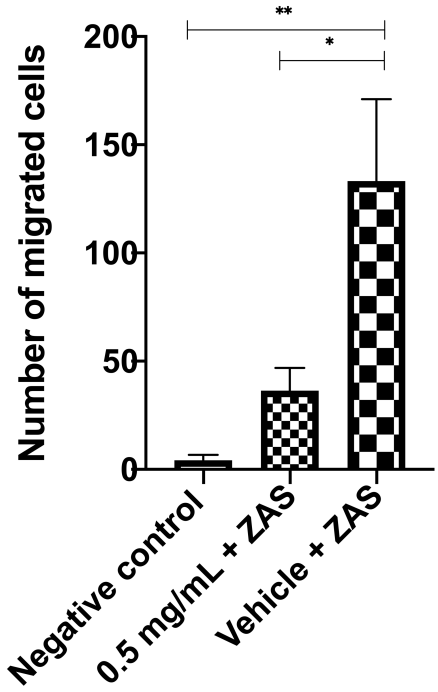


Fig. 2. Effects of citrus pectin (CP) on chicken monocytes chemotaxis. Citrus pectin (0.5 mg/mL) and vehicle control (only medium) treated cells were activated with Zymosan Activated Serum (ZAS), while negative control cells were not. Data are means ± SEM of five independent experiments. All experiments were carried out using duplicates for each treatment. Significance was declared for $P < 0.05$ (*).

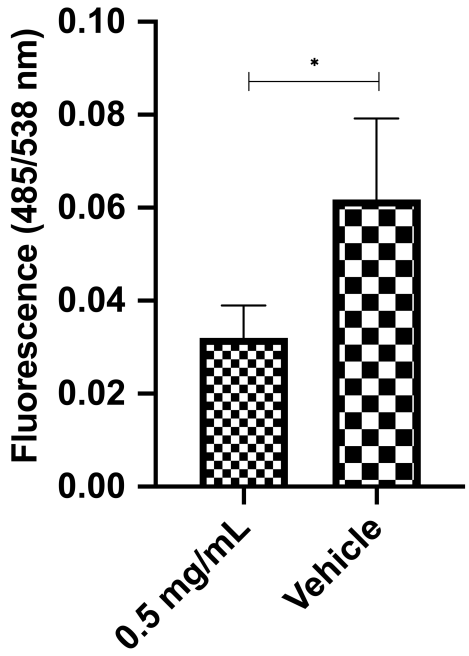


Fig. 3. Phagocytosis of fluorescein-labelled *Escherichia coli* bioparticles by chicken monocytes after 20 h incubation with citrus pectin (0.5 mg/mL). Cells treated with only medium (vehicle) were considered as control. Data are means ± SEM of six independent experiments. All experiments were carried out using duplicates for each treatment. Significance was declared for $P < 0.05$ (*).

Manuscript draft 5: Conjugated Linoleic Acid (CLA) modulates bovine peripheral blood mononuclear cells (PBMC) proteome *in vitro*

Conjugated Linoleic Acid (CLA) modulates bovine peripheral blood mononuclear cells (PBMC) proteome *in vitro*

G. Ávila[a], F. Ceciliani[a], D. Viala[b,c], S. Dejean[d], , D. Pravettoni[a] , G. Sala[a], C. Lecchi[a], M.Bonnet[b]*

[a]*Department of Veterinary Medicine and Animal Sciences, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy*

[b] *INRAE, Université Clermont Auvergne, Vetagro Sup, UMRH, 63122, Saint-Genès-Champanelle, France*

[c] *INRAE, Metabolomic and Proteomic Exploration Facility (PFEM), F-63122 Saint-Genès-Champanelle, France*

[d] *Institut de Mathématiques de Toulouse, Université de Toulouse, CNRS, UPS, UMR 5219,31062 Toulouse, France*

[*]Corresponding author: Gabriela Ávila Morales

E-mail address: gabriela.avila@unimi.it

Department of Veterinary Medicine, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy

Tel: 0039 02 50334531

ABSTRACT

Conjugated linoleic acid (CLA) is a group of naturally isomers of the n-6 polyunsaturated fatty acid (PUFA) linoleic acid, commonly used in cattle nutrition due to its beneficial properties on cows' milk production, metabolism, fertility and immunity. In our previous *in vitro* study, the mixture in equal proportions (50:50) of the two main isomers of CLA (cis-9, trans-11 and trans-10, cis-12) has shown to exert enhanced and synergic immunomodulatory effects on bovine monocytes, as it decreased the cells' apoptosis and increased their reactive oxygen species (ROS) production under pro-inflammatory conditions. However, no studies on the exact molecular mechanisms underlying these immunomodulatory effects on bovine mononuclear cells are available so far. Therefore, this study aimed to assess the effects of CLA (50:50) mixture on bovine peripheral blood mononuclear cells (PBMC) proteome. PBMC purified from peripheral blood of seven healthy Holstein-Friesian cows were incubated with 50 μM of the CLA (50:50) mixture isomers or with 0.014% ethanol (vehicle) or only medium (untreated) as controls. Proteins were extracted, concentrated and analyzed by nano-LC-MS/MS. A total of 1608 of quantifiable proteins was identified, and the supervised multivariate statistical analysis, sparse variant partial least squares – discriminant analysis (sPLS-DA) for paired data, was applied to identify discriminant proteins (DP) that contributed to a clear separation between the CLA (50:50) mixture, vehicle and untreated groups. A total of 407 DP was identified, and a global Gene Ontology (GO) analysis was performed using ProteINSIDE. The main enriched GO terms in the biological process (BP) category were mainly related to RNA splicing and processing, metabolic processes, immune system process and negative regulation of proteolysis. To further elucidate the specific role of the DP identified for each group, DP with the highest abundance in CLA group, vehicle group and untreated group were selected and again mined with ProteINSIDE. DP with higher abundance in CLA (50:50) mixture group were mainly involved in negative regulation of proteolysis and endopeptidases activity, acute-phase response, complement activation, lipid metabolism, oxidative stress response and ROS metabolism. Remarkably, proteins with anti-apoptotic (SERPINA3-7 and ITIH4) and anti-oxidant effects (HMOX1 and HMOX2), and involved in the host innate immune defense (C3, C4A, agp and A2M) were found annotated in those enriched BP. In conclusion, these results suggest that CLA (50:50) mixture does modulate bovine PBMC proteome, support the antiapoptotic and immunomodulatory effects observed in our previous *in vitro* study on bovine monocytes,

and propose a potential cytoprotective role of CLA (50:50) mixture against oxidative stress.

***Keywords**:* conjugated linoleic acid, bovine PBMC proteome, anti-apoptotic, immunomodulation, SERPINA3-7, ITIH4, HMOX1

3. Introduction

Conjugated linoleic acid (CLA) is a group of naturally occurring positional and geometrical isomers of the essential n-6 polyunsaturated fatty acid (PUFA) linoleic acid, characterized by conjugated double bonds in either cis or trans configuration. The cis-9,trans-11 (c9,t11) and the trans-10,cis-12 (t10,c12) are the two most abundant and biologically relevant CLA isomers [1,2]. Each isomer seems to exert differential biological effects when used separately, but enhanced and synergic effects were demonstrated when they are combined in equal amounts [3]. Indeed, most of the animal studies reporting CLA benefits and commercially available CLA supplements use a mixture of these two isomers in roughly equal amounts [4,5]. CLA has been commonly used as dietary supplement in cattle nutrition as it has shown to increase milk yield, reduce milk fat, thus, enhancing whole body energy utilization, and increase reproductive performance in early lactation [6–8]. Moreover, a potential role of CLA in mitigating the pro-inflammatory status associated with the oxidative stress around calving in dairy cows has been proposed [9], as it has been shown for other PUFA [10]. *In vitro*, *ex vivo* and *in vivo* studies have also shown that CLA isomers separately or in mixture can modulate bovine immune response, by affecting peripheral blood mononuclear cells (PBMC) proliferation and cytokine expression, PBMC stimulation ability and increasing different PBMC subsets (e.g. T and B cells) in mesenteric lymph nodes and ileal lamina propria, respectively [11–13]. In addition, in our previous in *vitro* study, only the CLA (50:50) mixture was able to decrease bovine monocytes apoptosis, and increase their ROS production under experimentally induced pro-inflammatory conditions [14]. However, the exact molecular mechanisms underlying these immunomodulatory effects have not been identified so far.

The implementation of OMIC technologies – the set of high-throughput technologies that detect genes (genomics), RNA (transcriptomics), proteins (proteomics), lipids (lipidomics) and metabolites (metabolomics) – has become a widely used approach in livestock nutrition as it helps to understand better the molecular effects of novel feed

additives in a comprehensive, holistic and systematic way [15]. Specifically, proteomics – large scale study of the protein profile in a given biological system – could help us to identify the molecular signature of CLA (50:50) mixture and, thus, to improve our understanding on the potential biological role that CLA could exert in cattle immunity [16]. In a quite recent study, the *ex vivo* bovine PBMC proteome of postpartum dairy cows supplemented with omega-3 fatty acids was determined [17]. Nonetheless, at the best of our knowledge, the *in vitro* impact of CLA, and specially of the CLA (50:50) mixture on bovine PBMC proteome has not been assessed yet. Therefore, in this study we aim at evaluating the capacity of CLA (50:50) mixture in modulating bovine PBMC proteome *in vitro*, using an untargeted proteomics approach.

4. Materials and methods

5.1. Purification of bovine PBMC from blood

Bovine PBMC were isolated from peripheral blood through Ficoll density gradient centrifugation, as previously described for bovine [14] with some minor modifications. Briefly, peripheral blood from 7 healthy pluriparous late lactation Holstein-Friesian cows was collected during routine slaughtering procedures at a local slaughterhouse, in sterile flasks containing 1.8 mg K_2EDTA per mL of blood as anticoagulant. Blood was first centrifuged (without breaks) to collect the buffy coat that was then diluted with sterile PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA (Sigma-Aldrich, St. Louis, MO, USA), layered carefully on Ficoll (1.077 g/mL; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and centrifuged again without breaks. The PBMC ring was recovered at the interface, washed with sterile PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA, and treated with red blood cell lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany) for red blood cell elimination. Finally, the PBMC were counted, their viability assessed with trypan blue exclusion (>90%) and resuspended at the desired concentration in complete medium (RPMI 1640 Medium + 25 mM HEPES + L-Glutamine, supplemented with 1% of Non-essential Amino Acid Solution 100X and 1% Penicillin Streptomycin Solution 100X and 1% FBS (Sigma-Aldrich)).

5.2. Bovine PBMC stimulation with CLA (50:50) mixture

Firstly, the two CLA isomers 9(E),11(Z)-octadecadienoic acid (c9,t11) and 10(E),12(Z)-octadecadienoic acid (t10,c12) (Matreya LLC, State College, PA, USA) were used to

prepare the CLA (50:50) mixture as previously described for bovine monocytes [14] with some minor modifications. Briefly, the mixture containing both CLA isomers in a 50:50 proportion at a final concentration of 50 μM, was prepared in complete medium (1% FBS) when needed for each experiment and it was set as the working concentration for *in vitro* the stimulation of bovine PBMC. Secondly, a total of $15x10^6$ bovine PBMC (1.5 mL) was seeded in triplicates in Corning® tissue-culture treated culture 60 mm dishes (Corning Inc., Costar, Kennebunk, ME, USA) and incubated with 50 μM of CLA (50:50) mixture (100 μL) or 0.014% ethanol (vehicle) as control - this being the same concentration of ethanol found in the 50 μM CLA (50:50) mixture that was used for the two isomers solubilization - overnight at 39 °C in a humified atmosphere with 5% CO_2. Complete medium (1% FBS) was then added to each dish to reach a final volume of 3 mL. Cells treated with only complete medium (1% FBS) (untreated cells) were also used as negative control to evaluate the effects of 0.014% ethanol on the cells' proteome. Finally, according to the results of our previous study, ethanol at this concentration did not affect the cells viability as assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetra-zolium bromide (MTT)-based assay [14].

5.3. Bovine PBMC collection and protein extraction

The cells' supernatant (3mL) that contain the cells in suspension (lymphocytes), was collected into two tubes of 1.5 mL. Cells were centrifuged at 500 x g for 7 min at 4°C to pellet the lymphocytes, which were then washed twice with 500 μL sterile-PBS without Ca^{2+} and Mg^{2+} (room temperature) and again centrifuged twice at 500 x g for 7 min at 4°C to remove the rest of medium and of FBS that could interfere with the proteomic analysis. In the meantime, three washes with 2 mL of sterile-PBS without Ca^{2+} and Mg^{2+} (room temperature) were performed in the 60 mm dishes to wash the adhered monocytes, and remove dead cells and rest of contaminant medium and FBS. When both, the lymphocytes and monocytes were washed, 500 μL of Igepal buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA and EGTA, 100 mM NaF, 4 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 2 mM Na_3VO_4, 1% Triton X100, 0.5% IGEPAL CA-630 (Sigma-Aldrich), 1 pill for 50 mL of total volume of Protease Inhibitor Cocktail (Roche)) was added to the lymphocytes pellet, the cells were resuspended and then collected and added directly to the monocytes in the 60 mm dishes. The dishes were then carefully mixed to make sure all of the surface was covered with Igepal buffer and incubated for 1 h at 4°C with regular shaking to lyse the cells. After incubation, the cells were scraped using sterile Corning Cell scrapers

(blade L 1.8 cm, handle L 25 cm; Corning Inc.), the cell lysate was collected in 1.5 mL tubes and sonicated for 10 min in an ultrasonic bath (Branson 2200, Danbury, CT, USA) to complete the cells' lysis and homogenization. Finally, the tubes with the cell lysate were centrifuged for 10 min at 6000 x g at 4°C to remove the cell debris, and the supernatant containing the extracted proteins of all 3 technical replicates for each biological replicate was pooled in 1.5 mL tubes, aliquoted and stored at -80 °C for further analyses.

5.4. Sample preparation for proteomic analysis

Total protein concentration was first determined with the Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's instructions. For peptide preparation, extracted proteins were digested and concentrated using the Filter Aided Sample Preparation (FASP) method, following the manufacturer's instructions with some minor modifications. Briefly, fifty µg of proteins lysate were put into Milipore 10 kDa MWCO filters and 100 µl of Urea 8 M in 0.1 M Tris-HCl pH 8.5 were added for solubilization. The detergent together with other contaminant components within the protein lysate were removed by repeated filtration upon aid of centrifugation. After discarding the flow-through, proteins were reduced with 100 µl DTT (10 mM) for 46 min and centrifuged at 14000 rpm for 10 min. Then, 200 µl of 25 mM iodoacetamide solution were added to the filters and incubated for 20 min at room temperature for protein alkylation. After performing several washes of the filters with Urea 8 M and 50 mM of ammonium bicarbonate, proteins were digested with 200 µl of 0.25 mg/mL trypsin (50:1 ratio of protein:enzyme) and mixed at 600 rpm in a thermomixer for 1 min. Filters were then transferred to new collection tubes, incubated in a wet chamber at 37 °C overnight. Finally, peptides were eluted by adding 200 µl of 50 mM of ammonium bicarbonate and the filtrate lyophilized in a SpeedVac and then resuspended in 100 µl of 1% formic acid.

5.5.Nano-LC-MS/MS analysis

For each sample 50µg of proteins was used according to the FASP preparation. Final volume was adjusted exactly to 300 µL with a recovery solution (H_2O/ACN/TFA – 94.95/5/0.05). After passing through the ultrasonic bath (10 min), the entire supernatant was transferred to an HPLC vial prior to LC-MS/MS analysis. Peptides mixtures were

analysed by nano-LC-MS/MS (ThermoFisher Scientific) using an Ultimate 3000 system coupled to a QExactive HF-X mass spectrometer (MS) with a nanoelectrospray ion source. Five µL of hydrolyzate was first preconcentrated and desalted at a flow rate of 30 µl/mn on a C18 pre-column 5 cm length x 100 µm (Acclaim PepMap 100 C18, 5µm, 100A nanoViper) equilibrated with Trifluoroacetic Acid 0.05% in water. After 6 min, the concentration column was switched online with a nanodebit analytical C18 column (Acclaim PepMap 100 - 75 µm inner diameter × 25 cm length; C18 - 3 µm -100Å - SN 20106770) operating at 400 nL/min equilibrated with 96 % solvent A (99.9 % H2O, 0.1 % formic acid). The peptides were then separated according to their hydrophobicity thanks to a gradient of solvent B (99.9 % acetonitrile, 0.1 % formic acid) of 4 to 25% in 60 minutes. For MS analysis, eluted peptides were electro sprayed in positive-ion mode at 1.6 kV through a Nano electrospray ion source heated to 250°C. The mass spectrometer operated in data dependent mode: the parent ion is selected in the orbitrap cell (FTMS) at a resolution of 60,000 and each MS analysis is succeeded by 18 MS/MS with analysis of the MS/MS fragments at a resolution of 15,000).

2.5. Processing of raw mass spectrometry data

At the end of the LC-MS/MS analysis, for raw data processing, MS/MS ion search was carried out with Mascot v2.5.1 (http://www.matrixscience.com) against the bovine database (i.e. ref_bos_taurus 20210114-37,512 sequences). The following parameters were used during the request: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C), oxidation (M) and deamidation (NQ) set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identity above Mascot scores with a False Discovery Rate of 1%. Ions score is -10 log(P), where P is the probability that the observed match is a random event. For the bovine proteome, the Mascot score was 32 with a False Discovery Rate (FDR) of 1%. The adjusted p-value was 0.02561. Finally, for label-free protein quantification analysis, LC-Progenesis QI software (version 4.2, Nonlinear Dynamics, Newcastle upon Tyne, UK) was used with the same identification parameters described above with the phenotypic data among all matrix. All unique validated peptides of an identified protein were included and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. LC-Progenesis analysis yielded a total of 1608 unique quantifiable

proteins, having at least two peptides and two unique peptides. Finally, for sample 1 (corresponding to animal 1) from the untreated group it was not possible to quantify any proteins, so for further statistical and bioinformatic analyses this sample was eliminated.

5.6.Statistical analyses

2.7.1. Univariate analysis

To identify the differential abundant proteins a univariate statistical approach was performed on all 1608 quantified proteins, using an R package for proteomic analysis (available on GitHub with the DOI https://doi.org/10.5281/zenodo.2539329) as previously described [18]. Briefly, data normality distribution was assessed by the Shapiro-Wilk-Test. Then, either a paired Student's t-test (normally distributed data) or a paired Wilcoxon test (not normally distributed data) was applied to determine the p-value between the different treatment groups comparisons (CLA vs Vehicle, CLA vs. Untreated and Vehicle vs Untreated). Finally, the obtained p-values were corrected using different adjustment methods (e.g. Benjamini & Hochberg or FDR, Hochberg and Bonferroni). For all of these analyses R version 3.6.3 was used.

2.7.2. Multivariate analysis

As no differentially abundant proteins were obtained after performing the p-value corrections, an alternative supervised multilevel sparse variant partial least square discriminant analysis (sPLS-DA) was applied, using the mixOmics package in R. The multilevel sPLS-DA enables the selection of the most predictive or discriminative proteins in the data of the two components (PC1 and PC2) to classify or cluster the samples between the three different treatment groups [19].

2.8. Bioinformatic analysis of discriminant proteins selected by sPLS-DA

First, before performing the bioinformatic and functional annotation analyses, the accession numbers from all 1608 quantified proteins were converted into Gene ID using UniProt retrieve/ID mapping online tool. For undefined proteins, the Gene ID of human orthologs of bovine proteins were assigned. Second, only for the selected discriminant proteins a gene ontology (GO) enrichment analysis, focused on the Biological Processes (BP) was performed using ProteINSIDE online tool version 2.0 (available at https://umrh-bioinfo.clermont.inrae.fr/ProteINSIDE_2/index.php?page=upload.php), as previously described [20], and using *Bos Taurus* as reference specie. Only significant (FDR < 0.05)

GO BP terms were considered as enriched and were used for further interpretation and figure creation. The enriched GO BP terms were summarized and their enrichments expressed as -log10 (FDR) for visualization on horizontal bar graphs plotted using GraphPad Prism 9.1.2 for Mac OS X, GraphPad Software (San Diego, California, USA). Finally, a hierarchical clustering heat map analysis was performed to illustrate the relative abundance of all the 407 DP selected by sPLS-DA using the clustered image maps (cim) function from mixOmics package in R (http://mixomics.org/graphics/cim/). Hierarchical clustering of both proteins and samples was performed, and dendrograms were used along the left and upper axes to show how each protein and sample clusters based on their similarity and the hierarchical clustering method used. Pearson correlation and Ward methods were used as the distance and cluster methods, respectively.

Finally, the average log fold-change (log FC) of the raw abundances of all the 407 DP from the CLA mixture samples was calculated respect only to those of the vehicle group, and plotted using R version 3.6.3.

6. Results

6.1. The proteome of bovine PBMC and identification of the molecular signature of CLA (50:50) mixture

A total of 1608 proteins, with at least two unique peptides, were identified and quantified in bovine PBMC (data not shown). A classical univariate statistical analysis (paired t-test or Wilcoxon test) was applied and no differentially abundant proteins were detected between the CLA (50:50) mixture and the other treatment groups after adjusting the p-value (FDR and Bonferroni). However, in order to retrieve meaningful biological information, the multivariate sPLS-DA analysis was applied to identify the molecular signature of CLA (50:50) mixture treatment. The sPLS-DA model selected a total of 407 DP (approximately 25% of the total proteins identified) from both components (data not shown), which contributed to a good clustering between the different treatment groups (Fig. 1). From the 407 DP, only the proteins from the sPLS-DA component 1 that strongly correlated together (data not shown) for each treatment group were selected to perform separate GO BP enrichment analyses. From these proteins, a total of 85 proteins had the highest abundance in the CP group, while 96 and 27 in the vehicle (ethanol) and untreated groups, respectively (data not shown).

6.2. GO enrichment analyses of total DP identified by sPLS-DA

In order to identify the global function of all the 407 DP a GO enrichment analysis was performed. These proteins were annotated by 237 enriched (FDR < 0.05) GO terms within the BP category. The main enriched BP were related to cellular RNA splicing (GO:0008380), mRNA processing (GO:0006397), organonitrogen compound metabolic process (GO:1901564), negative regulation of proteolysis (GO:0045861), metabolic process (GO:0008152), mitochondrial electron transport, NADH to ubiquinone (GO:0006120), immune system process (GO:0002376), negative regulation of endopeptidase (GO:0010951) and catalytic activity (GO:0043086) (Fig. 2). Among the most annotated BP were: metabolic process (269), organonitrogen compound metabolic process (91), immune system process (86) and negative regulation of catalytic activity (34) (Supplementary Table S1).

6.3. GO enrichment analyses of DP with highest abundance in CLA (50:50) mixture group

To further elucidate the specific role of the DP with highest abundance in each group, separate GO enrichment analyses were performed using only those DP selected in component 1 from the sPLS-DA that strongly correlated. The 85 proteins with the highest abundance in CLA (50:50) mixture group were annotated by 314 enriched (FDR < 0.05) GO terms within the BP category. These proteins were mainly involved in BP such as: negative regulation of endopeptidase (GO:0010951) and peptidase activity (GO:0010466), negative regulation of proteolysis (GO:0045861) and of hydrolase (GO:0051346) and catalytic activity (GO:0043086), acute-phase response (GO:0006953), complement activation (GO:0006956), lipid metabolic process (GO:0006629), response to oxidative stress (GO:0006979) and reactive oxygen species metabolic process (GO:0072593). From these enriched BP, the ones with the highest number of annotated proteins were: negative regulation of catalytic activity (20), of proteolysis (18) and of endopeptidase, peptidase and hydrolase activity (17) (Fig. 3). ITIH1, ITIH2, ITIH4, SERPINA3-7, SERPINA7, SERPINC1, SERPIND1 SERPING1, A2M, C3, C4B were some of the annotated proteins in all of the most enriched BP. Proteins ITIH4, agp and F2 were found in acute-phase response; and C3, C4A, CFB were also found in complement activation. C3, CAT, APOA4, APOE, ACADVL and CPT1A were annotated in lipid metabolism, while APOA4, APOE and CAT were also annotated in the response to oxidative stress and reactive oxygen species metabolic process. Finally,

HMOX1 and HMOX2 were exclusively annotated in the response to oxidative stress (Supplementary Table S2).

6.4. GO enrichment analyses of DP with highest abundance in vehicle group

In total, 96 DP were found to be more abundant in the vehicle group. These proteins were annotated by a total of 66 enriched (FDR < 0.05) BP GO terms (Supplementary Table S6). These enriched GO terms were mainly related to: RNA processing (GO:0006396) and splicing (GO:0008380), mRNA metabolic process (GO:0016071), mRNA splicing, via spliceosome (GO:0000398), protein-containing complex subunit organization (GO:0043933), gene expression (GO:0010467), regulation of cytokine production (GO:0001817), translation (GO:0006412) and regulation of interleukin-6 production (GO:0032675) (Fig. 4). The enriched BP with the highest number of annotated proteins were gene expression (47), protein-containing complex subunit organization (24) and RNA processing (22). AIF1 was found in the two most annotated BP, gene expression and protein-containing complex subunit organization, but also in regulation of cytokine production and the regulation of interleukin-6 production, among other proteins (Supplementary Table S3).

6.5.GO enrichment analyses of DP with highest abundance in untreated group

In the untreated group, 27 DP were found to be more abundant and were annotated by a total of 41 enriched (FDR < 0.05) BP GO terms. These enriched GO terms were mainly related to: RNA processing (GO:0006396), RNA splicing (GO:0008380), snoRNA localization (GO:0048254), mRNA metabolic process (GO:0016071), mRNA processing (GO:0006397), positive regulation of double-strand break repair via nonhomologous end joining (GO:2001034), cellular aromatic compound metabolic process (GO:0006725), nucleic acid metabolic process (GO:0090304) and ribosome biogenesis (GO:0042254) (Fig. 5). The enriched BP with the highest number annotated proteins were related with nucleic acid metabolic processes (14), cellular aromatic compound metabolic process (14) and RNA processing (9) (Supplementary Table S4).

6.6. Hierarchical clustering heat map analysis of all the DP identified with sPLS-DA

A hierarchical clustering heat map analysis was performed to illustrate the relative abundance of all 407 DP (Fig. 7). Both, proteins and samples were clustered by similarity as indicated by the left and upper dendrograms, respectively. Samples were sorted in two main clusters (upper dendrogram), the first cluster contained mainly samples from the CLA group (green) with the exception of two vehicle group (orange) samples (Cows 3 and Cow 5) that also clustered with them, while the second cluster contained most of all the samples from vehicle and untreated groups (blue), except for one from CLA group (Cow 5). Moreover, the 407 DP proteins were also sorted in two main clusters (left dendrogram), cluster 1 (red) contained 250 DP (data not shown) that showed higher abundance (red) mainly in the CLA group; while cluster 2 (light blue) had 157 DP (data not shown) that showed a lower abundance in the CLA group respect to the vehicle and untreated groups. Altogether, these results approved the DP selected by sPLS-DA, as overall different protein abundance profiles were observed between the CLA group and the vehicle and untreated groups, that in the other hand had similar protein abundance profiles.

6.7. GO enrichment analyses of the DP clustered by hierarchical clustering heat map analysis

Separate GO enrichment analyses were again performed using the DP from the two clusters generated by the hierarchical clustering analysis to further confirm their differential role. In cluster 1, the 250 DP were annotated by a total of 342 enriched (FDR < 0.05) BP GO terms. The enriched GO terms were very similar to the previous ones observed for the 85 DP - coming only from component 1 - with highest abundance in CLA group (Fig. 3), as they were mainly related to: negative regulation of endopeptidase (GO:0010951) and peptidase activity (GO:0010466), and of proteolysis (GO:0045861), immune system process (GO:0002376) and lipid metabolic process (GO:0006629), among others (Fig. 8A). On the contrary, in cluster 2, the 157 DP were annotated by a total of 164 enriched (FDR < 0.05) BP GO terms, and those BP were very similar to those found previously for the DP with the highest abundance in both, vehicle and untreated groups (Fig. 5 and Fig. 6). They were mainly related to: regulation of RNA splicing (GO:0043484), regulation of mRNA metabolic process (GO:1903311), regulation of mRNA processing (GO:0050684), cellular amide metabolic process (GO:0043603), translation (GO:0006412), ribosome biogenesis (GO:0042254), gene expression (GO:0010467) and interleukin-6 production (GO:0032635) (Fig. 8B) (Supplementary

Tables S5 and S6). In conclusion, these results further elucidated and confirmed the specific role of the DP selected by sPLS-DA.

6.8. Average log fold-change (log FC) of the abundances of the DP identified with sPLS-DA

The changes (*logFC*) in the abundance of all the 407 DP between the CLA (50:50) mixture and vehicle group is illustrated in Fig. 6. The protein with the greatest change in abundance (logFC > 20) was PLIN2. Other proteins with higher abundances in CLA group were found, including CD36 (logFC > 5); VCAN, SERPINA3-7, SDS, LUM (logFC > 2). On the other hand, proteins such as APOA2, PNKP, GBP2 and RBM1B showed the lowest abundance in CLA group (logFC < 1).

7. Discussion

The synergic immunomodulatory and anti-apoptotic effects of the CLA (50:50) mixture on bovine mononuclear cells, specifically monocytes, has been previously reported *in vitro* [14]. However, the exact molecular mechanisms underlying such effects have still not been identified. This study presents for the first time the capacity of CLA (50:50) mixture in modulating bovine PBMC proteome and we propose a potential molecular signature of CLA treatment. Our main findings were that CLA (50:50) mixture caused an enrichment in BP related to negative regulation of proteolysis and endopeptidases activity, potentially explaining the anti-apoptotic activity observed in our previous *in vitro* study on bovine monocytes [14]. Moreover, CLA mixture also enriched immune-related processes, such as acute-phase response, complement activation, response to oxidative stress and ROS metabolism, suggesting a potential role in modulating bovine immune defense response.

7.1. Global biological processes enriched by the DP selected by sPLS-DA

In the first part of the study, we aimed at identifying the molecular signature of CLA treatment, using the sPLS-DA method that allows the selection of the most discriminative proteins in the data to cluster the samples [19]. In other words, in our study the sPLS-DA selected 407 discriminant proteins that allowed an adequate clustering of the samples from the CLA, vehicle and untreated groups, and therefore a clear separation between

them was observed. Once the DP were identified, a GO enrichment analysis was performed to determine their global function, and it revealed that the proteins were involved in both, very general BP processes related to RNA splicing, processing and several metabolic processes, but also related to immune system processes such as negative regulation of proteolysis, endopeptidase and catalytic activity. Proteolysis – the intracellular protein degradation – is critical for the immune response and elimination of invading pathogens [21]. Indeed, endopeptidases - a class of proteolytic enzymes – have shown to play important roles in inducing robust immune responses [22].

7.2. Biological processes enriched by DP with the highest abundance in CLA (50:50) mixture group on bovine PBMC

In the second part of the study, the specific function of the DP identified with the highest abundance in each treatment group was investigated by performing separate GO enrichment analyses. The 85 DP with the highest abundance in the CLA (50:50) mixture group caused an enrichment in BP related mostly to immune system processes such as: negative regulation of proteolysis, and of endopeptidase, hydrolytic and catalytic activity, acute-phase response, complement activation, response to oxidative stress and ROS metabolism.

Proteolysis, as previously mentioned is essential for many cell functions, including the elimination of defective proteins, supply of free amino acids as energy source or for synthesis of essential proteins, immune response, cell proliferation and apoptosis [21]. Indeed, the proteolytic cleavage of specific cellular proteins is a recognized central biochemical feature of apoptosis [23]. Caspases are the main proteolytic executioner enzymes of apoptosis [24]. Caspases are aspartate-specific cysteine endopeptidases that hydrolyze peptide bonds in a reaction that depends on catalytic cysteine residues in the caspase active site and occurs only after certain aspartic acid residues in the substrate proteins [25]. These endopeptidases can be found expressed in most cells as an inactive proenzyme, and once they activate they can also activate other caspases starting a proteolytic cascade, amplifying the apoptotic signaling pathway in an irreversible manner [23]. However, it is also known that non-caspase proteins like other cysteine proteases (e.g. calpains), serine proteases, granzymes and the proteasome-ubiquitin pathway can modulate apoptosis [26,27].

Remarkably, all of these BP related to proteolysis and the activity of proteolytic enzymes like endopeptidases were negatively regulated after CLA (50:50) mixture, which could

potentially explain the anti-apoptotic effects of CLA mixture that we previously observed on bovine monocytes *in vitro* [14]. Indeed, most of the proteins annotated in these BP, including, SERPINA3-7, SERPINA7, SERPINC1, SERPIND1, SERPING1, ITIH1, ITIH2, ITIH4 and A2M are indeed known for their serine endopeptidases inhibitor activity.

Serpins are a superfamily of proteins found in all organisms that are recognized by their common and principal function as serine protease inhibitors [28]. The large serpin family is divided into nine first clades (A-I) based on their sequence similarities, with a variety of members being in each clade. The clades with the largest number of members are the serpin A and B, known as antitrypsin-like and ovalbumin-like serpins, respectively [29]. These functionally diverse proteins are ubiquitously expressed and are involved in multiple processes such as blood coagulation [30], hormone transport [31], immune and inflammatory response (e.g. acute-phase response and complement system activation) [32], and apoptosis [33,34]. Indeed, the α1-Antichymotrypsin protein (SERPINA3) – one of the most well studied and abundant serpins- is known to play important role in modulating the immune and inflammatory response, such as inhibiting cytotoxic T-cells activity, enhancing antibody response and inhibiting serine proteases involved in inflammation and leukocyte-derived proteases [35–38]. In humans, this protein is encoded by the single SERPINA3 gene, but in cattle it is encoded by a cluster of eight closely related genes (SERPINA3-1 to SERPINA3-8) [29]. In the present study, SERPINA3-7 – also known as endopin 2– was identified as one of the DP with higher abundance in CLA group. Interestingly, previous studies have confirmed the inhibitory role of this protein towards serine and cysteine proteases in chromaffin cells [39,40], suggesting a potential anti-apoptotic effect, which has indeed already been attributed to other bovine SERPINA3 proteins (SERPINA3-1 and SERPINA3-3), as caspase 8 and 3 inhibitors [41].

Several members of another family of plasma serine endopeptidase inhibitors (ITIH1, ITIH2 and ITIH4) were also found with higher abundance after CLA mixture treatment. The inter-alpha (globulin) inhibitor (ITI) family proteins – also known as inter-alpha-trypsin inhibitors – besides exerting the protease inhibitor functions, have also shown to contribute to extracellular matrix stabilization, cell signaling, immune response, inflammation, carcinogenesis and apoptosis [42,43]. Indeed, some of ITIH proteins have been demonstrated to be both negative and positive acute-phase proteins in humans [44]. In cattle, the inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) – also known as IHRP –

is a well-known positive acute-phase protein that its expression increases after bacterial and viral infections, and it might have a role in modulating cell migration and proliferation during the acute-phase response [45], which explains why it was found also annotated in the enriched BP of acute-phase response. Moreover, in a previous study this protein inhibited actin polymerization and thus the phagocytic capacity of polymorphonuclear cells (PMN), suggesting anti-inflammatory roles [46]. Finally, ITIH4 has also shown to have anti-apoptotic effects on alveolar epithelial cells submitted to air pollution, as its inhibition hampered apoptosis [47]. The potential anti-apoptotic role of this protein has also been indirectly assumed previously, as it often, together with other ITIH proteins, are downregulated in many types of human cancer [42,43].

The DP with the highest abundances after CLA (50:50) mixture also enriched critical processes for the host immune defense such as: acute-phase, complement activation, response to oxidative stress and ROS metabolism. The acute phase response is the immediate innate immune defense against acute illness (e.g., infection or tissue damage), and is mediated by acute-phase proteins (APP), a group of approximately 40 plasma that share a common protective role from injury, which concentration can increase (positive APP) or decrease (negative APP) during inflammation [48]. Among the annotated proteins found in these first two BP were recognized several positive APP proteins such as: alpha-1-acid glycoprotein (agp), prothrombin (F2), complement 3 (C3), complement component 4A (C4A) and component factor B (CFB), among others [48,49]. The protein α2-macroglobulin (A2M), another known positive APP was not annotated in these BP, but was found in those related with negative regulation of proteolysis and of proteases activity, which makes sense as it is a recognized proteases inhibitor that has shown to exert anti-apoptotic effects in macrophages infected with *T.cruzi in vitro* [50,51]. Overall, all of these proteins play different important roles in the immune defense, including immune cell recruitment, proteolytic enzymes inactivation, complement activation, opsonization and elimination of infectious agents and limit tissue damage by scavenging free radicals and giving negative feedback to the inflammatory response [48,52]. Our results agree with previous studies in pigs supplemented with CLA diets, which increased the abundance of APP such agp [53], suggesting that CLA might play an important role in bovine immune and inflammatory response.

Finally, lipid metabolic process and other two critical immune related processes, response to oxidative stress and ROS metabolic process were also found enriched. In the lipid metabolic process, proteins involved with fatty acid beta oxidation, lipid binding,

transport and lipid absorption, including the very long-chain specific acyl-CoA dehydrogenase, mitochondrial (ACADVL), carnitine O-palmitoyltransferase (CPT1A), apolipoprotein A-IV (APOA4), apolipoprotein E (APOE) and catalase (CAT), were found annotated, respectively [54–57]. In the oxidative stress response and ROS metabolism, besides APOA4, APOE and CAT, also HMOX1 and HMOX2, were found among other proteins. The fact that proteins involved in lipid metabolism are also annotated in these GO BP terms could be explained by the potential that both mitochondrial and peroxisomal β-oxidation of fatty acids have to generate ROS (e.g. H_2O_2) [58]. Finally, heme oxygenase 1 (HMOX1) and 2 (HMOX2) are microsomal enzymes with known anti-oxidant and anti-inflammatory roles involved in heme group degradation. HMOX1 can be induced by biological, chemical and physical stresses, making it directly involved in the oxidative stress response to try to counteract or reduce inflammation, while HMOX2 is constitutively expressed [59]. HMOX1, has indeed demonstrated to have anti-inflammatory roles in macrophages *in vivo* and *in vitro* [60]. In addition, HMOX1 overexpression has shown to have anti-apoptotic and cytoprotective effects on cancer cells [61], fibroblasts [62] and endothelial cells [60]. The increase in abundance of anti-oxidant, anti-apoptotic and cytoprotective proteins against oxidative stress its remarkable, especially as in our previous *in vitro* study on bovine monocytes treated with CLA (50:50) mixture an increase in ROS production was observed [14].

Altogether, these results suggest that CLA (50:50) mixture might exert anti-apoptotic, anti-oxidant and cytoprotective effects on bovine PBMC, and that it modulates the cells immune response by upregulating the abundance of proteins involved in the negative regulation of proteolysis and endopeptidases activity, response to oxidative stress and ROS metabolism, acute-phase response and complement activation.

7.3.Biological processes enriched by DP *with the highest abundance in vehicle and untreated groups on bovine PBMC*

The 96 and 27 DP with highest abundances in vehicle and untreated group, respectively were mined using ProteINSIDE to determine the most enriched GO BP terms. Most of the enriched BP in both groups were shared or similar, and included very unspecific and basic GO terms, related mainly with RNA processing and splicing, mRNA and other metabolic processes, gene expression, translation and ribosome biogenesis, suggesting that both control treatments exerted a similar effect on bovine PBMC. Overall, we could assume that no major modulation in the immune response was caused by both treatments,

as almost no BP related with immune system processes were found, with the exception in the vehicle treated group where the regulation of interleukin-6 (IL-6) production was found enriched. IL-6 is a cytokine with pleiotropic functions in inflammation and immune response, acting as both a pro-inflammatory and anti-inflammatory cytokine [63,64]. Its abundance seems to increase during ethanol consumption, and it has shown to act as a potential endogenous cytoprotective cytokine, protecting murine hepatocytes against ethanol-induced apoptosis *in vivo* [65]. One of the proteins annotated by this BP is the allograft inflammatory factor 1 (AIF1), a cytoplasmic protein that is involved mainly in the activation of macrophages and enhancing the production of pro-inflammatory cytokines. This protein seems to be expressed in other immune cells such as T cells, neutrophils and microglia [66]. In human PBMC and murine macrophages, AIF1 expression has been positively correlated with IL-6 and chemokines secretion, suggesting an important role in the activation and inflammatory response of these cells [67,68]. Moreover, our results agree to those reported in other models such as in microglia cells – the only macrophage population in the central nervous system – from ethanol treated mice, where an increase in the mRNA levels of AIF1 was found [69].

In conclusion, these results made evident that after CLA (50:50) mixture treatment there was a functional switch from very basic BP to others highly related with different immune related processes, and that even though in both vehicle and untreated groups mostly redundant functions from the DP were found, ethanol treatment did cause a differential effect on bovine PBMC immune response.

7.4. Biological processes enriched by DP clustered by hierarchical clustering heat map analysis

To further illustrate the abundance profiles of all the 407 DP, a hierarchical clustering analysis was performed. Overall, two main clusters of the samples were appreciated: in the first one mainly CLA treated samples were grouped together with the exception of two vehicle treated samples, while in the second mostly the untreated and vehicle treated samples grouped together with the exception of one CLA sample. These results made evident that mostly two differential protein abundance patterns were observed between the CLA group and the other two control groups, vehicle and untreated groups, which were more similar to each other. Indeed, these results further confirmed the functional similarity or redundancy that was previously observed on the separate GO enrichment analyses between the vehicle and untreated groups, and the difference when compared to

the CLA group. However, it is thought that the clustering of the samples was not completely perfect, as all the 407 DP, selected in both components from sPLS-DA, were used and not only the ones that strongly correlated between each other in component 1 as it was done with the first separate GO enrichment analyses. Moreover, the high biological variability between the animals could have also played a role in it.

Nevertheless, to further confirm the differential roles of the DP that were grouped in two main clusters, separate GO analyses were performed again. Remarkably, the 250 DP from cluster 1, which had mostly a higher abundance in the CLA group, enriched GO BP related to negative regulation of proteolysis and endopeptidase activity, immune system process and lipid metabolism. On the contrary, the 157 DP from cluster 2, which had mostly a higher abundance in vehicle and untreated groups, enriched BP related to regulation of RNA splicing, mRNA processing and metabolic process, ribosome biogenesis and interleukin-6 production, among others. These results just further confirmed the differential effects that CLA (50:50) mixture caused on bovine PBMC already described herein.

7.5. Key DP with greater changes in abundance after CLA (50:50) mixture treatment

Lastly, to further elucidate the effects of CLA (50:50) mixture on bovine PBMC proteome and determine its molecular signature, we aimed at identifying key DP that showed greater changes in their abundances (logFC) when treated with CLA respect only to vehicle group. This decision was taken because the vehicle group corresponds to the most 'immediate' control group as it has the same percentage of ethanol used for solubilizing the CLA isomers, and no major functional differences were detected when compared to the untreated group.

The protein with the highest abundances after CLA treatment were perilipin-2 (PLIN2) and platelet glycoprotein 4 or scavenger receptor-class B (CD36). PLIN2 is one of the major lipid droplet-associated proteins that is expressed ubiquitously [70]. Lipid droplets work not exclusively as lipid storage organelles, but also important roles in immunity and inflammation have been attributed as reviewed elsewhere [71,72]. Additionally, other roles of PLIN2 in immunity have been reported previously, such as in the innate immune response against bacterial infections and modulating chemokines production on monocytes [73,74]. Its expression has shown to be upregulated in different cell types supplied with fatty acids, and it is known that peroxisome proliferator-activated receptor α (PPARα), γ (PPARγ) and δ (PPARδ) – key regulators of lipid metabolism and

inflammation - are the responsible for its induction and regulation of expression [75–77]. In macrophages, PPAR γ and PPARδ activation indeed demonstrated to regulate of the expression of PLIN2 and of CD36, and PPARγ of PLIN2 in human monocytes [76,78,79]. CD36, is a scavenger receptor that plays a role in the uptake or import of long-chain fatty acids, with similar expression regulatory mechanisms of PLIN2 [78,79]. Moreover, it is well known that CLA is among the most potent agonists of PPAR, specially of PPARα and PPARγ that are mostly active in macrophages [80]. Indeed, several of the immunomodulatory and anti-inflammatory effects of CLA seem to be mediated by the PPAR signaling pathways [80,81]. Our results agree with a previous study where an increase in the mRNA abundance of PLIN2 and a modulation of lipid droplet formation was observed after caprine monocytes were treated *in vitro* with two omega-3 long-chain polyunsaturated fatty acids (PUFA), namely DHA and EPA [82]. Moreover, in human monocytes the addition of γ-linolenic acid (omega-6 PUFA) and EPA also increased the mRNA and protein expression of PLIN2 [78]. Similarly, preadipocytes stimulated with long-chain fatty acids also exhibited an increase the expression of both PLIN2 and CD36 [83,84]. In *in vivo* studies, the t10,c12 CLA isomer increased the gene expression of PLIN2 and CD36 in CLA-fed rats [85], while no effects on their expression in the mammary tissues of t10,c12 CLA-fed cows were observed [86]. However, at the best of our knowledge no reports on the effects of CLA isomers alone or in mixture on the abundance of these two proteins on bovine PBMC are available yet.

More discrete increases on other proteins abundance were observed such as: lumican (LUM) and versican core protein (VCAN), both extracellular matrix proteins involved in the regulation of innate immune responses and inflammation [87,88]; L-serine dehydratase/L-threonine deaminase (SDS), a cytosolic enzyme involved in gluconeogenesis and lipid metabolism [89]; and the aforementioned SERPINA3-7 that is involved in the inhibition of endopeptidases activity [29].

On the contrary, proteins that showed a lower abundance in the CLA group were: apolipoprotein A-II (APOA2); involved in lipid transport and metabolism, which expression has shown to increase after alcohol ingestion [90]; guanylate binding protein 2 (GBP2), is a interferon (IFN)-inducible GTPase that is an important mediator of host immune defenses and inflammation [91]; RNA binding motif protein 12B (RBM12B), a RNA binding protein involved in RNA splicing; and polynucleotide kinase 3'-phosphatase (PNKP), a DNA binding protein involved in DNA repair [92].

8. Conclusions

The results of this study demonstrate for the first time that the mixture (50:50) of the two main CLA isomers (c9, t11 and t10, c12) does modulate bovine PBMC proteome *in vitro*, and elucidate the molecular signature of CLA treatment. The GO analysis revealed that CLA (50:50) mixture caused an enrichment in biological processes related mainly to negative regulation of proteolysis and of endopeptidases activity, which are critical biochemical features of apoptosis, acute-phase response, complement activation, oxidative stress response and ROS metabolism, confirming the anti-apoptotic and immunomodulatory effects observed in our previous *in vitro* study on bovine monocytes. Moreover, our results suggest that CLA (50:50) mixture might also exert potential cytoprotective effects against oxidative stress, by increasing the abundance of proteins with anti-oxidant capacity. By revealing some of the molecular mechanisms underlying the immunomodulatory effects of CLA mixture, we pretend to have a clearer idea of its potential biological significance *in vivo,* thus allowing a better management for its inclusion in nutrition strategies to enhance the animals immune and health status. However, to deepen the knowledge on the overall impact of CLA (50:50) mixture on bovine immunity, it is necessary to perform further molecular pathway enrichment and protein-protein interaction analyses, and integration of proteomics data with other OMIC technologies.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper's content.

Ethics statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The procedures for the blood collection were carried out during routine slaughtering procedures.

Acknowledgements

The authors acknowledge A. Delavaud (INRAE, Herbivore Research Unit) for his technical assistance in protein extraction, quantification, and concentration for mass spectrometry analyses, and also Arnaud Cougoul, Jeremy Tournayre and Céline Boby (INRAE, Herbivore Research Unit) for their assistance in the statistical and bioinformatic

analyses, respectively. We acknowledge also Valentina Zamarian (Department of Veterinary Medicine and Animal Science, Università Degli Studi di Milano, Lodi, Italy) for her valuable support in the proteins' extraction from bovine PBMC.

Funding

This study was supported by the European Union's Horizon 2020 research and innovation programme H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate) [Grant agreement nº: 765423, 2017] – MANNA.

References

[1] A. Bhattacharya, J. Banu, M. Rahman, J. Causey, G. Fernandes, Biological effects of conjugated linoleic acids in health and disease, J. Nutr. Biochem. 17 (2006) 789–810. doi:https://doi.org/10.1016/j.jnutbio.2006.02.009.

[2] M. Viladomiu, R. Hontecillas, J. Bassaganya-Riera, Modulation of inflammation and immunity by dietary conjugated linoleic acid, Eur. J. Pharmacol. 785 (2016) 87–95. doi:https://doi.org/10.1016/j.ejphar.2015.03.095.

[3] M.W. Pariza, Y. Park, M.E. Cook, The biologically active isomers of conjugated linoleic acid, Prog. Lipid Res. 40 (2001) 283–298. doi:https://doi.org/10.1016/S0163-7827(01)00008-X.

[4] H.-J. Song, I. Grant, D. Rotondo, I. Mohede, N. Sattar, S.D. Heys, K.W.J. Wahle, Effect of CLA supplementation on immune function in young healthy volunteers, Eur. J. Clin. Nutr. 59 (2005) 508–517. doi:10.1038/sj.ejcn.1602102.

[5] L. Renner, J. Pappritz, R. Kramer, S. Kersten, G. Jahreis, S. Dänicke, Fatty acid profile and proliferation of bovine blood mononuclear cells after conjugated linoleic acid supplementation, Lipids Health Dis. 11 (2012) 63. doi:10.1186/1476-511X-11-63.

[6] E. Galamb, V. Faigl, M. Keresztes, Z. Csillik, A. Tröscher, P. Elek, M. Kulcsár, G. Huszenicza, H. Fébel, F. Husvéth, Effect of pre- and post-partum supplementation with lipid-encapsulated conjugated linoleic acid on milk yield and metabolic status in multiparous high-producing dairy cows, J. Anim. Physiol. Anim. Nutr. (Berl). 101 (2017) 1026–1035. doi:https://doi.org/10.1111/jpn.12544.

[7] K.T. Selberg, A.C. Lowe, C.R. Staples, N.D. Luchini, L. Badinga, Production and Metabolic Responses of Periparturient Holstein Cows to Dietary Conjugated

Linoleic Acid and trans-Octadecenoic Acids*, J. Dairy Sci. 87 (2004) 158–168. doi:https://doi.org/10.3168/jds.S0022-0302(04)73153-7.

[8] M.J. de Veth, D.E. Bauman, W. Koch, G.E. Mann, A.M. Pfeiffer, W.R. Butler, Efficacy of conjugated linoleic acid for improving reproduction: A multi-study analysis in early-lactation dairy cows1, J. Dairy Sci. 92 (2009) 2662–2669. doi:https://doi.org/10.3168/jds.2008-1845.

[9] N. Hanschke, M. Kankofer, L. Ruda, M. Höltershinken, U. Meyer, J. Frank, S. Dänicke, J. Rehage, The effect of conjugated linoleic acid supplements on oxidative and antioxidative status of dairy cows, J. Dairy Sci. 99 (2016) 8090–8102. doi:10.3168/jds.2015-10685.

[10] L.M. Sordillo, Nutritional strategies to optimize dairy cattle immunity1, J. Dairy Sci. 99 (2016) 4967–4982. doi:https://doi.org/10.3168/jds.2015-10354.

[11] L. Renner, S. Kersten, A. Duevel, H.-J. Schuberth, S. Dänicke, Effects of cis-9,trans-11 and trans-10,cis-12 Conjugated Linoleic Acid, Linoleic Acid, Phytanic Acid and the Combination of Various Fatty Acids on Proliferation and Cytokine Expression of Bovine Peripheral Blood Mononuclear Cells, Nutr. . 5 (2013). doi:10.3390/nu5072667.

[12] S. Dänicke, J. Kowalczyk, L. Renner, J. Pappritz, U. Meyer, R. Kramer, E.-M. Weber, S. Döll, J. Rehage, G. Jahreis, Effects of conjugated linoleic acids fed to dairy cows during early gestation on hematological, immunological, and metabolic characteristics of cows and their calves, J. Dairy Sci. 95 (2012) 3938–3953. doi:https://doi.org/10.3168/jds.2011-4879.

[13] W. Liermann, T. Viergutz, K.L. Uken, L. Vogel, M. Gnott, D. Dannenberger, A. Tuchscherer, H. Kienberger, M. Rychlik, A. Tröscher, H.M. Hammon, Influences of Maternal Conjugated Linoleic Acid and Essential Fatty Acid Supply During Late Pregnancy and Early Lactation on T and B Cell Subsets in Mesenteric Lymph Nodes and the Small Intestine of Neonatal Calves , Front. Vet. Sci. . 7 (2020). https://www.frontiersin.org/article/10.3389/fvets.2020.604452.

[14] G. Ávila, C. Catozzi, D. Pravettoni, G. Sala, P. Martino, G. Meroni, C. Lecchi, F. Ceciliani, In vitro effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes, J. Dairy Sci. 103 (2020) 8554–8563. doi:10.3168/jds.2020-18659.

[15] J.D. Lippolis, E.J. Powell, T.A. Reinhardt, T.C. Thacker, E. Casas, Symposium review: Omics in dairy and animal science—Promise, potential, and pitfalls*, J.

Dairy Sci. 102 (2019) 4741–4754. doi:https://doi.org/10.3168/jds.2018-15267.

[16] A. Pardanani, E.D. Wieben, T.C. Spelsberg, A. Tefferi, Primer on medical genomics part IV: expression proteomics, in: Mayo Clin. Proc., Elsevier, 2002: pp. 1185–1196.

[17] G. Kra, N. Nemes-Navon, J.R. Daddam, L. Livshits, S. Jacoby, Y. Levin, M. Zachut, U. Moallem, Proteomic analysis of peripheral blood mononuclear cells and inflammatory status in postpartum dairy cows supplemented with different sources of omega-3 fatty acids, J. Proteomics. 246 (2021) 104313. doi:https://doi.org/10.1016/j.jprot.2021.104313.

[18] J. Bazile, B. Picard, C. Chambon, A. Valais, M. Bonnet, Pathways and biomarkers of marbling and carcass fat deposition in bovine revealed by a combination of gel-based and gel-free proteomic analyses, Meat Sci. 156 (2019) 146–155. doi:https://doi.org/10.1016/j.meatsci.2019.05.018.

[19] K.-A. Lê Cao, S. Boitard, P. Besse, Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems, BMC Bioinformatics. 12 (2011) 253. doi:10.1186/1471-2105-12-253.

[20] N. Kaspric, B. Picard, M. Reichstadt, J. Tournayre, M. Bonnet, ProteINSIDE to Easily Investigate Proteomics Data from Ruminants: Application to Mine Proteome of Adipose and Muscle Tissues in Bovine Foetuses, PLoS One. 10 (2015) e0128086–e0128086. doi:10.1371/journal.pone.0128086.

[21] L. Combaret, D. Taillandier, C. Polge, D. Béchet, D. Attaix, Chapter 3 - Cellular Mechanisms of Protein Degradation Among Tissues, in: D.B.T.-T.M.N. of A.A. and P. Dardevet (Ed.), Academic Press, Boston, 2016: pp. 27–37. doi:https://doi.org/10.1016/B978-0-12-802167-5.00003-7.

[22] S. Maschalidi, S. Hässler, F. Blanc, F.E. Sepulveda, M. Tohme, M. Chignard, P. van Endert, M. Si-Tahar, D. Descamps, B. Manoury, Asparagine Endopeptidase Controls Anti-Influenza Virus Immune Responses through TLR7 Activation, PLOS Pathog. 8 (2012) e1002841. https://doi.org/10.1371/journal.ppat.1002841.

[23] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495–516. doi:10.1080/01926230701320337.

[24] P.J. Utz, P. Anderson, Life and death decisions: regulation of apoptosis by proteolysis of signaling molecules, Cell Death Differ. 7 (2000) 589–602. doi:10.1038/sj.cdd.4400696.

[25] D.R. McIlwain, T. Berger, T.W. Mak, Caspase functions in cell death and disease,

Cold Spring Harb. Perspect. Biol. 5 (2013) a008656–a008656. doi:10.1101/cshperspect.a008656.

[26] K.L. Moffitt, S.L. Martin, B. Walker, The emerging role of serine proteases in apoptosis, Biochem. Soc. Trans. 35 (2007) 559–560. doi:10.1042/BST0350559.

[27] A.R. O'Connell, C. Stenson-Cox, A more serine way to die: Defining the characteristics of serine protease-mediated cell death cascades, Biochim. Biophys. Acta - Mol. Cell Res. 1773 (2007) 1491–1499. doi:https://doi.org/10.1016/j.bbamcr.2007.08.002.

[28] A. Sánchez-Navarro, I. González-Soria, R. Caldiño-Bohn, N.A. Bobadilla, An integrative view of serpins in health and disease: the contribution of SerpinA3, Am. J. Physiol. Physiol. 320 (2020) C106–C118. doi:10.1152/ajpcell.00366.2020.

[29] A. Péré-Brissaud, X. Blanchet, D. Delourme, P. Pélissier, L. Forestier, A. Delavaud, N. Duprat, B. Picard, A. Maftah, L. Brémaud, Expression of SERPINA3s in cattle: focus on bovSERPINA3-7 reveals specific involvement in skeletal muscle, Open Biol. 5 (2022) 150071. doi:10.1098/rsob.150071.

[30] A.H. Schmaier, Serpin targets in hemostasis/kinin formation, Blood. 134 (2019) 1566–1568. doi:10.1182/blood.2019002713.

[31] G.A. Silverman, P.I. Bird, R.W. Carrell, F.C. Church, P.B. Coughlin, P.G.W. Gettins, J.A. Irving, D.A. Lomas, C.J. Luke, R.W. Moyer, P.A. Pemberton, E. Remold-O'Donnell, G.S. Salvesen, J. Travis, J.C. Whisstock, The Serpins Are an Expanding Superfamily of Structurally Similar but Functionally Diverse Proteins, J. Biol. Chem. 276 (2001) 33293–33296. doi:10.1074/jbc.R100016200.

[32] C. Heit, B.C. Jackson, M. McAndrews, M.W. Wright, D.C. Thompson, G.A. Silverman, D.W. Nebert, V. Vasiliou, Update of the human and mouse SERPINgene superfamily, Hum. Genomics. 7 (2013) 22. doi:10.1186/1479-7364-7-22.

[33] L. Vidalino, A. Doria, S. Quarta, M. Zen, A. Gatta, P. Pontisso, SERPINB3, apoptosis and autoimmunity, Autoimmun. Rev. 9 (2009) 108–112. doi:https://doi.org/10.1016/j.autrev.2009.03.011.

[34] P.I. Bird, Serpins and Regulation of Cell Death BT - Apoptosis: Mechanisms and Role in Disease, in: S. Kumar (Ed.), Springer Berlin Heidelberg, Berlin, Heidelberg, 1998: pp. 63–89. doi:10.1007/978-3-540-69185-3_4.

[35] D.C. Hoffmann, C. Textoris, F. Oehme, T. Klaassen, A. Goppelt, A. Römer, B. Fugmann, J.M. Davidson, S. Werner, T. Krieg, S.A. Eming, Pivotal Role for

α1-Antichymotrypsin in Skin Repair * , J. Biol. Chem. 286 (2011) 28889–28901. doi:10.1074/jbc.M111.249979.

[36] N. Kalsheker, S. Morley, K. Morgan, Gene regulation of the serine proteinase inhibitors α1-antitrypsin and α1-antichymotrypsin, Biochem. Soc. Trans. 30 (2002) 93–98. doi:10.1042/bst0300093.

[37] M. Matsumoto, M. Tsuda, T. Kusumi, S. Takada, T. Shimamura, T. Katsunuma, Enhancement by α-1-antichymotrypsin of antibody response in vivo, Biochem. Biophys. Res. Commun. 100 (1981) 478–482. doi:https://doi.org/10.1016/S0006-291X(81)80121-0.

[38] P. Gravagna, E. Gianazza, P. Arnaud, M. Neels, E.W. Ades, Modulation of the immune response by plasma protease inhibitors III. Alpha 1-antichymotrypsin inhibits human natural killing and antibody-dependent cell-mediated cytotoxicity., J. Reticuloendothel. Soc. 32 (1982) 125–130.

[39] S.-R. Hwang, B. Steineckert, T. Toneff, R. Bundey, A. V Logvinova, P. Goldsmith, V.Y.H. Hook, The Novel Serpin Endopin 2 Demonstrates Cross-Class Inhibition of Papain and Elastase: Localization of Endopin 2 to Regulated Secretory Vesicles of Neuroendocrine Chromaffin Cells, Biochemistry. 41 (2002) 10397–10405. doi:10.1021/bi020088o.

[40] S.-R. Hwang, R. Bundey, T. Toneff, V. Hook, Endopin Serpin Protease Inhibitors Localize with Neuropeptides in Secretory Vesicles and Neuroendocrine Tissues, Neuroendocrinology. 89 (2009) 210–216. doi:10.1159/000162916.

[41] C.H. Herrera-Mendez, S. Becila, X. Blanchet, P. Pelissier, D. Delourme, G. Coulis, M.A. Sentandreu, A. Boudjellal, L. Bremaud, A. Ouali, Inhibition of human initiator caspase 8 and effector caspase 3 by cross-class inhibitory bovSERPINA3-1 and A3-3, FEBS Lett. 583 (2009) 2743–2748.

[42] M.S. Lord, J. Melrose, A.J. Day, J.M. Whitelock, The Inter-α-Trypsin Inhibitor Family: Versatile Molecules in Biology and Pathology, J. Histochem. Cytochem. 68 (2020) 907–927. doi:10.1369/0022155420940067.

[43] A. Hamm, J. Veeck, N. Bektas, P.J. Wild, A. Hartmann, U. Heindrichs, G. Kristiansen, T. Werbowetski-Ogilvie, R. Del Maestro, R. Knuechel, E. Dahl, Frequent expression loss of Inter-alpha-trypsin inhibitor heavy chain (ITIH) genes in multiple human solid tumors: a systematic expression analysis, BMC Cancer. 8 (2008) 25. doi:10.1186/1471-2407-8-25.

[44] M. Daveau, P. Rouet, M. Scotte, L. Faye, M. Hiron, J.P. Lebreton, J.P. Salier,

Human inter-α-inhibitor family in inflammation: simultaneous synthesis of positive and negative acute-phase proteins, Biochem. J. 292 (1993) 485–492. doi:10.1042/bj2920485.

[45] M. Pineiro, M. Andres, M. Iturralde, S. Carmona, J. Hirvonen, S. Pyorala, P.M.H. Heegaard, K. Tjørnehøj, F. Lampreave, A. Pineiro, ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4) is a new acute-phase protein isolated from cattle during experimental infection, Infect. Immun. 72 (2004) 3777–3782. doi:10.1128/IAI.72.7.3777-3782.2004.

[46] N.-H. Choi-Miura, K. Takahashi, M. Yoda, K. Saito, M. Hori, H. Ozaki, T. Mazda, M. Tomita, The novel acute phase protein, IHRP, inhibits actin polymerization and phagocytosis of polymorphonuclear cells, Inflamm. Res. 49 (2000) 305–310. doi:10.1007/PL00000211.

[47] X.-Y. Chen, P.-H. Feng, C.-L. Han, Y.-T. Jheng, C.-D. Wu, H.-C. Chou, Y.-Y. Chen, S.-M. Wu, K.-Y. Lee, H.-P. Kuo, K.F. Chung, T.-C. Hsiao, K.-Y. Chen, S.-C. Ho, T.-Y. Chang, H.-C. Chuang, Alveolar epithelial inter-alpha-trypsin inhibitor heavy chain 4 deficiency associated with senescence-regulated apoptosis by air pollution, Environ. Pollut. 278 (2021) 116863. doi:https://doi.org/10.1016/j.envpol.2021.116863.

[48] I. Serrano, A. Luque, J.M. Aran, Exploring the Immunomodulatory Moonlighting Activities of Acute Phase Proteins for Tolerogenic Dendritic Cell Generation , Front. Immunol. . 9 (2018). https://www.frontiersin.org/article/10.3389/fimmu.2018.00892.

[49] C. Gabay, I. Kushner, Acute-Phase Proteins and Other Systemic Responses to Inflammation, N. Engl. J. Med. 340 (1999) 448–454. doi:10.1056/NEJM199902113400607.

[50] J. Vandooren, Y. Itoh, Alpha-2-Macroglobulin in Inflammation, Immunity and Infections , Front. Immunol. . 12 (2021). https://www.frontiersin.org/article/10.3389/fimmu.2021.803244.

[51] E.M. De Souza, M. Meuser-Batista, D.G. Batista, B.B. Duarte, T.C. Araújo-Jorge, M.N.C. Soeiro, Trypanosoma cruzi: Alpha-2-macroglobulin regulates host cell apoptosis induced by the parasite infection in vitro, Exp. Parasitol. 118 (2008) 331–337. doi:https://doi.org/10.1016/j.exppara.2007.09.004.

[52] S. Jain, V. Gautam, S. Naseem, Acute-phase proteins: As diagnostic tool, J. Pharm. Bioallied Sci. 3 (2011) 118–127. doi:10.4103/0975-7406.76489.

[53] J. Bassaganya-Riera, R. Hontecillas-Magarzo, K. Bregendahl, M.J. Wannemuehler, D.R. Zimmerman, Effects of dietary conjugated linoleic acid in nursery pigs of dirty and clean environments on growth, empty body composition, and immune competence, J. Anim. Sci. 79 (2001) 714–721. doi:10.2527/2001.793714x.

[54] C.Y. Park, S.N. Han, Chapter 8 - Lipid Pathway in Liver Cells and Its Modulation by Dietary Extracts, in: V.B.B.T.-T.M.N. of F. Patel (Ed.), Academic Press, 2019: pp. 103–116. doi:https://doi.org/10.1016/B978-0-12-811297-7.00008-1.

[55] J. Qu, C.-W. Ko, P. Tso, A. Bhargava, Apolipoprotein A-IV: A Multifunctional Protein Involved in Protection against Atherosclerosis and Diabetes, Cells. 8 (2019) 319. doi:10.3390/cells8040319.

[56] J.R. Pérez-Estrada, D. Hernández-García, F. Leyva-Castro, J. Ramos-León, O. Cuevas-Benítez, M. Díaz-Muñoz, S. Castro-Obregón, R. Ramírez-Solís, C. García, L. Covarrubias, Reduced lifespan of mice lacking catalase correlates with altered lipid metabolism without oxidative damage or premature aging, Free Radic. Biol. Med. 135 (2019) 102–115. doi:https://doi.org/10.1016/j.freeradbiomed.2019.02.016.

[57] Y. Huang, R.W. Mahley, Apolipoprotein E: structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases, Neurobiol. Dis. 72 Pt A (2014) 3–12. doi:10.1016/j.nbd.2014.08.025.

[58] Q. Chen, L. Tang, G. Xin, S. Li, L. Ma, Y. Xu, M. Zhuang, Q. Xiong, Z. Wei, Z. Xing, H. Niu, W. Huang, Oxidative stress mediated by lipid metabolism contributes to high glucose-induced senescence in retinal pigment epithelium, Free Radic. Biol. Med. 130 (2019) 48–58. doi:https://doi.org/10.1016/j.freeradbiomed.2018.10.419.

[59] S.C. Funes, M. Rios, A. Fernández-Fierro, C. Covián, S.M. Bueno, C.A. Riedel, J.P. Mackern-Oberti, A.M. Kalergis, Naturally Derived Heme-Oxygenase 1 Inducers and Their Therapeutic Application to Immune-Mediated Diseases , Front. Immunol. . 11 (2020). https://www.frontiersin.org/article/10.3389/fimmu.2020.01467.

[60] S. Brouard, L.E. Otterbein, J. Anrather, E. Tobiasch, F.H. Bach, A.M.K. Choi, M.P. Soares, Carbon Monoxide Generated by Heme Oxygenase 1 Suppresses Endothelial Cell Apoptosis, J. Exp. Med. 192 (2000) 1015–1026. doi:10.1084/jem.192.7.1015.

[61] S. Tanaka, T. Akaike, J. Fang, T. Beppu, M. Ogawa, F. Tamura, Y. Miyamoto, H. Maeda, Antiapoptotic effect of haem oxygenase-1 induced by nitric oxide in experimental solid tumour, Br. J. Cancer. 88 (2003) 902–909. doi:10.1038/sj.bjc.6600830.

[62] I. Petrache, L.E. Otterbein, J. Alam, G.W. Wiegand, A.M.K. Choi, Heme oxygenase-1 inhibits TNF-α-induced apoptosis in cultured fibroblasts, Am. J. Physiol. Cell. Mol. Physiol. 278 (2000) L312–L319. doi:10.1152/ajplung.2000.278.2.L312.

[63] T. Tanaka, M. Narazaki, T. Kishimoto, IL-6 in inflammation, immunity, and disease, Cold Spring Harb. Perspect. Biol. 6 (2014) a016295–a016295. doi:10.1101/cshperspect.a016295.

[64] E. Karatayli, R.A. Hall, S.N. Weber, S. Dooley, F. Lammert, Effect of alcohol on the interleukin 6-mediated inflammatory response in a new mouse model of acute-on-chronic liver injury, Biochim. Biophys. Acta - Mol. Basis Dis. 1865 (2019) 298–307. doi:https://doi.org/10.1016/j.bbadis.2018.11.008.

[65] F. Hong, W.-H. Kim, Z. Tian, B. Jaruga, E. Ishac, X. Shen, B. Gao, Elevated interleukin-6 during ethanol consumption acts as a potential endogenous protective cytokine against ethanol-induced apoptosis in the liver: involvement of induction of Bcl-2 and Bcl-xL proteins, Oncogene. 21 (2002) 32–43. doi:10.1038/sj.onc.1205016.

[66] K. Piotrowska, S. Słuczanowska-Głabowska, M. Kurzawski, V. Dziedziejko, P. Kopytko, E. Paczkowska, D. Rogińska, K. Safranow, B. Machaliński, A. Pawlik, Over-Expression of Allograft Inflammatory Factor-1 (AIF-1) in Patients with Rheumatoid Arthritis, Biomolecules. 10 (2020) 1064. doi:10.3390/biom10071064.

[67] M. Kadoya, A. Yamamoto, M. Hamaguchi, H. Obayashi, K. Mizushima, M. Ohta, T. Seno, R. Oda, H. Fujiwara, M. Kohno, Y. Kawahito, Allograft inflammatory factor-1 stimulates chemokine production and induces chemotaxis in human peripheral blood mononuclear cells, Biochem. Biophys. Res. Commun. 448 (2014) 287–291. doi:https://doi.org/10.1016/j.bbrc.2014.04.106.

[68] K. Watano, K. Iwabuchi, S. Fujii, N. Ishimori, S. Mitsuhashi, M. Ato, A. Kitabatake, K. Onoé, Allograft inflammatory factor-1 augments production of interleukin-6, -10 and -12 by a mouse macrophage line, Immunology. 104 (2001) 307–316. doi:10.1046/j.1365-2567.2001.01301.x.

[69] B. Dukay, F.R. Walter, J.P. Vigh, B. Barabási, P. Hajdu, T. Balassa, E. Migh, A.

Kincses, Z. Hoyk, T. Szögi, E. Borbély, B. Csoboz, P. Horváth, L. Fülöp, B. Penke, L. Vígh, M.A. Deli, M. Sántha, M.E. Tóth, Neuroinflammatory processes are augmented in mice overexpressing human heat-shock protein B1 following ethanol-induced brain injury, J. Neuroinflammation. 18 (2021) 22. doi:10.1186/s12974-020-02070-2.

[70] H. Itabe, T. Yamaguchi, S. Nimura, N. Sasabe, Perilipins: a diversity of intracellular lipid droplet proteins, Lipids Health Dis. 16 (2017) 83. doi:10.1186/s12944-017-0473-y.

[71] W. Zhang, L. Xu, L. Zhu, Y. Liu, S. Yang, M. Zhao, Lipid Droplets, the Central Hub Integrating Cell Metabolism and the Immune System , Front. Physiol. . 12 (2021). https://www.frontiersin.org/article/10.3389/fphys.2021.746749.

[72] P.T. Bozza, K.G. Magalhães, P.F. Weller, Leukocyte lipid bodies — Biogenesis and functions in inflammation, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1791 (2009) 540–551. doi:https://doi.org/10.1016/j.bbalip.2009.01.005.

[73] K.A. Mattos, F.A. Lara, V.G.C. Oliveira, L.S. Rodrigues, H. D'Avila, R.C.N. Melo, P.P.A. Manso, E.N. Sarno, P.T. Bozza, M.C. V Pessolani, Modulation of lipid droplets by Mycobacterium leprae in Schwann cells: a putative mechanism for host lipid acquisition and bacterial survival in phagosomes, Cell. Microbiol. 13 (2011) 259–273.

[74] M.M. Wurfel, W.Y. Park, F. Radella, J. Ruzinski, A. Sandstrom, J. Strout, R.E. Bumgarner, T.R. Martin, Identification of High and Low Responders to Lipopolysaccharide in Normal Subjects: An Unbiased Approach to Identify Modulators of Innate Immunity, J. Immunol. 175 (2005) 2570 LP – 2578. doi:10.4049/jimmunol.175.4.2570.

[75] K.T. Dalen, S.M. Ulven, B.M. Arntsen, K. Solaas, H.I. Nebb, PPARα activators and fasting induce the expression of adipose differentiation-related protein in liver, J. Lipid Res. 47 (2006) 931–943. doi:10.1194/jlr.M500459-JLR200.

[76] M. Schubert, S. Becher, M. Wallert, M.B. Maeß, M. Abhari, K. Rennert, A.S. Mosig, S. Große, R. Heller, M. Grün, S. Lorkowski, The Peroxisome Proliferator–Activated Receptor (PPAR)-γ Antagonist 2-Chloro-5-Nitro-N-Phenylbenzamide (GW9662) Triggers Perilipin 2 Expression via PPARδ and Induces Lipogenesis and Triglyceride Accumulation in Hum, Mol. Pharmacol. 97 (2020) 212 LP – 225.

doi:10.1124/mol.119.117887.

[77] S. Tian, P. Lei, C. Teng, Y. Sun, X. Song, B. Li, Y. Shan, Targeting PLIN2/PLIN5-PPARγ: Sulforaphane Disturbs the Maturation of Lipid Droplets, Mol. Nutr. Food Res. 63 (2019) 1900183.

[78] C. Buechler, M. Ritter, C.Q. Duong, E. Orso, M. Kapinsky, G. Schmitz, Adipophilin is a sensitive marker for lipid loading in human blood monocytes, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1532 (2001) 97–104. doi:https://doi.org/10.1016/S1388-1981(01)00121-4.

[79] H. Vosper, L. Patel, T.L. Graham, G.A. Khoudoli, A. Hill, C.H. Macphee, I. Pinto, S.A. Smith, K.E. Suckling, C.R. Wolf, C.N.A. Palmer, The Peroxisome Proliferator-activated Receptor δ Promotes Lipid Accumulation in Human Macrophages *, J. Biol. Chem. 276 (2001) 44258–44265. doi:10.1074/jbc.M108482200.

[80] E. Stachowska, M. Baśkiewicz-Masiuk, V. Dziedziejko, I. Gutowska, I. Baranowska-Bosiacka, M. Marchlewicz, B. Dołęgowska, B. Wiszniewska, B. Machaliński, D. Chlubek, Conjugated linoleic acid increases intracellular ROS synthesis and oxygenation of arachidonic acid in macrophages, Nutrition. 24 (2008) 187–199. doi:https://doi.org/10.1016/j.nut.2007.10.018.

[81] S. McClelland, C. Cox, R. O'Connor, M. de Gaetano, C. McCarthy, L. Cryan, D. Fitzgerald, O. Belton, Conjugated linoleic acid suppresses the migratory and inflammatory phenotype of the monocyte/macrophage cell, Atherosclerosis. 211 (2010) 96–102. doi:10.1016/j.atherosclerosis.2010.02.003.

[82] C. Lecchi, G. Invernizzi, A. Agazzi, S. Modina, P. Sartorelli, G. Savoini, F. Ceciliani, Effects of EPA and DHA on lipid droplet accumulation and mRNA abundance of PAT proteins in caprine monocytes, Res. Vet. Sci. 94 (2013) 246–251. doi:https://doi.org/10.1016/j.rvsc.2012.09.019.

[83] Z. Sfeir, A. Ibrahimi, E. Amri, P. Grimaldi, N. Abumrad, CD36 antisense expression in 3T3-F442A preadipocytes, in: Lipid Bind. Proteins within Mol. Cell. Biochem., Springer, 1999: pp. 3–8.

[84] J. Gao, H. Ye, G. Serrero, Stimulation of adipose differentiation related protein (ADRP) expression in adipocyte precursors by long-chain fatty acids, J. Cell. Physiol. 182 (2000) 297–302.

[85] J. Li, S. Viswanadha, J.J. Loor, Hepatic Metabolic, Inflammatory, and Stress-Related Gene Expression in Growing Mice Consuming a Low Dose of Trans-10,

cis-12-Conjugated Linoleic Acid, J. Lipids. 2012 (2012) 571281. doi:10.1155/2012/571281.

[86] K.J. Harvatine, Y.R. Boisclair, D.E. Bauman, Time-dependent effect of trans-10,cis-12 conjugated linoleic acid on gene expression of lipogenic enzymes and regulators in mammary tissue of dairy cows, J. Dairy Sci. 101 (2018) 7585–7592. doi:https://doi.org/10.3168/jds.2017-13935.

[87] H. Shao, S.-G. Scott, C. Nakata, A.R. Hamad, S. Chakravarti, Extracellular Matrix Protein Lumican Promotes Clearance and Resolution of Pseudomonas aeruginosa Keratitis in a Mouse Model, PLoS One. 8 (2013) e54765. https://doi.org/10.1371/journal.pone.0054765.

[88] T.N. Wight, I. Kang, S.P. Evanko, I.A. Harten, M.Y. Chang, O.M.T. Pearce, C.E. Allen, C.W. Frevert, Versican—A Critical Extracellular Matrix Regulator of Immunity and Inflammation, Front. Immunol. 11 (2020). https://www.frontiersin.org/article/10.3389/fimmu.2020.00512.

[89] I. López-Flores, J.B. Barroso, R. Valderrama, F.J. Esteban, E. Martínez-Lara, F. Luque, M.Á. Peinado, H. Ogawa, J.A. Lupiáñez, J. Peragón, Serine dehydratase expression decreases in rat livers injured by chronic thioacetamide ingestion, Mol. Cell. Biochem. 268 (2005) 33–43. doi:10.1007/s11010-005-2995-2.

[90] C. Rosales, B.K. Gillard, A.M. Gotto Jr, H.J. Pownall, The Alcohol-High-Density Lipoprotein Athero-Protective Axis, Biomolecules. 10 (2020) 987. doi:10.3390/biom10070987.

[91] P. Yu, Y. Li, Y. Li, Z. Miao, M.P. Peppelenbosch, Q. Pan, Guanylate-binding protein 2 orchestrates innate immune responses against murine norovirus and is antagonized by the viral protein NS7, J. Biol. Chem. 295 (2020) 8036–8047. doi:10.1074/jbc.RA120.013544.

[92] A. Leal, S. Bogantes-Ledezma, A.B. Ekici, S. Uebe, C.T. Thiel, H. Sticht, M. Berghoff, C. Berghoff, B. Morera, M. Meisterernst, A. Reis, The polynucleotide kinase 3′-phosphatase gene (PNKP) is involved in Charcot-Marie-Tooth disease (CMT2B2) previously related to MED25, Neurogenetics. 19 (2018) 215–225. doi:10.1007/s10048-018-0555-7.

Figures

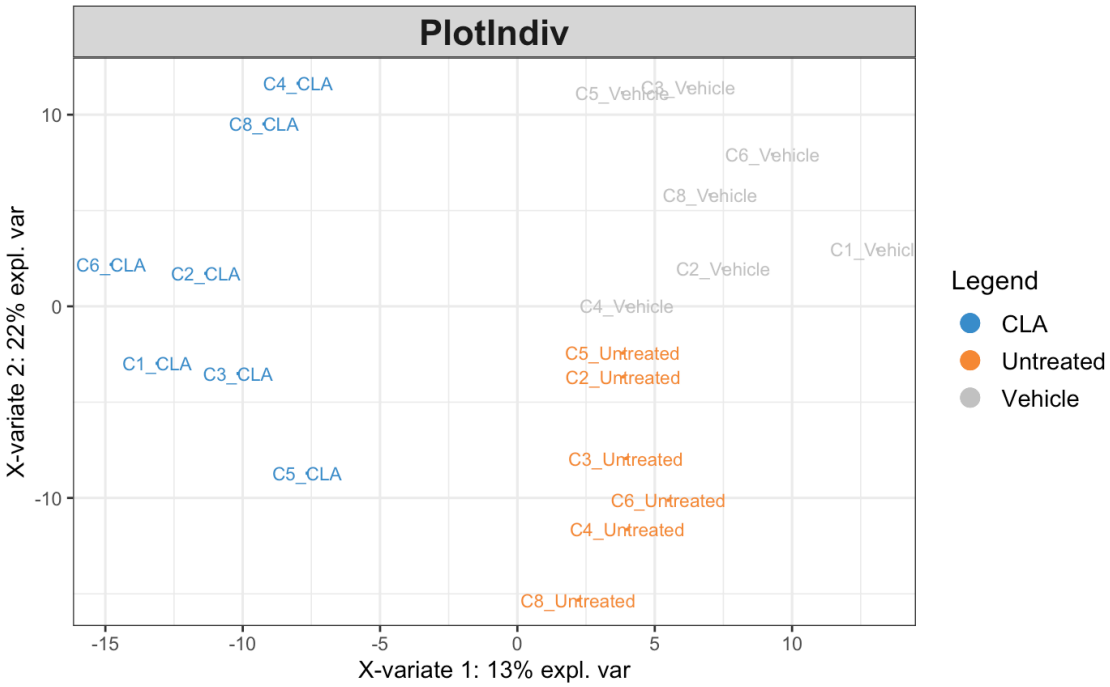


Fig. 1. Sparse partial least squares discriminant analysis (sPLS-DA) for paired data individual plot. The individual ploy shows the similarities and relationship (clustering) between samples of the conjugated linoleic acid (CLA) mixture (50:50) (blue), vehicle (ethanol) (gray), and untreated (orange) treatment groups.

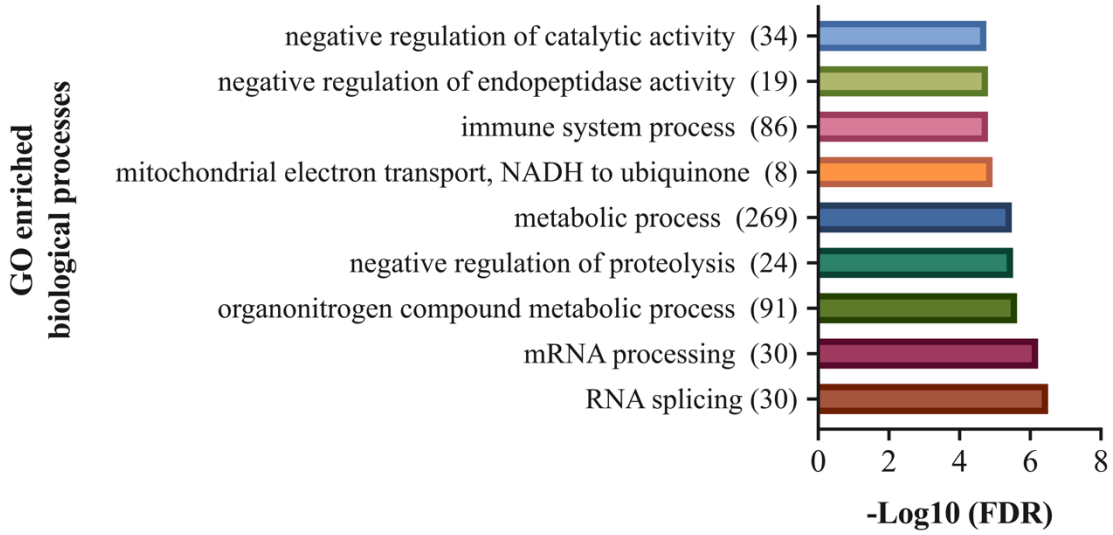


Fig. 2. Global enriched (P<0.05) Gene ontology (GO) terms within the biological processes (BP) category that have annotated all 407 discriminant proteins (DP). GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

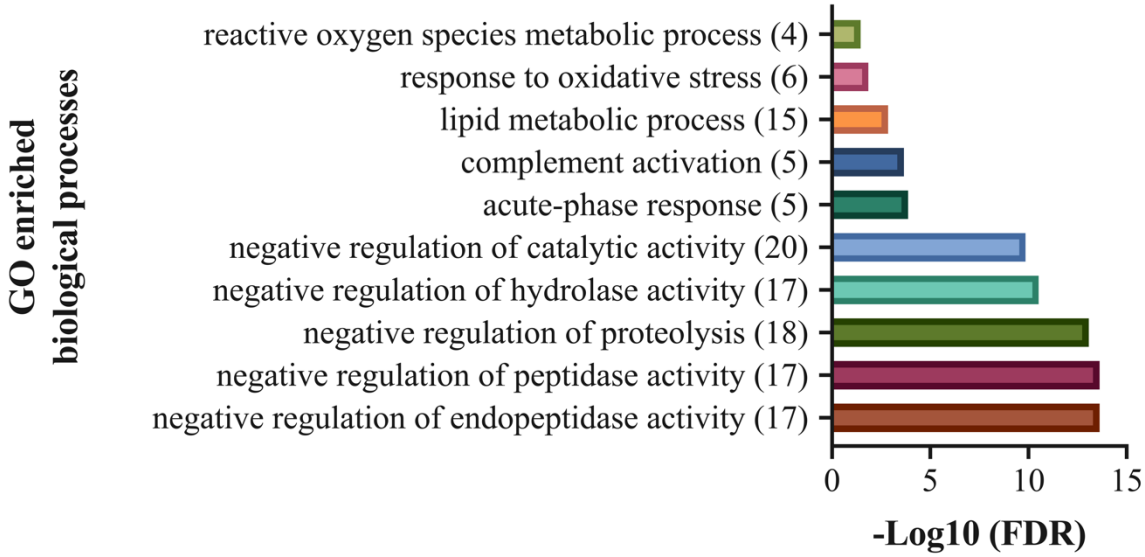


Fig. 3. Enriched (P<0.05) Gene ontology (GO) terms within the biological processes category that have annotated the 85 discriminant proteins (DP) with the highest abundance in the conjugated linoleic acid (CLA) mixture (50:50) group. GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

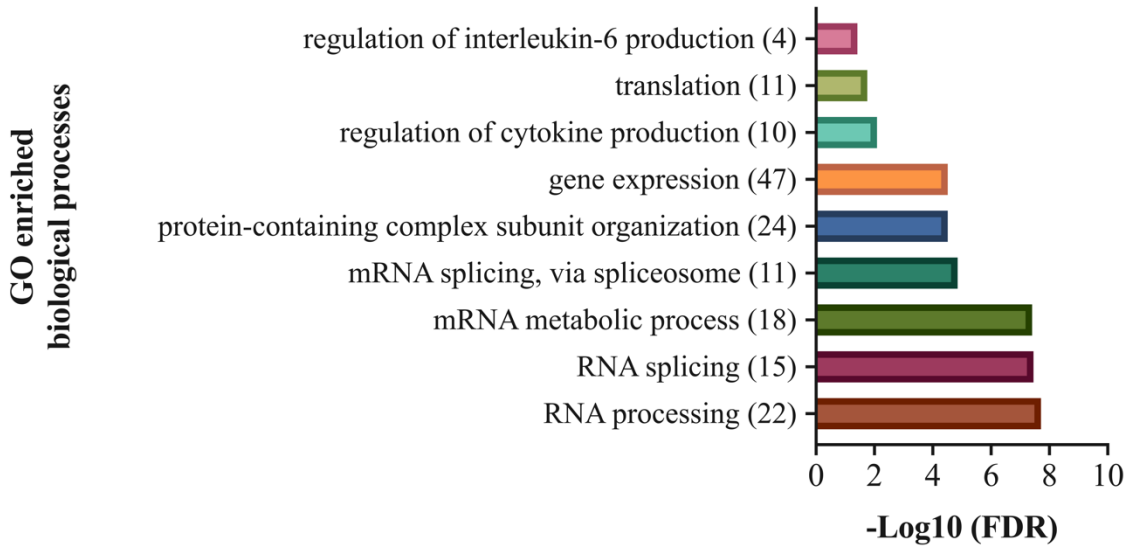


Fig. 4. Enriched (P<0.05) Gene ontology (GO) terms within the biological processes category that have annotated the 96 discriminant proteins (DP) with the highest abundance in the vehicle (ethanol) group. GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

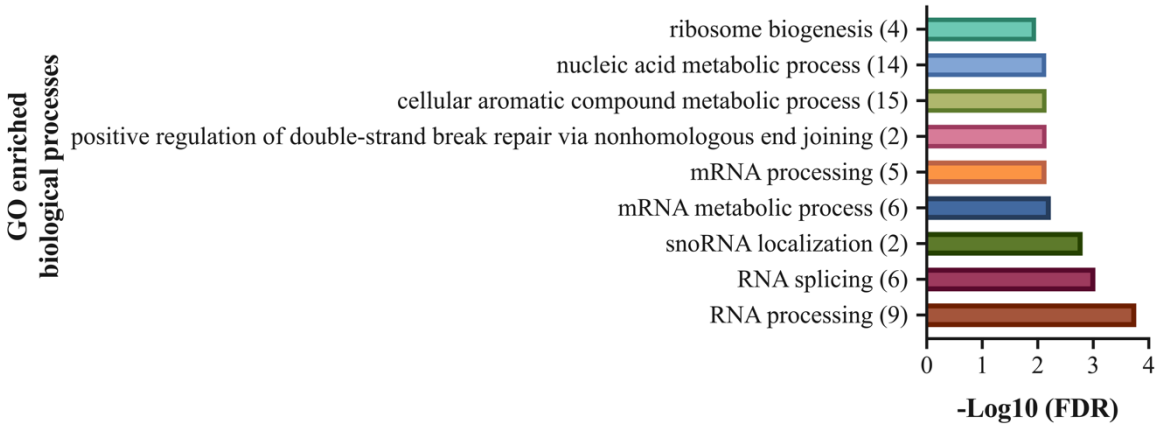


Fig. 5. Enriched (P<0.05) Gene ontology (GO) terms within the biological processes category that have annotated the 27 discriminant proteins (DP) with the highest abundance in the untreated group (only medium treated cells). GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

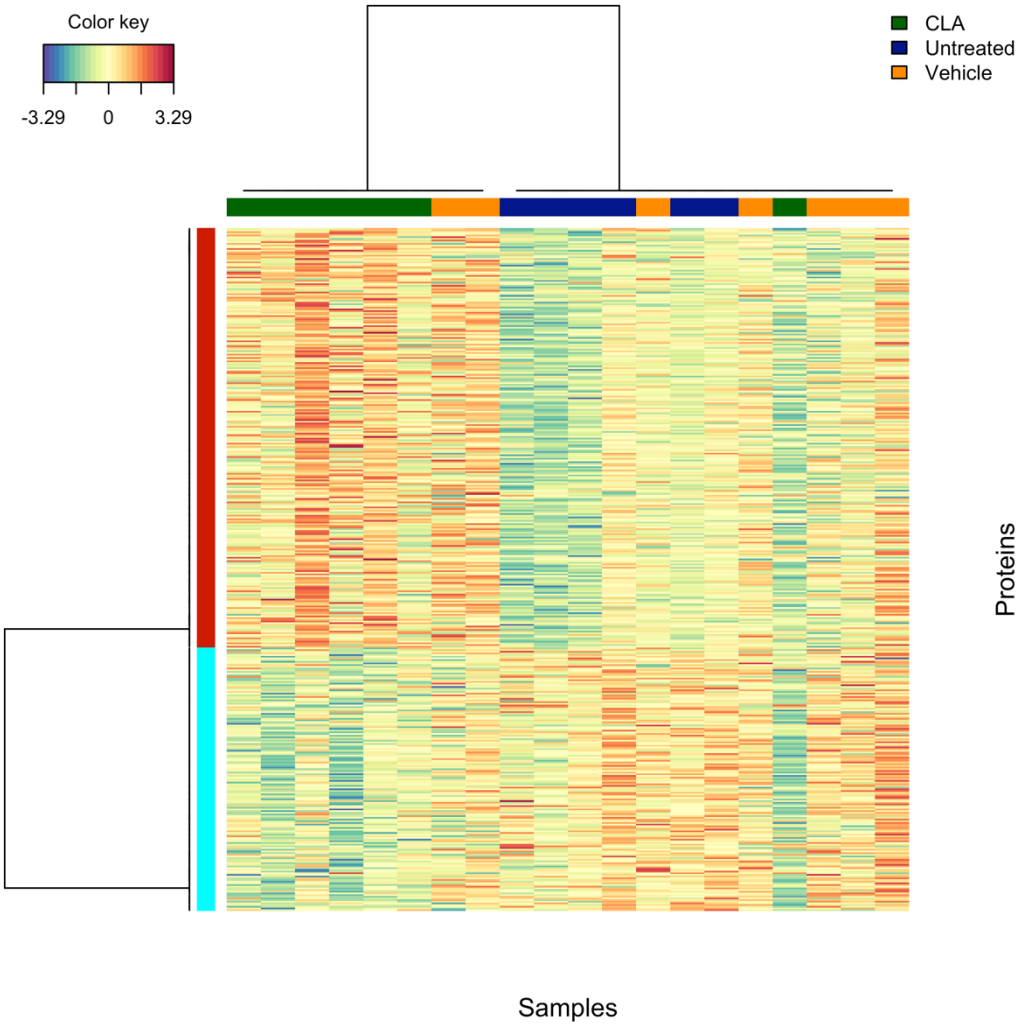


Fig. 6. Hierarchical clustering heat map analysis of discriminant proteins (DP) selected by sPLS-DA. Rows and columns are sorted by similarity as indicated by the left (proteins) and top (samples) dendrograms. Red and blue represent increased and decreased protein abundance, respectively. Samples from the three treatment groups were represented with different colors: green = CLA (50:50) mixture; orange = vehicle, and blue = untreated. A total of 250 DP was sorted in Cluster 1 (red), while 157 in Cluster 2 (light blue).

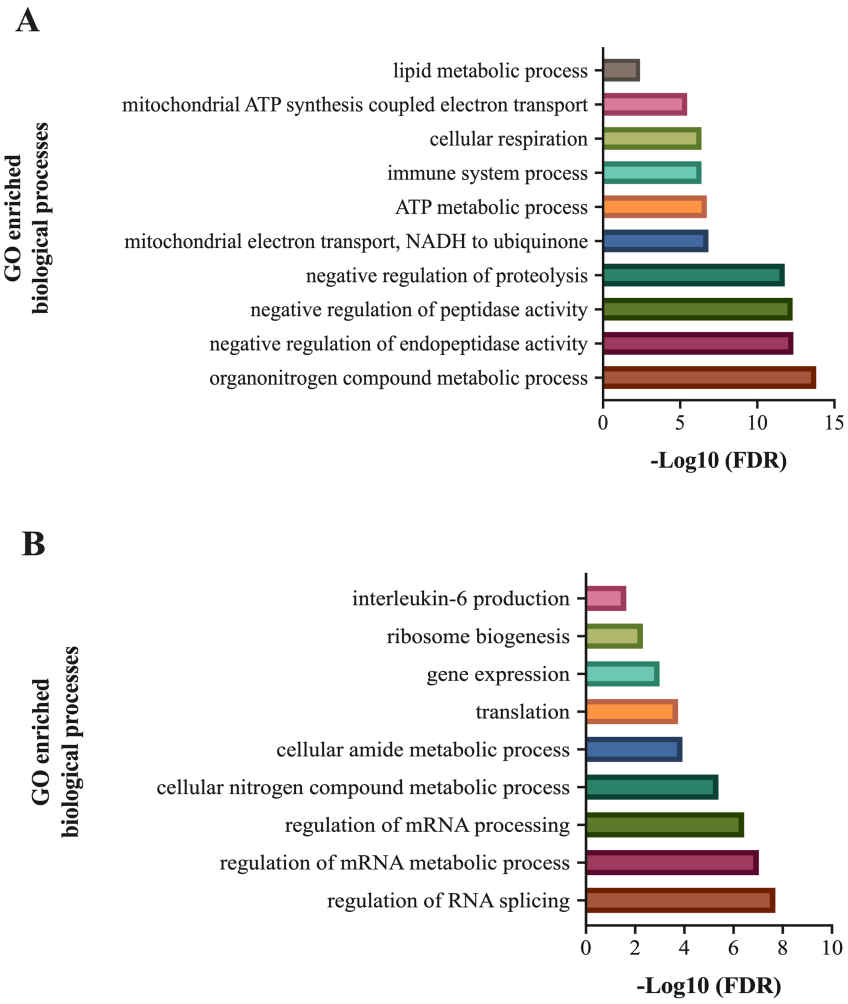


Fig. 7. Enriched (P<0.05) Gene ontology (GO) terms within the biological processes category that have annotated the discriminant proteins (DP) from (A) cluster 1; and (B) cluster 2 generated in the hierarchical clustering heat map analysis. GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs.

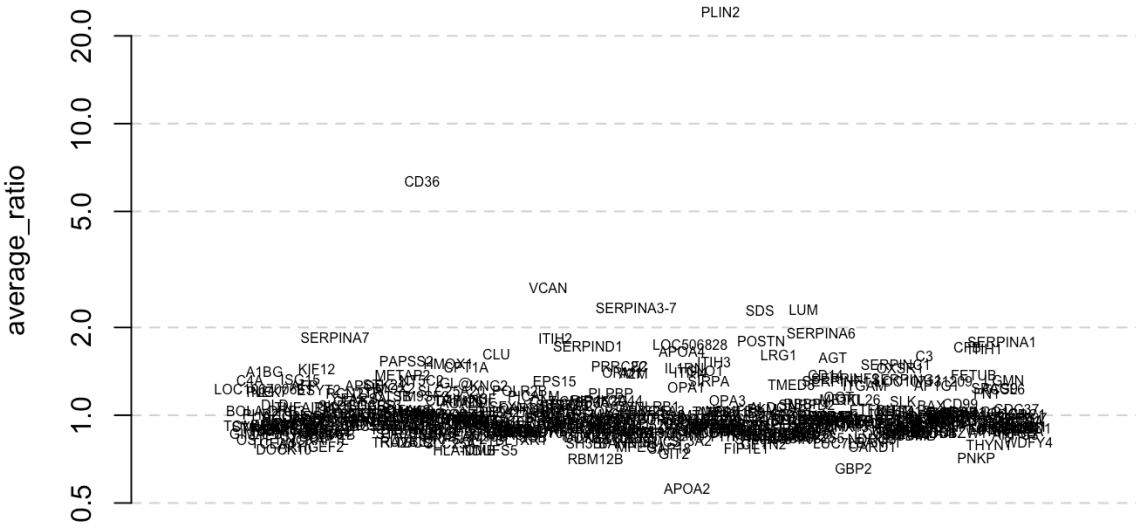


Fig. 8. Average log fold-change (logFC) of protein raw abundances of the 407 discriminant proteins (DP) selected by sPLS-DA. The y axis shows the average logFC (average ratio) of the raw abundances from all the seven different biological replicates (samples) after CLA (50:50) mixture treatment, respect to vehicle group.

Supplementary material

Supplementary Table S1. List of global enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by all 407 discriminant proteins (DP) selected the sPLS-DA.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0008380	RNA splicing	0,00000031	6,51	AHNAK CELF1 CELF2 DAZAP1 DCPS DDX39A HNRNPA1 HNRNPK HNRNPM KHDRBS1 LGALS2 LGALS3 LSM4 LUC7L MBNL1 PRPF31 PTBP1 RBM12B RNPS1 RTCB SF3A2 SF3A3 SNRNP200 SNRNP70 SREK1 SRSF1 SRSF10 STRAP TRA2A WBP11
GO:0006397	mRNA processing	0,00000060	6,22	CELF1 CELF2 DAZAP1 DCPS DDX39A FIP1L1 HNRNPA1 HNRNPK HNRNPM KHDRBS1 LSM4 LUC7L MBNL1 PRPF31 PTBP1 RNPS1 RPRD1B SF3A2 SF3A3 SLTM SNRNP200 SNRNP70 SRRM1 SRRT SRSF1 SRSF10 STRAP SUPT5H TRA2A WBP11
GO:1901564	organonitrogen compound metabolic process	0,00000236	5,63	A2M ABCE1 ACADM ACO1 ACSL6 ADD1 AGT AIP ALDOA APOA2 APOA4 APOE ARHGEF2 ARL6IP5 ARRB1 ATP5F1A ATP5ME ATP5PO BANK1 C3 C4A CAT CBFB CD44 CDC37 CFB CKB CPT1A CTSA CTSB CTSC DCPS DDOST DDX3X DLD DPP3 EEF1G EIF1AX EIF3G EIF3L EIF4B EIF5A ERAP1 F2 FAM98B FARSA FBL FETUB FKBP5 FN1 GABARAPL2 GARS1 GOT1 GOT2 GPX1 GRK2 HARS1 HIBADH HMOX1 HMOX2 HNRNPD HPRT1 PRMT1 HRMT1L2 HSP90AA1 HUWE1 ISG15 ITIH1 ITIH2 ITIH3 ITIH4 KHDRBS1 KIF12 LACTB LAMP2 LAMTOR2 LAP3 LGMN LOC107131209 LOC506828 MAP2K1

				MAP4K1 METAP2 MPC2 MTHFD1L MYDGF NAGA NARS1 NAXE NCKAP1L NEK7 NFKB1 NME2 NSF OARD1 OGT OPA1 OXSR1 PAPSS1 PAPSS2 PFDN1 PFKP PHB2 PHGDH PICALM PKM PKN1 PLG PNKP PNP POGLUT1 PPIH PPM1F PPP1CA PRDX3 PRDX4 PRKAR2A PRPSAP2 PSMA1 PSMA3 PSMA4 PSMA6 PSMB1 PSMB2 PSMB3 PSMB9 PTPN1 PTPRC PYCR3 QARS1 RAD23A RAP2B RPL11RPL3 RPL9 RPN1 RPN2 RPRD1B RPS14 RPS17 RPS20 RPS24 RPS27 RPS28 RUVBL2 SDCBP SDS SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1 SKP1 SLK SNX12 SPAG9 SPTLC2 STK24 STRAP TBL1XR1 TCOF1 TRIM25 TTLL12 TXN UBA1 UCHL3 VNN2 VPS35 WARS1 XPO1 YARS1
GO:0045861	negative regulation of proteolysis	0,00000305	5,52	A2M C3 C4A CD44 DDX3X F2 FETUB GABARAPL2 GPX1 ITIH1 ITIH2 ITIH4 KIF12 LOC107131209 LOC506828 OGT PICALM RPL11 SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1 SNX12
GO:0008152	metabolic process	0,00000333	5,48	A2M ABCE1 ACADM ACADVL ACO1 ACO2 ACSL6 ADD1 AFP AGT AHNAK AIF1 AIP AK3 ALDH3A2 ALDOA APOA2 APOA4 APOE ARHGEF2 ARL6IP5 ARRB1 ATP1A1 ATP5F1A ATP5ME ATP5PO ATP6V1B2 BANF1 BANK1 C3 C4A CAT CBFB CD14 CD44 CD47 CDC37 CELF1 CELF2 CETN2 CFB CKB CPT1A CTSA CTSB CTSC CYBC1 DAZAP1 DCPS DDOST DDX39A DDX3X DLD DPP3 EEF1G EIF1AX EIF3G EIF3L EIF4B EIF5A ERAP1 ETFDH F2 FAM98B FARSA FBL FETUB FIP1L1 FKBP5 FN1 GABARAPL2 GARS1 GBE1 GOT1 GOT2 GPX1 GRK2 GSDMD HARS1 HIBADH HMGB2 HMOX1 HMOX2 HNRNPA1 HNRNPD HNRNPK HNRNPM HPRT1 PRMT1 HRMT1L2 HSP90AA1

				HUWE1 IL1RN ISG15 ITGAM ITIH1 ITIH2 ITIH3 ITIH4 KHDRBS1 KIF12 LACTB LAMP2 LAMTOR2 LANCL1 LAP3 LGALS2 LGALS3 LGMN LOC107131209 LOC506828 LSM4 LUC7L LUM MAP2K1 MAP4K1 MBNL1 MCM5 MDH2 METAP2 MOB1A MPC2 MTHFD1L MYBBP1A MYDGF NAGA NARS1 NAXE NCKAP1L NDUFA10 NDUFB8 NDUFS1 NDUFS3 NDUFS6 NDUFS7 NDUFV2 NEK7 NFKB1 NFYC NME2 NPM1 NSF OARD1 OGT OPA1 OXSR1 PAPSS1 PAPSS2 PCCA PDCD4 PFDN1 PFKP PHB2 PHGDH PICALM PIP4K2A PKM PKN1 PLBD2 PLG PNKP PNP POGLUT1 POLR2B PPIH PPM1F PPP1CA PRDX3 PRDX4 PRKAR2A PRPF31 PRPSAP2 PSMA1 PSMA3 PSMA4 PSMA6 PSMB1 PSMB2 PSMB3 PSMB9 PTBP1 PTER PTPN1 PTPRC PYCR3 QARS1 QKI RAD23A RAP2B RBM12B REL RNPS1 RPL11RPL3 RPL9 RPN1 RPN2 RPRD1B RPS14 RPS17 RPS20 RPS24 RPS27 RPS28 RTCB RUVBL2 SDCBP SDHA SDS SEPHS1 SERPINA3-7 SERPINA6 SERPINA7 SERPINC1 SERPIND1 SERPINF2 SERPING1 SF3A2 SF3A3 SFXN1 SKP1 SLK SLTM SMC3 SMCHD1 SNRNP200 SNRNP70 SNX1 SNX12 SPAG9 SPTLC2 SREK1 SRRM1 SRRT SRSF1 SRSF10 SSB STAT1 STK24 STRAP SUPT5H SYNCRIP TBL1XR1 TCEA1 TCOF1 TFAM TKFC TRA2A TRIM25 TTLL12 TTR TXN TYROBP UBA1 UCHL3 UQCRC1 VDAC1 VNN2 VPS35 WARS1 WAS WBP11 XPO1 YARS1
GO:0006120	mitochondrial electron transport, NADH to ubiquinone	0,00001173	4,94	DLD NDUFA10 NDUFB8 NDUFS1 NDUFS3 NDUFS6 NDUFS7 NDUFV2

GO:0002376	immune system process	0,00001565	4,81	ADD1 AIF1 AP1G1 APOA2 APOA4 APOE ARHGEF2 BANK1 BOLA-DOB BoLA-DOB BoLA DR-ALPHA BOLA-DRA BoLA-DRA BoLA-DRB2 BOLA-DRB2 BOLA-DRB3 BoLA-DRB3 BOLA-NC1 C3 C4A CBFB CD14 CD247 CD36 CD44 CD47 CD99 CDC37 CFB CTSC CYBC1 DDX3X ERAP1 F2 GBP2 GNLY GPX1 GSDMD HLA-DMB HMGB2 HMOX1 PRMT1 HRMT1L2 IL1RN IPO7 ISG15 ITGAM KHDRBS1 LGALS2 LGALS3 LGMN LOC107131209 LOC512486 MAP2K1 MYO1C NCKAP1L agp ORM1 OXSR1 PHB2 PICALM PIP4K2A PKN1 PLG PRDX3 PRRC2C PSMA1 PSMB9 PTPRC RAB10 REL RPS24 SERPING1 SFXN1 SIRPA STAT1 STX7 TKFC TNFAIP8L2 TRIM25 TTLL12 TYROBP WAS WDFY4
GO:0010951	negative regulation of endopeptidase activity	0,00001565	4,81	A2M C3 C4A CD44 DDX3X FETUB GPX1 ITIH1 ITIH2 ITIH4 KIF12 LOC107131209 LOC506828 PICALM SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1
GO:0043086	negative regulation of catalytic activity	0,00001753	4,76	A2M ABCE1 AGT APOA2 APOE C3 C4A CD44 DDX3X FETUB GPX1 ITIH1 ITIH2 ITIH4 KIF12 LOC107131209 LOC506828 NFKB1 PICALM PIP4K2A PKN1 PPM1F PRDX3 PRKAR2A PTPN1 PTPRC QARS1 RPL11 SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1 WARS1

Supplementary Table S2. List of enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins (DP) selected by the sPLS-DA with the highest abundance in CLA (50:50) mixture group.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0010951	negative regulation of endopeptidase activity	0,0000000	13,62	A2M C3 C4A CD44 FETUB ITIH1 ITIH2 ITIH4 KIF12 LOC107131209 LOC506828 PICALM SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1
GO:0010466	negative regulation of peptidase activity	0,0000000	13,62	A2M C3 C4A CD44 FETUB ITIH1 ITIH2 ITIH4 KIF12 LOC107131209 LOC506828 PICALM SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1
GO:0045861	negative regulation of proteolysis	0,0000000	13,07	A2M C3 C4A CD44 F2 FETUB ITIH1 ITIH2 ITIH4 KIF12 LOC107131209 LOC506828 PICALM SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1
GO:0051346	negative regulation of hydrolase activity	0,000000	10,52	A2M C3 C4A CD44 FETUB ITIH1 ITIH2 ITIH4 KIF12 LOC107131209 LOC506828 PICALM SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1
GO:0043086	negative regulation of catalytic activity	0,000000	9,85	A2M AGT APOE C3 C4A CD44 FETUB ITIH1 ITIH2 ITIH4 KIF12 LOC107131209 LOC506828 PICALM PIP4K2A SERPINA3-7 SERPINA7 SERPINC1 SERPIND1 SERPING1
GO:0006953	acute-phase response	0,0001328	3,88	F2 FN1 ITIH4 ORM1 agp
GO:0006956	complement activation	0,0002195	3,66	C3 C4A CFB LOC107131209 SERPING1

GO:0006629	lipid metabolic process	0,001383	2,86	ACADVL ACSL6 AFP AGT APOA4 APOE C3 CAT CPT1A F2 IL1RN PIP4K2A QKI SERPINA6 TTR
GO:0006979	response to oxidative stress	0,01417	1,85	APOA4 APOE CAT HMOX1 HMOX2 TXN
GO:0072593	reactive oxygen species metabolic process	0,03523	1,45	APOA4 CAT F2 ITGAM

Supplementary Table S3. List of enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins (DP) selected by the sPLS-DA with the highest abundance in vehicle (ethanol) group.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR	Annotated Proteins (Gene Name)
GO:0006396	RNA processing	0,0000	7,70	CELF2 DCPS FIP1L1 HNRNPA1 HNRNPK HNRNPM LUC7L RBM12B RNPS1 RPL11 RPS14 RTCB SLTM SNRNP200 SNRNP70 SREK1 SRRM1 SRRT SRSF1 SSB STRAP WBP11
GO:0008380	RNA splicing	0,0000	7,45	CELF2 DCPS HNRNPA1 HNRNPK HNRNPM LUC7L RBM12B RNPS1 RTCB SNRNP200 SNRNP70 SREK1 SRSF1 STRAP WBP11
GO:0016071	mRNA metabolic process	0,0000	7,40	CELF2 DCPS FIP1L1 HNRNPA1 HNRNPD HNRNPK HNRNPM LUC7L RNPS1 SLTM SNRNP200 SNRNP70 SRRM1 SRRT SRSF1 SSB STRAP WBP11

GO:0000398	mRNA splicing, via spliceosome	0,0000	4,85	CELF2 DCPS HNRNPK HNRNPM LUC7L RNPS1 SNRNP200 SNRNP70 SRSF1 STRAP WBP11
GO:0043933	protein-containing complex subunit organization	0,0000	4,52	ADD3 AIF1 APOA2 ARHGEF2 ARPC3 BOLA-DRA BoLA DR-ALPHA BoLA-DRA CELF2 DDX3X EIF5A GSDMD HRMT1L2 PRMT1 LUC7L MCM5 NCKAP1L NDUFA10 NDUFS5 RPL11 RPS10 RPS14 SNRNP200 SRSF1 STRAP WARS1
GO:0010467	gene expression	0,0000	4,52	AIF1 APOA2 ARHGEF2 BANK1 CELF2 DCPS DDX3X EIF1AX EIF5A FIP1L1 GPX1 GSDMD HMGB2 HNRNPA1 HNRNPD HNRNPK HNRNPM LUC7L NARS1 NCKAP1L NFYC NPM1 PDCD4 PHGDH RBM12B REL RNPS1 RPL11 RPL9 RPS14 RTCB RUVBL2 SLTM SNRNP200 SNRNP70 SNX12 SREK1 SRRM1 SRRT SRSF1 SSB STAT1 STRAP TBL1XR1 TFAM WARS1 WBP11
GO:0001817	regulation of cytokine production	0,0081	2,09	AIF1 APOA2 ARHGEF2 BANK1 DDX3X GSDMD NCKAP1L PDCD4 REL STAT1
GO:0006412	translation	0,0175	1,76	BANK1 DCPS DDX3X EIF1AX EIF5A HNRNPD NARS1 RPL11 RPL9 RPS14 WARS1
GO:0032675	regulation of interleukin-6 production	0,03805	1,42	AIF1 ARHGEF2 BANK1 NCKAP1L

Supplementary Table S4. List of enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins (DP) selected by the sPLS-DA with the highest abundance in untreated group.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0006396	RNA processing	0,00017	3,78	AHNAK DAZAP1 FBL LSM4 MBNL1 PTBP1 RPS27 RPS28 TRA2A
GO:0008380	RNA splicing	0,00092	3,04	AHNAK DAZAP1 LSM4 MBNL1 PTBP1 TRA2A
GO:0048254	snoRNA localization	0,00156	2,81	FBL NOP58
GO:0016071	mRNA metabolic process	0,00579	2,24	DAZAP1 LSM4 MBNL1 PTBP1 SYNCRIP TRA2A
GO:0006397	mRNA processing	0,00693	2,16	DAZAP1 LSM4 MBNL1 PTBP1 TRA2A
GO:2001034	positive regulation of double-strand break repair via nonhomologous end joining	0,00693	2,16	PNKP SMCHD1
GO:0006725	cellular aromatic compound metabolic process	0,00693	2,16	AHNAK CETN2 DAZAP1 FBL LSM4 MBNL1 MYBBP1A PNKP PRPSAP2 PTBP1 RPS27 RPS28 SMCHD1 SYNCRIP TRA2A
GO:0090304	nucleic acid metabolic process	0,00706	2,15	AHNAK CETN2 DAZAP1 FBL LSM4 MBNL1 MYBBP1A PNKP PTBP1 RPS27 RPS28 SMCHD1 SYNCRIP TRA2A
GO:0042254	ribosome biogenesis	0,01074	1,97	FBL NOP58 RPS27 RPS28

Supplementary Table S5. List of enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins (DP) in cluster 1 after the hierarchical clustering heat map analysis.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:1901564	organonitrogen compound metabolic process	0,0000000	13,82	A2M ABCE1 ACADM ACSL6 ADD1 AGT AIP AK3 ALDOA APOA4 APOE ARL6IP5 ATP5F1A ATP5ME ATP5PO BAX C3 C4A CAT CBFB CD36 CD44 CFB CKB CLU CPT1A CTSA CTSB CTSC DDOST DLD DPP3 EEF1G EIF3G EIF3L EIF4B ERAP1 F2 FAM98B FETUB FN1 GARS1 GOT1 GOT2 GRK2 HARS1 HIBADH HMOX1 HMOX2 HPRT1 PRMT1 HRMT1L2 HUWE1 ISG15 ITIH1 ITIH2 ITIH3 ITIH4 KIF12 LACTB LAMP2 LGMN LOC107131209 LOC506828 MAP2K1 METAP2 MOB1A MPC2 MYDGF NAGA NAXE NFKB1 NME2 NSF NT5C2 OGT PAPSS2 PFKP PHB2 PICALM PKM PLG PNP POGLUT1 PPM1F PPP1CA PRDX3 PRDX4 PRKAR2A PSMA1 PSMA3 PSMA4 PSMA6 PSMB1 PSMB2 PSMB3 PSMB9 PSMD13 PTPN1 PTPRC PYCR3 QARS1 QDPR QKI RAD23A RAP2B RPN1 RPN2 RPS24 RUVBL2 SDCBP SDS SERPINA1 SERPINA3-7 SERPINA6 SERPINA7 SERPINC1 SERPIND1 SERPINF1 SERPINF2 SERPING1 SLK SPTLC2 STK24 TBL1XR1 TCOF1 TRIM25 TTR TXN UBA1 UCHL3 VPS35 XPO1 YARS1
GO:0010951	negative regulation of endopeptidase activity	0,0000000	12,34	A2M AGT C3 C4A CD44 FETUB ITIH1 ITIH2 ITIH3 ITIH4 KIF12 LOC107131209 LOC506828 PICALM PRDX3 SERPINA1 SERPINA3-7 SERPINA6 SERPINA7 SERPINC1 SERPIND1 SERPINF1 SERPINF2 SERPING1

GO:0010466	negative regulation of peptidase activity	0,0000000	12,30	A2M AGT C3 C4A CD44 FETUB ITIH1 ITIH2 ITIH3 ITIH4 KIF12 LOC107131209 LOC506828 PICALM PRDX3 SERPINA1 SERPINA3-7 SERPINA6 SERPINA7 SERPINC1 SERPIND1 SERPINF1 SERPINF2 SERPING1
GO:0045861	negative regulation of proteolysis	0,0000000	11,78	A2M AGT C3 C4A CD44 F2 FETUB ITIH1 ITIH2 ITIH3 ITIH4 KIF12 LOC107131209 LOC506828 OGT PICALM PRDX3 SERPINA1 SERPINA3-7 SERPINA6 SERPINA7 SERPINC1 SERPIND1 SERPINF1 SERPINF2 SERPING1
GO:0006120	mitochondrial electron transport, NADH to ubiquinone	0,0000001	6,83	DLD NDUFA10 NDUFB8 NDUFS1 NDUFS3 NDUFS6 NDUFS7 NDUFV2
GO:0046034	ATP metabolic process	0,0000002	6,73	ALDOA ATP5F1A ATP5ME ATP5PO ATP6V1B2 COX4I1 DLD NDUFA10 NDUFB8 NDUFS1 NDUFS3 NDUFS6 NDUFS7 NDUFV2 OGT PFKP PKM UQCRC1
GO:0002376	immune system process	0,0000004	6,39	ADD1 AP1G1 APOA4 APOE BoLA-DOB BOLA-DOB BOLA-DRB3 BoLA-DRB3 BOLA-NC1 C3 C4A CBFB CD14 CD247 CD36 CD44 CD47 CD99 CFB CLU CTSC CXCR4 CYBC1 ERAP1 F2 FN1 HMOX1 PRMT1 HRMT1L2 HSP90AA1 IL1RN IPO7 ISG15 ITGAM LGALS2 LGALS3 LGMN LOC107131209 MAP2K1 MYO1C agp ORM1 PHB2 PICALM PIP4K2A PLD4 PLG PRDX3 PRRC2C PSMA1 PSMB9 PTPRC RAB10 S100A13 SERPING1 SFXN1 STX7 TRIM25 TYROBP WAS
GO:0045333	cellular respiration	0,0000004	6,39	ACO2 ATP5F1A ATP5PO CAT COX4I1 DLD ETFDH NDUFA10 NDUFB8 NDUFS1 NDUFS3 NDUFS6 NDUFS7 NDUFV2 SDHA UQCRC1

GO:0042775	mitochondrial ATP synthesis coupled electron transport	0,0000035	5,45	COX4I1 DLD NDUFA10 NDUFB8 NDUFS1 NDUFS3 NDUFS6 NDUFS7 NDUFV2 UQCRC1
GO:0006629	lipid metabolic process	0,0039790	2,40	ACADM ACADVL ACSL6 AFP AGT ALDH3A2 APOA4 APOE ATP1A1 ATP5F1A C3 CAT CPT1A ETFDH F2 IL1RN LACTB NAGA NFKB1 PCCA PHB2 PIP4K2A PLBD2 PLD4 QKI SERPINA6 SPTLC2 TTR

Supplementary Table S6. List of enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins (DP) in cluster 2 after the hierarchical clustering heat map analysis.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0043484	regulation of RNA splicing	0,00000002	7,70	AHNAK CELF1 CELF2 DAZAP1 FAM50A LOC100848353 HNRNPA1 HNRNPK KHDRBS1 MBNL1 PTBP1 RBM12B RNPS1 TRA2A
GO:1903311	regulation of mRNA metabolic process	0,00000009	7,03	CELF1 CELF2 DAZAP1 DCPS LOC100848353 HNRNPA1 HNRNPD HNRNPK HNRNPM KHDRBS1 MBNL1 PTBP1 RNPS1 SLTM SYNCRIP TRA2A
GO:0050684	regulation of mRNA processing	0,00000037	6,43	CELF1 CELF2 DAZAP1 LOC100848353 HNRNPA1 HNRNPK KHDRBS1 MBNL1 PTBP1 RNPS1 SLTM TRA2A
GO:0034641	cellular nitrogen compound metabolic process	0,00000410	5,39	ACO1 ACOT9 AHNAK ARHGEF2 ARRB1 BANF1 BANK1 CELF1 CELF2 CETN2 DAZAP1 DCPS DDX39A DDX3X EDF1 EIF1AX EIF5A FAM50A FARSA FBL FIP1L1 GPX1 GSTP1 HMGB2 LOC100848353 HNRNPA1 HNRNPD HNRNPK HNRNPM KHDRBS1 LSM4 LUC7L MBNL1 MCM5 MTAP MTHFD1L MYBBP1A

				NARS1 NEK7 NPM1 OARD1 OPA1 PAPSS1 PDCD4 PKN1 PNKP POLR2B PPIH PRPF31 PRPSAP2 PTBP1 RBM12B REL RNPS1 RPL11 RPL3 RPL9 RPRD1B RPS14 RPS17 RPS20 RPS27 RPS28 RTCB SF3A2 SLTM SMCHD1 SNRNP200 SNRNP70 SNRPD2 SREK1 SRRM1 SRRT SRSF1 SRSF10 SSB STAT1 STRAP SYNCRIP TCEA1 TFAM TRA2A VNN2 WARS1 WBP11
GO:0043603	cellular amide metabolic process	0,00012000	3,92	ACO1 ACOT9 BANK1 DCPS DDX3X EIF1AX EIF5A FARSA GPX1 GSTP1 HNRNPD KHDRBS1 NARS1 RPL11 RPL3 RPL9 RPS14 RPS17 RPS20 RPS27 RPS28 VNN2 WARS1
GO:0006412	translation	0,00018000	3,74	ACO1 BANK1 DCPS DDX3X EIF1AX EIF5A FARSA HNRNPD KHDRBS1 NARS1 RPL11 RPL3 RPL9 RPS14 RPS17 RPS20 RPS27 RPS28 WARS1
GO:0010467	gene expression	0,00101500	2,99	ACO1 AHNAK AIF1 APOA2 ARHGEF2 ARRB1 BANK1 CDC37 CELF1 CELF2 DAZAP1 DCPS DDX39A DDX3X EDF1 EIF1AX EIF5A FAM50A FARSA FBL FIP1L1 GPX1 GSDMD HMGB2 LOC100848353 HNRNPA1 HNRNPD HNRNPK HNRNPM KHDRBS1 LSM4 LUC7L MBNL1 MYBBP1A NARS1 NCKAP1L NPM1 PDCD4 PHGDH PKN1 POLR2B PPIH PRPF31 PTBP1 RBM12B REL RNPS1 RPL11 Rpl3 RPL3 RPL9 RPRD1B RPS14 RPS17 RPS20 RPS27 RPS28 RTCB SF3A2 SLTM SMCHD1 SNRNP200 SNRNP70 SNRPD2 SNX12 SREK1 SRRM1 SRRT SRSF1 SRSF10 SSB STAT1 STRAP SYNCRIP TCEA1 TFAM TRA2A WARS1 WBP11
GO:0032635	interleukin-6 production	0,02296000	1,64	AIF1 ARHGEF2 ARRB1 BANK1 NCKAP1L
GO:0042254	ribosome biogenesis	0,00481500	2,32	DDX3X FBL NOP56 NOP58 NPM1 RPL11 RPL3 RPS14 RPS27 RPS28

Manuscript draft 6: Porcine milk exosomes modulate porcine peripheral blood mononuclear cell (PBMC) proteome *in vitro*

Porcine milk exosomes modulate porcine peripheral blood mononuclear cells (PBMC) proteome *in vitro*

G. Ávila[a], M. Bonnet[b], D. Viala[b,c], S. Dejean[d], A. Agazzi[a], M. Comi[e], C. Lecchi[a], F. Ceciliani[a]*

[a]Department of Veterinary and Animal Sciences, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy

[b]INRAE, University of Clermont Auvergne, Vetagro Sup, UMRH, 63122, Saint-Genès-Champanelle, France

[c]INRAE, Metabolomic and Proteomic Exploration Facility (PFEM), F-63122 Saint-Genès-Champanelle, France

[d]Institute of Mathematics of Toulouse, University of Toulouse, CNRS, UPS, UMR 5219,31062 Toulouse, France

[e]Department of Human Science and Quality of Life Promotion, Università Telematica San Raffaele, Via di Val Cannuta 247, 00166 Roma, Italy

[*]Corresponding author: Gabriela Ávila Morales

E-mail address: gabriela.avila@unimi.it

Department of Veterinary and Animal Science, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy

Tel: 0039 02 50334531

ABSTRACT

Exosomes are a subtype of nano-sized extracellular vesicles that mediate intercellular communication. By transferring their cargo (nucleic acids, proteins, lipids, and metabolites) into recipient cells, they can influence the cells' biological response. Exosomes can be found in different body fluids, including milk. Porcine milk exosomes are internalized by monocytes and modulate their immune functions *in vitro*, by decreasing their phagocytosis and chemotaxis, while increasing their oxidative burst. No information on the molecular mechanisms underlying the immunomodulatory effects on porcine mononuclear cells is available so far. Thus, this study aimed to assess the effects of porcine milk exosomes on the porcine peripheral blood mononuclear cells (PBMC) proteome. Cells were purified from the peripheral blood of seven healthy pigs (TOPIGS) and incubated with porcine milk exosomes (200 exosomes/cell) or medium as a control for 22 h at 39°C. Extracted proteins were then analyzed using nano-LC-MS/MS. A total of 1584 quantifiable proteins were identified, and the supervised multivariate statistical analysis, sparse variant partial least squares – discriminant analysis (sPLS-DA) for paired data, was applied to identify discriminant proteins (DP) that contributed to a clear separation between the porcine milk exosomes and control groups. A total of 384 DP from both components were selected. To elucidate the differential role of the identified DP for each treatment group, 54 proteins with the highest abundance in milk exosomes and 142 in the control group were selected to perform a Gene Ontology (GO) enrichment analysis with ProteINSIDE. DP with a higher abundance in porcine milk exosomes were mainly involved in innate immune-related and exosome uptake processes. Proteins such as TLR2, APOE, CD36, MFGE8 and CLU were found annotated in those enriched BP. In conclusion, these results provide a proteomics background to the immunomodulatory activity of porcine milk exosomes and to the potential mechanisms used by immune cells to internalize them

***Keywords**:* milk exosomes, pigs, PBMC proteome, immunomodulation, TLR2, CD36, uptake, phagocytosis

1. Introduction

Exosomes are nano-sized extracellular vesicles (30 - 160 nm) with an endosome-derived limiting membrane that mediate intercellular communication in both physiological and

pathological conditions [1,2]. They can be produced and released to the extracellular space by all cell types through exocytosis [3]. Exosomes carry a wide range of regulatory molecules (cargo) such as proteins, lipids, DNA, RNA (mRNA, miRNA, non-coding RNA) and metabolites [1]. These nanovesicles mediate near long-distance intercellular communication by targeting and transferring their cargo into recipient cells, altering their function [4,5].

Over the last two decades, it has been demonstrated that exosomes can induce, amplify and suppress both innate and adaptive immune responses. Indeed, exosomes have been shown to modulate natural killer (NK) cells activation, macrophage and T cell differentiation and monocytes chemotaxis [6–8]. Anti-inflammatory roles of exosomes in human peripheral blood mononuclear cells (PBMC) and T cells have also been reported [9].

Exosomes can be found in different body fluids, including milk [10]. Milk exosomes are also thought to exert potential immunomodulatory effects, as both human and bovine milk exosomes that contain functional RNA are taken up by human macrophages [11,12]. Porcine milk exosomes and their cargo have previously shown can also accumulate in piglets and mice peripheral tissues rich in immune cells (e.g. liver, spleen, lung and the small intestine) after suckling or oral administration [13], suggesting potential immunomodulation. Moreover, porcine milk exosomes demonstrated in our previous *in vitro* study to exert immunomodulatory effects on porcine monocytes, by decreasing their phagocytosis and chemotaxis and increasing their reactive oxygen species (ROS) production (See Manuscript draft 3, pages 103-133). However, the molecular mechanisms underlying the immunomodulatory activity of porcine milk exosomes on porcine mononuclear cells are still unknown.

The use of OMIC technologies, specifically proteomics – the large-scale study of the protein profile in a sample – provides a great option to unravel the molecular impact of porcine milk exosomes on porcine PBMC immune response [14]. In previous studies, porcine PBMC proteome has been investigated, mainly in the context of infectious diseases [15], stress [16], pregnancy [17] and characterization of the cell's protein profile [18]. However, no information on the *in vitro* impact of porcine milk exosomes on porcine PBMC proteome is available yet. Therefore, this study investigated for the first time the immunomodulatory capacity of porcine milk exosomes on porcine PBMC proteome *in vitro*, using an untargeted proteomics approach,

2. Materials and methods

2.1. Purification of porcine PBMC from blood

Porcine PBMC were isolated from peripheral blood through Ficoll density gradient centrifugation, as previously described for bovine blood, with some minor modifications [23]. Briefly, 100 mL of blood from seven 60-100 kg healthy pigs (TOPIGS) was collected during routine slaughtering procedures in sterile flasks containing 0.2% of EDTA (Sigma-Aldrich, St. Louis, MO, USA) as an anticoagulant. Blood was then centrifuged at 1260 x g for 30 min at 18 °C to obtain the buffy coat. The buffy coat was diluted 1:5 in cold sterile Dulbecco's PBS without Ca^{2+} and Mg^{2+} + 2 mM EDTA (Sigma-Aldrich), carefully layered onto Ficoll-Paque Plus (1.077g/mL) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and centrifuged at 1700 x g (without brakes) for 30 min at 4 °C to obtain the PBMC ring. PBMC were collected at the interface, washed with cold sterile PBS without Ca^{2+} and Mg^{2+} + 2 mM EDTA, centrifuged at 500 x g for 7 min at 4 °C and incubated with Red Blood Cell Lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany) for 3 min at room temperature to remove the red blood cells. Washes with cold sterile PBS+ 2 mM EDTA were performed to remove contaminant platelets. Finally, the PBMC were counted using an automatic cell counter (TC20TM, BioRad), their viability assessed with trypan blue exclusion, and resuspended at the desired concentration in complete medium (RPMI 1640 Medium + 25 mM HEPES + L-Glutamine, supplemented with 1% of Non-essential Amino Acid Solution 100X and 1% Penicillin Streptomycin Solution 100X and 1% exosome-depleted Fetal Bovine Serum (FBS) (Sigma-Aldrich).

2.2. Porcine PBMC stimulation with porcine milk exosomes

First, porcine milk exosomes were purified from sows' milk and characterized by means of their concentration, size, morphology/structure, and presence of exosome marker protein, as previously reported (See Manuscript draft 3, pages 103-133). For each experiment, an aliquot of LPS-depleted porcine milk exosomes (6.2 x 10^{10} exosomes/mL) was thawed and used. Briefly, a total of 15 x 10^6 porcine PBMC (1.5 mL) were seeded in triplicates in Corning® tissue-culture treated culture 60 mm dishes (Corning Inc., Costar, Kennebunk, ME, USA) and incubated with 3 x 10^9 (48.4 µL) porcine milk exosomes (in a ratio of 200 exosomes/cell) for 22 h at 39 °C in a humified atmosphere with 5% CO_2.

Complete medium (1% exosome-depleted FBS) was then added to each dish to reach a final volume of 3 mL.

2.3. Porcine PBMC collection and protein extraction

After incubation time, around 3mL of the cells in suspension (lymphocytes) were collected and centrifuged at 500 x g for 7 min at 4°C. The lymphocytes were then washed twice with 500 µL sterile-PBS without Ca^{2+} and Mg^{2+} (room temperature) and again centrifuged twice at 500 x g for 7 min at 4°C to remove the remaining medium and FBS. In the meantime, three washes with 2 mL of sterile-PBS without Ca^{2+} and Mg^{2+} (room temperature) were performed in the 60 mm dishes to wash the adhered monocytes and remove dead cells. When both, the lymphocytes pellet and the adhered monocytes were washed, 500 µL of Igepal buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA and EGTA, 100 mM NaF, 4 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 2 mM Na_3VO_4, 1% Triton X100, 0.5% IGEPAL CA-630 (Sigma-Aldrich), 1 pill for 50 mL of the total volume of Protease Inhibitor Cocktail (Roche)) was added to the lymphocytes pellet. The lymphocytes were resuspended and added directly to the 60 mm dishes containing the monocytes. The dishes containing the cells and the Igepal buffer were incubated for 1h at 4°C with regular shaking to allow the lysis of the cells. After incubation, the cells were scraped using sterile Corning Cell scrapers (blade L 1.8 cm, handle L 25 cm; Corning Inc.), the cell lysate was collected and sonicated for 10 min in an ultrasonic bath (Branson 2200, Danbury, CT, USA) to complete the cells' lysis and homogenization. Finally, the cell lysates were centrifuged for 10 min at 6000 x g at 4°C to remove the cell debris, and the supernatant containing the extracted proteins of all 3 technical replicates for each biological replicate was pooled in 1.5 mL tubes, aliquoted and stored at -80 °C for further analyses.

2.4. Sample preparation for proteomic analysis

The total protein concentration was determined with the Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's instructions. For peptide preparation, extracted proteins were digested and concentrated using the Filter Aided Sample Preparation (FASP) method, following the manufacturer's instructions with some minor modifications. Briefly, 50 µg of proteins lysate were put into Millipore 10 kDa MWCO filters and 100 µl of Urea 8 M in 0.1 M Tris-HCl pH 8.5 were added for solubilization. The detergent together with other contaminant components within the protein lysate were removed by repeated filtration by

centrifugation. After discarding the flow-through, proteins were reduced with 100 µl DTT (10 mM) for 46 min and centrifuged at 14000 rpm for 10 min. Then, 200 µl of 25 mM iodoacetamide solution were added to the filters and incubated for 20 min at room temperature for protein alkylation. After performing several washes of the filters with Urea 8 M and 50 mM of ammonium bicarbonate, proteins were digested with 200 µl of 0.25 mg/mL trypsin (50:1 ratio of protein:enzyme) and mixed at 600 rpm in a thermomixer for 1 min. Filters were then transferred to new collection tubes, incubated in a wet chamber at 37 °C overnight. Finally, peptides were eluted by adding 200 µl of 50 mM of ammonium bicarbonate and the filtrate lyophilized in a SpeedVac and then resuspended in 100 µl of 1% formic acid.

2.5.Nano-LC-MS/MS analysis

For each sample, 50µg of proteins were used according to the FASP preparation. The final volume was adjusted exactly to 300 µL with a recovery solution (H_2O/ACN/TFA – 94.95/5/0.05). After passing through the ultrasonic bath (10 min), the entire supernatant was transferred to an HPLC vial before LC-MS/MS analysis. Peptides mixtures were analyzed by nano-LC-MS/MS (ThermoFisher Scientific) using an Ultimate 3000 system coupled to a QExactive HF-X mass spectrometer (MS) with a nanoelectrospray ion source. Five µL of hydrolysate was first preconcentrated and desalted at a flow rate of 30 µl/mn on a C18 pre-column 5 cm length x 100 µm (Acclaim PepMap 100 C18, 5µm, 100A nanoViper) equilibrated with Trifluoroacetic Acid 0.05% in water. After 6 min, the concentration column was switched online with a nanodebit analytical C18 column (Acclaim PepMap 100 - 75 µm inner diameter × 25 cm length; C18 - 3 µm -100Å - SN 20106770) operating at 400 nL/min equilibrated with 96 % solvent A (99.9 % H2O, 0.1 % formic acid). The peptides were then separated according to their hydrophobicity thanks to a gradient of solvent B (99.9 % acetonitrile, 0.1 % formic acid) of 4 to 25% in 60 minutes. For MS analysis, eluted peptides were electro sprayed in positive-ion mode at 1.6 kV through a Nano electrospray ion source heated to 250°C. The mass spectrometer operated in data-dependent mode: the parent ion is selected in the orbitrap cell (FTMS) at a resolution of 60,000 and each MS analysis is succeeded by 18 MS/MS with analysis of the MS/MS fragments at a resolution of 15,000).

2.5. Processing of raw mass spectrometry data

At the end of the LC-MS/MS analysis, for raw data processing, MS/MS ion search was carried out with Mascot v2.5.1 (http://www.matrixscience.com) against the porcine database (i.e. ref_sus_scrofa 20210114-49,792 sequences). The following parameters were used during the request: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C), oxidation (M) and deamidation (NQ) set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identity above Mascot scores with a False Discovery Rate of 1%. Ions score is -10 log(P), where P is the probability that an observed match is a random event. For the porcine proteome, the Mascot score was 32 with a False Discovery Rate (FDR) of 1%. The adjusted p-value was 0.02747. Finally, for label-free protein quantification analysis, LC-Progenesis QI software (version 4.2, Nonlinear Dynamics, Newcastle upon Tyne, UK) was used with the same identification parameters described above with the phenotypic data among all matrices. All unique validated peptides of an identified protein were included and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. LC-Progenesis analysis yielded a total of 1584 unique quantifiable proteins, having at least two peptides and two unique peptides.

2.6.Statistical analyses

2.6.1. Univariate analysis

To identify the differential abundant proteins a univariate statistical approach was performed on all 1584 quantified proteins, using an R package for proteomic analysis (available on GitHub with the DOI https://doi.org/10.5281/zenodo.2539329) as previously described [25]. Briefly, data normality distribution was assessed by the Shapiro-Wilk-Test and then either a paired Student's t-test, for normally distributed data, or a paired Wilcoxon test for not normally distributed data, was applied to determine the p-value. Finally, the obtained p-values were corrected using different adjustment methods (e.g. Benjamini & Hochberg or FDR, Hochberg and Bonferroni). For all of these analyses, R version 3.6.3 was used.

2.6.2. Multivariate analysis

An alternative supervised multilevel sparse variant partial least square discriminant analysis (sPLS-DA) was applied as no differentially abundant proteins were detected. The multilevel sPLS-DA enables the selection of the most predictive or discriminative proteins in the data of the two components (PC1 and PC2) to classify or cluster the samples between the treatment groups [26]. The multivariate analysis was performed using the mixOmics package in R (http://mixomics.org).

2.7.Bioinformatic analysis of discriminant proteins (DP) selected by sPLS-DA

First, before performing the bioinformatic and functional annotation analyses, the accession numbers from all 1584 quantified proteins were converted into Gene ID using UniProt retrieve/ID mapping online tool. For undefined proteins, the Gene ID of human orthologs of porcine proteins was assigned. Second, only for the selected DP a gene ontology (GO) enrichment analysis, focused on the Biological Processes (BP) was performed using ProteINSIDE online tool version 2.0 (available at https://umrh-bioinfo.clermont.inrae.fr/ProteINSIDE_2/index.php?page=upload.php), as previously described [27], and using *Sus scrofa* as reference specie. Only significant (FDR < 0.05) GO BP terms were considered enriched and were used for further interpretation and figure creation. The enriched GO BP terms were summarized and their enrichments were expressed as -log10 (FDR) for visualization on horizontal bar graphs plotted using GraphPad Prism 9.1.2 for Mac OS X, GraphPad Software (San Diego, California, USA). Finally, the average log fold-change (logFC) of the raw abundances of all the DP identified with sPLS-DA from all the seven 7 biological replicates was calculated and plotted using R version 3.6.3.

3. Results

3.1. The proteome of porcine PBMC and identification of the molecular signature of porcine milk exosomes treatment

A total of 1584 proteins, with at least two unique peptides, were identified and quantified in porcine PBMC (data not shown). A classical univariate statistical analysis (paired t-test or Wilcoxon test) was applied and no differentially abundant proteins were detected between the treatment groups after adjusting the p-value (FDR and Bonferroni). However, to retrieve meaningful biological information, the multivariate sPLS-DA analysis was applied to identify the molecular signature of the porcine milk exosomes

treatment. The sPLS-DA model selected a total of 384 DP (approximately 25% of the total proteins identified) from both components (data not shown), which contributed to a good clustering between the two treatment groups (Fig. 1). From the 384 DP, only the proteins from the sPLS-DA component 1 that strongly correlated together (data not shown) for each treatment group were selected to perform separate GO BP enrichment analyses. From these proteins, a total of 54 proteins had the highest abundance in the CP group, while 142 in the control group (data not shown).

3.2. GO enrichment analyses of total DP identified by s-PLS-DA

To identify the global function of all the 384 DP a GO enrichment analysis was performed. These DP were annotated by 301 enriched (FDR < 0.05) GO terms within the BP category. The main enriched BP were related to cellular supramolecular fiber organization (GO:0097435), metabolic process (GO:0008152), actin cytoskeleton organization (GO:0030036), intracellular transport (GO:0046907), positive regulation of organelle organization (GO:0010638), translation (GO:0006412), regulation of vesicle-mediated transport (GO:0060627), gene expression (GO:0010467), immune system process (GO:0002376) and regulation of apoptotic signaling pathway (GO:2001233) (Fig. 2). Among the most annotated BP were: gene expression (142), metabolic processes (92), immune system process (70) and intracellular transport (50) (Supplementary Table S1).

3.3.GO enrichment analyses of DP with the highest abundance in the milk exosomes group

To further elucidate the specific role of the DP with the highest abundance in each group, separate GO enrichment analyses were performed. The 54 proteins with the highest abundance in the milk exosomes group were annotated by 130 enriched (FDR < 0.05) GO terms within the BP category. These proteins were mainly involved in BP such as cellular detoxification (GO:1990748), response to diacyl bacterial lipopeptide (GO:0071726), nitric oxide-mediated signal transduction (GO:0007263), cGMP-mediated signalling (GO:0019934), positive regulation of tumor necrosis factor production (GO:0032760), positive regulation of endocytosis (GO:0045807), secretion (GO:0071706), immune system process (GO:0002376), ERK1 and ERK2 cascade (GO:0070371), regulation of body fluid levels (GO:0050878) and phagocytosis, recognition (GO:0006910). From these enriched BP, the ones with the highest number

of annotated proteins were the immune system process with nine proteins, secretion with six proteins, and ERK1 and ERK2 cascade and regulation of body fluid with five proteins (Fig. 3). CD36 and APOE were mainly annotated by almost all of these enriched BP. SASH3, CLU and CTSH were found in the immune system process, but CLU and CTSH were also found in positive regulation of endocytosis, and ERK1 and ERK2 cascade, respectively. Moreover, TLR2 was found in the biological processes involved with the response to bacterial lipopeptide, immune system process and secretion. In the latter, and also in the regulation of body fluids, CSN2 and CSN3 were also found. Lastly, MFGE8 was only found in phagocytosis (Supplementary Table S2).

3.4. GO enrichment analyses of DP with the highest abundance in the control group

A higher number of DP (142) was found to be more abundant in the control group. These proteins were annotated by a total of 64 enriched (FDR < 0.05) BP GO terms. These enriched GO terms were mainly related to the catabolic process (GO:0009056), amide biosynthetic process (GO:0043604), cellular organization (GO:0051641), translation (GO:0006412), metabolic process (GO:0008152), vesicle budding from the membrane (GO:0006900), vesicle-mediated transport (GO:0016192), regulation of anoikis (GO:2000209) and interleukin-27-mediated signaling pathway (GO:0070106) (Fig. 4). The enriched BP with the highest number of annotated proteins were: metabolic processes (87), cellular localization (35) and catabolic process (34) (Supplementary Table S3).

3.5. Average log fold-change (logFC) of the abundances of the DP identified with sPLS-DA

Lastly, to identify and focus on key DP that presented greater changes in their abundance after porcine milk exosomes treatment, the average logFC of the raw abundances of the proteins from the cells treated with milk exosomes was calculated (Fig. 5). The protein with the greatest change in the abundance (logFC > 100) between the milk exosomes and control was CSN2. Other proteins with higher abundances in the milk exosomes group were found, including BTN1A1, CSN1S1, CSN1S2, MFGE8 and SLC34A2 (logFC > 5). On the other hand, FABP4 (logFC < 0.5) and LGALS3 (logFC < 1) showed the lowest abundance in the milk exosomes group.

4. Discussion

The present study describes the proteome of porcine PBMC after stimulation with milk exosomes. Interestingly, in our previous *in vitro* study, we demonstrated for the first time that porcine milk exosomes were internalized by monocytes and that they modulated three inflammatory functions of monocytes (See Manuscript draft 3, pages 129-165). However, to the best of our knowledge, the exact molecular mechanisms underlying such effects have not been yet elucidated. In this study, we present for the first time that porcine milk exosomes can modulate PBMC proteome *in vitro*, providing a molecular signature of their immunomodulatory activity. Our main findings were that porcine milk exosomes caused an enrichment in BP related to different immune system processes, such as response to diacyl bacterial lipopeptide, nitric oxide-mediated signal transduction, positive regulation of tumor necrosis factor and phagocytosis. Moreover, enrichment of processes related to exosome internalization or uptake by immune cells, including phagocytosis and endocytosis was also observed, suggesting a potential role of porcine milk exosomes in modulating immune defense response.

4.1. Global biological processes enriched by the DP selected by sPLS-DA

In the first part of the study, an sPLS-DA model was applied to select the most discriminative proteins that allow an adequate clustering of the samples from the milk exosomes and control groups [26]. The sPLS-DA selected 384 DP that enabled clear discrimination between the two treatment groups.

To determine globally the function of these DP, a GO enrichment analysis was performed, and it revealed that the DP was involved in both, very general BP processes, but also in other more specific ones. Among the general BP, metabolic process, intracellular transport, positive regulation of organelle organization, regulation of vesicle-mediated transport and translation and gene expression were enriched. These processes are essential for proper functioning and for maintaining the homeostasis of all cell types [29–31]. On the other hand, among the more specific we found BP related to immune system process, cellular supramolecular fiber organization, actin cytoskeleton organization and regulation of apoptotic signaling pathway were also found enriched. It is widely known that the actin cytoskeleton organization and fiber organization are two necessary processes for immune functions like chemotaxis, phagocytosis and cell communication [32–34]. Furthermore, the actin cytoskeleton has also been shown to be necessary for immune cells' crosstalk or communication, by playing a crucial role in the formation and

maintenance of the immunological synapse structure. Moreover, apoptosis – an ATP-dependent type of programmed cell death - is also another key feature of immunity, as it controls the immune response by eliminating autoreactive lymphocytes, or other immune cells like monocytes in the inflammatory environment, apoptosis rate being critical for a normal cell turnover and thus maintaining homeostasis.

4.2. Biological processes enriched by DP with the highest abundance in porcine milk exosomes *group*

In the second part of the study, to further elucidate the differential effects between the porcine milk exosomes and control groups, separate GO enrichment analyses were performed, using the DP with the highest abundance in each group. The 54 DP with the highest abundance in the porcine milk exosomes group enriched BP are involved in immune system process, including response to diacyl bacterial lipopeptide, nitric oxide-mediated signal transduction, positive regulation of tumor necrosis factor production, phagocytosis.

In the past decade, there has been increasing evidence of the immunomodulatory activity of exosomes coming from different sources, including milk [6–8,28]. Indeed, there is recent evidence that exosomes can modulate the innate immune response through the interaction with Toll-like receptors (TLR), by both triggering or inhibiting them [37]. TLR are a class of pathogen recognition receptor (PPR) expressed by innate immune cells (e.g. monocytes and macrophages), which upon recognition of the invading microorganism trigger an inflammatory response for its elimination [38].

Remarkably, in our study Toll-like receptor 2 protein (TLR2) -a membrane-bound PRR-, was among the DP found with a higher abundance in the porcine milk exosomes group, and was found annotated in BP such as response to diacyl bacterial lipopeptide and immune system process. Our results agree with those observed in other cellular models, where milk exosomes and other milk extracellular vesicles (EV) have also been shown to be able to interact with TLR. Specifically, human breast milk exosomes inhibited the activation of TLR4 in the intestinal epithelium, suggesting protective effects against necrotizing enterocolitis [41,42]. Schwan cells-derived exosomes also upregulated the expression of TLR2 in astrocytes through the NF-κB/PI3K signaling pathway, triggering the cells' inflammatory responses [39]. In addition, exosomes derived from *M.tuberculosis* infected cells have also been shown to suppress macrophages' response to IFN-γ, by affecting their expression of TLR2 [40].

The positive regulation of tumor necrosis factor production (TNF) was also enriched by the porcine milk exosomes and its production, together with other pro-inflammatory cytokines, is known to be dependent on TLR signaling pathways [43]. Our results agree with previous studies of exosomes coming from different sources. For example, exosomes isolated from systemic lupus erythematosus patients enhanced the production of TNF-α and other pro-inflammatory cytokines via the TLR1/2, TLR7, TLR9, and TLR4 pathways [44].

Another important process of the immune response is the production of nitric oxide (NO) and its mediated signaling transduction. Both NO-mediated signaling transduction and cGMP-mediated signaling were found enriched after milk exosomes treatment. NO production also occurs after TLR activation, which culminates in the upregulation of gene transcription of pro-inflammatory mediators such as the inducible nitric oxide synthase (iNOS), an enzyme that produces NO [46]. NO can also convert into different reactive nitrogen species (RNS) that can be highly toxic to invading microbes [47]. Besides exerting a protective role against pathogens, NO is also an important signaling molecule that acts via the activation of the NO sensitive guanylyl cyclase (NO-GC). This activation then leads to the formation of the second messenger cGMP that can further regulate some innate and adaptive immune functions [48,49]. These results are consistent previous studies on other cellular models, where exosomes derived from hypoxic cardiomyocytes promoted NO production in endothelial cells [50]. Moreover, the release of exosomes from senescent macrophages, containing iNOS has been reported; and bovine milk exosomes containing miR-155-5p – an essential regulator of eNOS and NO – increased eNOS in endothelial cells. Altogether, this suggests that exosomes cargo might also play an important role in modulating NO production [51,52].

The glycoprotein IIIb (CD36) was one of the proteins involved in almost all of the enriched BP, including the previously mentioned ones and in phagocytosis. CD36 is a membrane glycoprotein and a member of the class B scavenger receptor family, present on mononuclear phagocytes (e.g. monocytes and macrophages) among other cells. It functions as a scavenger receptor, and thus participates in the identification and phagocytosis of apoptotic cells, some pathogens, lipoproteins, and in general in the inflammatory response [53,54]. Moreover, it has been identified as a co-receptor of TLR2 in response to diacylated lipoproteins [55].

Another enriched BP was phagocytosis. Phagocytosis plays an important role in the uptake of exosomes by monocytes and macrophages [56,57]. However, different forms

of endocytosis have also been proposed as mechanisms of exosomes internalization, in which CD36 is also involved [57,58]. These results also agree with a previous study in which exosomes upregulated human macrophages' phagocytosis *in vitro,* and the proteomic analysis revealed that the identified exosome proteins were mainly related to Fcγ receptor-mediated phagocytosis [59]. Another protein that was found only annotated in the phagocytosis BP is the milk fat globule-EGF factor 8 (MFGE8) or lactadherin. MFGE8 is a phosphatidylserine-binding glycoprotein secreted and expressed by macrophages and other immune cells that promotes the engulfment of apoptotic cells and vesicles [60,61]. It is also one of the immune components found in milk, specifically in milk fat globules and highly enriched in milk exosomes [62,63]. Therefore, it is thought that MFGE8 might play a role in the intestinal immune system of newborns, as it has been shown to modulate intestinal dendritic cell (DC) activity and induce T cell differentiation [62]. This protein also plays an important part in maintaining cell homeostasis and tolerogenic immune responses, by eliminating apoptotic cells and inducing the secretion of regulatory T cells (Treg)-inducing cytokines [60]. Lastly, MFGE8 as a surface protein is involved in the targeting and internalization of EV, including exosomes, by specialized phagocytes (e.g. macrophages and monocytes) through the binding to the phosphatidylserine often present in these vesicles [65]. We can therefore hypothesize that exosomes can function as potential nanocarriers of immunomodulatory molecules (e.g. MFGE8) that participate in the transmission of immunity from the mother to the offspring, helping in the development of the newborn immune system and growth [64].

CD36 downstream signaling also involves the production of ROS and modulation of macrophage migration [53,66]. Specifically, it has been demonstrated that CD36 in response to oxidized low-density lipoproteins (LDL) can inhibit murine and human macrophage migration, by altering the cytoskeletal dynamics [66]. The fact that this DP showed a higher abundance in the porcine milk exosomes group, was of great interest, as in our previous *in vitro* study, we observed that porcine milk exosomes caused an increase in the ROS production and inhibition of the chemotaxis of porcine monocytes (See Manuscript draft 3, pages 103-133). Remarkably, a previous study using another model, confirmed these results, as extracellular vesicles (EV), including exosomes, inhibited endothelial cells migration and increased the ROS production via the CD36-mediated pathway [67]. Moreover, the fact that CD36 was involved and annotated in two of the main mechanisms (e.g. phagocytosis and endocytosis) of exosomes internalization used

by phagocytes, supports the uptake of porcine milk exosomes by porcine monocytes we observed in our previous *in vitro* study (See Manuscript draft 3, pages 103-133).

ERK1 and ERK2 cascade was another of the enriched BP after milk exosomes treatment. The extracellular signal-regulated kinase 1/2 (ERK1/2) cascade is a central signaling pathway that allows the intracellular communication between cell membrane receptors and their nuclear/cytoplasmic targets upon stimulation. It regulates a wide variety of stimulated cellular processes, including proliferation, differentiation, migration, survival, apoptosis and stress response [68]. Interestingly, in a previous study, it was shown that exosomes activated the ERK1/2 cascade and that its activity is necessary for an efficient exosome uptake via lipid raft-mediated endocytosis [69]. Moreover, it was previously found that exosomes and their miRNA cargo can modulate the ERK1/2 signaling pathway to induce immunomodulatory effects such as reducing macrophage migration [70]. Pro-cathepsin H (CTSH) - a lysosomal cysteine protease involved in protein degradation - was among the proteins annotated in this BP [71]. Cathepsins are key regulators of the innate and adaptive immune system [72]. Some cathepsins have been shown to regulate the migration of endothelial cells [73]. Cathepsins have also been found in EV and seem to be involved in their biogenesis and secretion [74].

Additionally, proteins such as APOE, CLU and SASH3 were found annotated in several of the immune-related processes previously described. Specifically, apolipoprotein E (APOE) - a major protein component of very-low and high-density lipoproteins – is mainly expressed by monocytes/macrophages and has shown to exert anti-inflammatory roles, including reduction of migration of some cells [75,76]. Our results are consistent with what has been previously reported on gastric cancer cells, where exosomes released by anti-inflammatory (M2) macrophages increased the levels of APOE in the cancer cells, and modulate their migratory capacity through the transfer of functional APOE [77]. The presence of APOE in porcine colostrum and milk exosomes has been also recently reported [24]. The function of APOE in modulating the migration of different cell types seems to be mediated in a MAPK/ERK1/2 dependent manner, which explains why this DP was also found annotated in the ERK1/2 cascade BP [78].

Clusterin (CLU) or also known as apolipoprotein J, is a ubiquitously expressed glycoprotein, and a known extracellular chaperone that has shown to exert immunomodulatory and anti-inflammatory effects such as modulation of antimicrobial responses, facilitation of apoptotic cell clearance and suppression of kidney macrophage

infiltration [79,80]. CLU can be found in both, milk and milk exosomes, and is one of the proteins that is implied to modulate the immune response of the offspring [81].

SAM and SH3 domain-containing 3 protein (SASH3) is an adaptor protein that plays an important role in the organization of signaling complexes and propagation of signal transduction cascades in lymphocytes. Moreover, it is involved in the positive regulation of the adaptive immune system, by modulating lymphocytes activation, proliferation and antibodies production, among other processes [82]. These results suggest that exosomes can modulate not only different aspects of innate immunity but also adaptive immunity, confirming their pleiotropic functions.

Lastly, other BP such as cellular detoxification, secretion and regulation of body fluids were also found enriched. In the first BP, proteins related to the removal of harmful toxins (AKR1A1 and ESD) were found. Cellular detoxification is a key feature of the proper function of the immune system [83]; and exosomes seem to play an important role in the detoxification of target cells by carrying and transferring drug or toxin-metabolizing enzymes, as has been previously observed in hepatocytes derived exosomes [84].

In the secretion and regulation of body fluids BP, proteins such as β-caseins and κ-casein (CSN2 and CSN3), among others were found. Caseins are among the most abundant proteins in milk and are involved in milk synthesis and the secretory process [85]. Moreover, caseins have also known to present antimicrobial properties such as it is the case of κ-casein, by inhibiting the adhesion of pathogenic bacteria to the mucosal lining [86]. Finally, in a previous study, it was demonstrated that caseins enhanced EV uptake *in vivo* in mice, altering the gene expression in blood cells [87].

Thus, altogether these results suggest that porcine milk exosomes can exert immunomodulatory effects by changing the abundance of some porcine PBMC proteins mostly related to innate immune responses and that might be mediated through the TLR2 signaling pathway. In addition, the enrichment of some BP and pathways involved with exosome uptake might indicate that exosomes internalization might be necessary for carrying out their immunomodulation.

4.3. Biological processes enriched by DP with the highest abundance in the control group

In contrast, to what was observed in the milk exosomes group, the 142 DP with a higher abundance in the control group were annotated mainly by very general BP such as different metabolic processes, cellular organization translation and vesicle-mediated

transport. These processes besides being the most enriched presented also the highest number of annotated proteins.

Other processes related to cell death and immune-related processes such as regulation of anoikis and interleukin-27-mediated signaling pathway were also identified, respectively. Anoikis is a subtype of apoptosis that is caused when cells lose their capacity to attach to the extracellular matrix (ECM) or their substrate and neighboring cells, which is required for normal cell function and cell turnover [88]. Anoikis is mostly seen in epithelial cells and cancer cells, however, previous studies have reported that monocytes can also undergo anoikis *in vitro* [89].

Interleukin-27 (IL-27) is a cytokine produced by monocytes, macrophages and DC, with both pro-and anti-inflammatory as well other immunoregulatory functions. IL-27-mediated signaling pathway regulates the transcription of genes involved in cell proliferation, differentiation and expression of cytokines [90].

Altogether, these results highlight that after porcine milk exosomes treatment, it occurred a functional switch from very general BP to others mostly related to immune response and exosome uptake processes.

4.4. DP with greater changes in abundance in the porcine milk exosomes group

T*o* identify key DP that presented greater changes in their raw abundances when after porcine milk exosomes treatment, their logFC was calculated. The protein with the highest abundance after treatment with milk exosomes was β-casein (CSN2), one of the most abundant proteins in milk. Caseins besides providing nourishment to the offspring, by being a major source of amino acids, is also known to have immunomodulatory effects. Specifically, β-casein and other caseins can be broken down into bioactive peptides that have shown to influence the activity of immune cells [91,92]. Alpha-s1-casein (CSN1S1) and Alpha-s2-casein (CSN1S2) were also found, but in lesser abundance than CSN2. All of these three caseins, but also CSN3, have been identified previously in isolated porcine milk exosomes, while only CSN2 and CSN1S1 were detected in bovine milk exosomes after proteomics analyses [24,93]. Interestingly, CSN1S1 was shown to be expressed outside the mammary gland and to be expressed by immune cells such as monocytes and T cells [94]. Moreover, this protein has shown to exert also immunomodulatory effects on monocytes such as: inducing the expression of IL-1β and the *in vitro* differentiation towards a macrophage-like phenotype [94,95].

Finally, BTN1A1, SLC34A2 and MFGE8 proteins also presented higher changes in their abundance in the milk exosomes group. Butyrophilin subfamily 1 member A1 (BTN1A1), is a member of the immunoglobulin superfamily that was first discovered in milk and is mainly associated with milk fat globules. Immunomodulatory roles of this protein have also been reported, mainly inhibitory effects on CD4+ T cell proliferation and T cell expression of cytokines associated with T cell activation; and some antibacterial properties as well [96]. The Na (+)-dependent phosphate cotransporter 2B protein (SLC34A2) is a multi-pass membrane protein that has been involved in the activation of the complement alternative pathway (C3 and C4b) [97,98]; while MFGE8 as previously described herein has been involved in the phagocytosis of apoptotic cells, modulation of DC and T cells activity, and more recently know in exosomes uptake by phagocytes [60,62,65].

On the contrary, lower abundances in the fatty acid-binding protein 4 (FABP4) and galectin-3 (LGALS3) were found. FABP4 is a protein known to play a crucial role in lipid transportation and that functions as a transmitter linking lipid metabolism to inflammation. LGALS3 is a carbohydrate-binding protein, known to be highly expressed in monocytes and macrophages and to be a strong regulator of cell migration and phagocytosis, among other immune and inflammation-related processes [99,100]. This finding, is remarkable, as it could potentially explain the suppressive effects of porcine milk exosomes we observed previously on porcine monocytes *in vitro* (See Manuscript draft 3, pages 103-133).

5. Conclusions

The results of this study demonstrate for the first time that porcine milk exosomes can modulate porcine PBMC proteome *in vitro*. The GO enrichment analysis revealed that the DP with higher abundance in the porcine milk exosomes group were mostly related to innate immune-related processes that are mediated in a TLR2 dependent manner; and to exosome uptake processes. Our results also suggest that porcine milk exosomes might exert pleiotropic immunomodulatory functions on porcine PBMC, by increasing the abundance of proteins with both, immune-enhancing and dampening properties. Therefore, these results confirm the suppressive and enhancing effects of porcine milk exosomes on porcine monocytes, observed in our previous *in vitro* study, where a decrease in the cells' phagocytosis and chemotaxis; and an increase in their oxidative burst was detected. Moreover, the detection of caseins and other milk proteins with

known immunomodulatory properties, which are also found in porcine milk exosomes, exemplifies how exosomes could fulfil their functions in intercellular communication by transferring their cargo to target cells. By providing a molecular background of porcine milk exosomes' immunomodulatory activity, we can have a better understanding of their potential role as novel dietary supplements, specifically in the sow-to-piglet transmission of regulatory molecules and immunomodulation. Finally, additional molecular pathway enrichment and protein-protein interaction analyses; and integration of proteomics data with other OMIC technologies should be performed to have a holistic view of the impact of porcine milk exosomes on porcine immunity.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper's content.

Ethics statement

The procedures for the blood collection were carried out during routine slaughtering procedures.

Acknowledgements

The authors acknowledge A. Delavaud (INRAE, Herbivore Research Unit) for his technical assistance in protein extraction, quantification, and concentration for mass spectrometry analyses, and also Arnaud Cougoul, Jeremy Tournayre and Céline Boby (INRAE, Herbivore Research Unit) for their assistance in the statistical and bioinformatic analyses, respectively.

Funding

This study was supported by the European Union's Horizon 2020 research and innovation programme H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate) [Grant agreement nº: 765423, 2017] – MANNA.

References

[1] R. Kalluri, V.S. LeBleu, The biology, function, and biomedical applications of exosomes, Science (80-.). 367 (2020). doi:10.1126/science.aau6977.

[2] A. Becker, B.K. Thakur, J.M. Weiss, H.S. Kim, H. Peinado, D. Lyden, Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis, Cancer Cell. 30 (2016) 836–848. doi:10.1016/j.ccell.2016.10.009.

[3] C. Wen, R.C. Seeger, M. Fabbri, L. Wang, A.S. Wayne, A.Y. Jong, Biological roles and potential applications of immune cell-derived extracellular vesicles, J. Extracell. Vesicles. 6 (2017) 1–19. doi:10.1080/20013078.2017.1400370.

[4] G. Raposo, W. Stoorvogel, Extracellular vesicles: Exosomes, microvesicles, and friends, J. Cell Biol. 200 (2013) 373–383. doi:10.1083/jcb.201211138.

[5] A. Chow, W. Zhou, L. Liu, M.Y. Fong, J. Champer, D. Van Haute, A.R. Chin, X. Ren, B.G. Gugiu, Z. Meng, W. Huang, V. Ngo, M. Kortylewski, S.E. Wang, Macrophage immunomodulation by breast cancer-derived exosomes requires Toll-like receptor 2-mediated activation of NF-κ B, Sci. Rep. 4 (2014). doi:10.1038/srep05750.

[6] P. Dalvi, B. Sun, N. Tang, L. Pulliam, Immune activated monocyte exosomes alter microRNAs in brain endothelial cells and initiate an inflammatory response through the TLR4/MyD88 pathway, Sci. Rep. 7 (2017) 1–12. doi:10.1038/s41598-017-10449-0.

[7] G. Raposo, H.W. Nijman, W. Stoorvogel, R. Liejendekker, C. V Harding, C.J. Melief, H.J. Geuze, B lymphocytes secrete antigen-presenting vesicles., J. Exp. Med. 183 (1996) 1161–1172. doi:10.1084/jem.183.3.1161.

[8] N. Chaput, C. Théry, Exosomes: Immune properties and potential clinical implementations, Semin. Immunopathol. 33 (2011) 419–440. doi:10.1007/s00281-010-0233-9.

[9] W. Chen, Y. Huang, J. Han, L. Yu, Y. Li, Z. Lu, H. Li, Z. Liu, C. Shi, F. Duan, Y. Xiao, Immunomodulatory effects of mesenchymal stromal cells-derived exosome, Immunol. Res. 64 (2016) 831–840. doi:10.1007/s12026-016-8798-6.

[10] C. Admyre, S.M. Johansson, K.R. Qazi, J.-J. Filén, R. Lahesmaa, M. Norman, E.P.A. Neve, A. Scheynius, S. Gabrielsson, Exosomes with Immune Modulatory Features Are Present in Human Breast Milk, J. Immunol. 179 (2007) 1969 LP – 1978.

[11] C. Lässer, V. Seyed Alikhani, K. Ekström, M. Eldh, P. Torregrosa Paredes, A. Bossios, M. Sjöstrand, S. Gabrielsson, J. Lötvall, H. Valadi, Human saliva, plasma and breast milk exosomes contain RNA: Uptake by macrophages, J. Transl. Med. 9 (2011) 9. doi:10.1186/1479-5876-9-9.

[12] H. Izumi, M. Tsuda, Y. Sato, N. Kosaka, T. Ochiya, H. Iwamoto, K. Namba, Y. Takeda, Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages, J. Dairy Sci. 98 (2015) 2920–2933. doi:10.3168/jds.2014-9076.

[13] S. Manca, B. Upadhyaya, E. Mutai, A.T. Desaulniers, R.A. Cederberg, B.R. White, J. Zempleni, Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns, Sci. Rep. 8 (2018) 1–11. doi:10.1038/s41598-018-29780-1.

[14] A. Pardanani, E.D. Wieben, T.C. Spelsberg, A. Tefferi, Primer on medical genomics part IV: expression proteomics, in: Mayo Clin. Proc., Elsevier, 2002: pp. 1185–1196.

[15] J. Sun, Z. Shi, H. Guo, C. Tu, Changes in the porcine peripheral blood mononuclear cell proteome induced by infection with highly virulent classical swine fever virus, J. Gen. Virol. 91 (2010) 2254–2262.

[16] D. Valent, L. Arroyo, R. Peña, K. Yu, R. Carreras, E. Mainau, A. Velarde, A. Bassols, Effects on pig immunophysiology, PBMC proteome and brain neurotransmitters caused by group mixing stress and human-animal relationship, PLoS One. 12 (2017) e0176928–e0176928. doi:10.1371/journal.pone.0176928.

[17] J.-I. Chae, J. Kim, S.G. Lee, M.W. Koh, Y.-J. Jeon, D.-W. Kim, S. mi Ko, K.S. Seo, H.K. Lee, N.-J. Choi, S.-K. Cho, J. Ryu, S. Kang, D.-S. Lee, H.-M. Chung, D.-B. Koo, Quantitative proteomic analysis of pregnancy-related proteins from peripheral blood mononuclear cells during pregnancy in pigs, Anim. Reprod. Sci. 134 (2012) 164–176. doi:https://doi.org/10.1016/j.anireprosci.2012.07.008.

[18] M. Ramirez-Boo, J.J. Garrido, S. Ogueta, J.J. Calvete, C. Gómez-Díaz, Á. Moreno, Analysis of porcine peripheral blood mononuclear cells proteome by 2-DE and MS: Analytical and biological variability in the protein expression level and protein identification, Proteomics. 6 (2006) S215–S225. doi:https://doi.org/10.1002/pmic.200500386.

[19] J. Korte, T. Fröhlich, M. Kohn, B. Kaspers, G.J. Arnold, S. Härtle, 2D DIGE analysis of the bursa of Fabricius reveals characteristic proteome profiles for different stages of chicken B-cell development., Proteomics. 13 (2013) 119–33. doi:10.1002/pmic.201200177.

[20] Z. Sekelova, O. Polansky, H. Stepanova, R. Fedr, M. Faldynova, I. Rychlik, L. Vlasatikova, Different roles of CD4, CD8 and γδ T-lymphocytes in naive and

vaccinated chickens during Salmonella Enteritidis infection., Proteomics. 17 (2017). doi:10.1002/pmic.201700073.

[21] X. Deng, Y. Cong, R. Yin, G. Yang, C. Ding, S. Yu, X. Liu, C. Wang, Z. Ding, Proteomic analysis of chicken peripheral blood mononuclear cells after infection by Newcastle disease virus, J Vet Sci. 15 (2014) 511–517. https://doi.org/10.4142/jvs.2014.15.4.511.

[22] C.A. Deeg, R.L. Degroote, I.M. Giese, S. Hirmer, B. Amann, M. Weigand, C. Wiedemann, S.M. Hauck, CD11d is a novel antigen on chicken leukocytes., J. Proteomics. 225 (2020) 103876. doi:10.1016/j.jprot.2020.103876.

[23] F. Ceciliani, V. Pocacqua, A. Miranda-Ribera, V. Bronzo, C. Lecchi, P. Sartorelli, α1-Acid glycoprotein modulates apoptosis in bovine monocytes, Vet. Immunol. Immunopathol. 116 (2007) 145–152. doi:10.1016/j.vetimm.2007.01.006.

[24] R.F. Ferreira, T. Blees, F. Shakeri, A. Buness, M. Sylvester, G. Savoini, A. Agazzi, V. Mrljak, H. Sauerwein, Comparative proteome profiling in exosomes derived from porcine colostrum versus mature milk reveals distinct functional proteomes, J. Proteomics. (2021) 104338. doi:https://doi.org/10.1016/j.jprot.2021.104338.

[25] J. Bazile, B. Picard, C. Chambon, A. Valais, M. Bonnet, Pathways and biomarkers of marbling and carcass fat deposition in bovine revealed by a combination of gel-based and gel-free proteomic analyses, Meat Sci. 156 (2019) 146–155. doi:https://doi.org/10.1016/j.meatsci.2019.05.018.

[26] K.-A. Lê Cao, S. Boitard, P. Besse, Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems, BMC Bioinformatics. 12 (2011) 253. doi:10.1186/1471-2105-12-253.

[27] N. Kaspric, B. Picard, M. Reichstadt, J. Tournayre, M. Bonnet, ProteINSIDE to Easily Investigate Proteomics Data from Ruminants: Application to Mine Proteome of Adipose and Muscle Tissues in Bovine Foetuses, PLoS One. 10 (2015) e0128086–e0128086. doi:10.1371/journal.pone.0128086.

[28] S. Komine-Aizawa, S. Ito, S. Aizawa, T. Namiki, S. Hayakawa, Cow milk exosomes activate NK cells and γδT cells in human PBMCs in vitro, Immunol. Med. 43 (2020) 161–170. doi:10.1080/25785826.2020.1791400.

[29] E. Brown, J. Van Weering, T. Sharp, J. Mantell, P. Verkade, Chapter 10 - Capturing Endocytic Segregation Events with HPF-CLEM, in: T. Müller-Reichert, P.B.T.-M. in C.B. Verkade (Eds.), Correl. Light Electron Microsc., Academic Press, 2012: pp. 175–201. doi:https://doi.org/10.1016/B978-0-12-416026-

2.00010-8.

[30] J. Nunnari, P. Walter, Regulation of organelle biogenesis, Cell. 84 (1996) 389–394.

[31] J.A. van der Knaap, C.P. Verrijzer, Undercover: gene control by metabolites and metabolic enzymes, Genes Dev. 30 (2016) 2345–2369. doi:10.1101/gad.289140.116.

[32] R.C. May, L.M. Machesky, Phagocytosis and the actin cytoskeleton, J. Cell Sci. 114 (2001) 1061–1077. doi:10.1242/jcs.114.6.1061.

[33] M. Affolter, C.J. Weijer, Signaling to Cytoskeletal Dynamics during Chemotaxis, Dev. Cell. 9 (2005) 19–34. doi:https://doi.org/10.1016/j.devcel.2005.06.003.

[34] N.H. Roy, J.K. Burkhardt, The Actin Cytoskeleton: A Mechanical Intermediate for Signal Integration at the Immunological Synapse , Front. Cell Dev. Biol. . 6 (2018). https://www.frontiersin.org/article/10.3389/fcell.2018.00116.

[35] C. Feig, M.E. Peter, How apoptosis got the immune system in shape., Eur. J. Immunol. 37 Suppl 1 (2007) S61-70. doi:10.1002/eji.200737462.

[36] F. Ceciliani, G. Ávila Morales, G. De Matteis, F. Grandoni, R. Furioso Ferreira, P. Roccabianca, C. Lecchi, Methods in isolation and characterization of bovine monocytes and macrophages, Methods. 186 (2021) 22–41. doi:https://doi.org/10.1016/j.ymeth.2020.06.017.

[37] H.-Y. Guo, A.-C. Cheng, M.-S. Wang, Z.-Q. Yin, R.-Y. Jia, Exosomes: Potential Therapies for Disease via Regulating TLRs, Mediators Inflamm. 2020 (2020) 2319616. doi:10.1155/2020/2319616.

[38] J. Yang, L. Zhang, C. Yu, X.F. Yang, H. Wang, Monocyte and macrophage differentiation: Circulation inflammatory monocyte as biomarker for inflammatory diseases, Biomark. Res. 2 (2014) 1–9. doi:10.1186/2050-7771-2-1.

[39] D. Pan, Y. Li, F. Yang, Z. Lv, S. Zhu, Y. Shao, Y. Huang, G. Ning, S. Feng, Increasing toll-like receptor 2 on astrocytes induced by Schwann cell-derived exosomes promotes recovery by inhibiting CSPGs deposition after spinal cord injury, J. Neuroinflammation. 18 (2021) 172. doi:10.1186/s12974-021-02215-x.

[40] P.P. Singh, C. LeMaire, J.C. Tan, E. Zeng, J.S. Schorey, Exosomes Released from M.tuberculosis Infected Cells Can Suppress IFN-γ Mediated Activation of Naïve Macrophages, PLoS One. 6 (2011) e18564. https://doi.org/10.1371/journal.pone.0018564.

[41] M. Good, C.P. Sodhi, C.E. Egan, A. Afrazi, H. Jia, Y. Yamaguchi, P. Lu, M.F.

Branca, C. Ma, T. Prindle, S. Mielo, A. Pompa, Z. Hodzic, J.A. Ozolek, D.J. Hackam, Breast milk protects against the development of necrotizing enterocolitis through inhibition of Toll-like receptor 4 in the intestinal epithelium via activation of the epidermal growth factor receptor, Mucosal Immunol. 8 (2015) 1166–1179. doi:10.1038/mi.2015.30.

[42] M.I. Zonneveld, M.J.C. van Herwijnen, M.M. Fernandez-Gutierrez, A. Giovanazzi, A.M. de Groot, M. Kleinjan, T.M.M. van Capel, A.J.A.M. Sijts, L.S. Taams, J. Garssen, E.C. de Jong, M. Kleerebezem, E.N.M. Nolte-'t Hoen, F.A. Redegeld, M.H.M. Wauben, Human milk extracellular vesicles target nodes in interconnected signalling pathways that enhance oral epithelial barrier function and dampen immune responses, BioRxiv. (2020) 2020.04.29.068841. doi:10.1101/2020.04.29.068841.

[43] Y. Lai, R.L. Gallo, Toll-like receptors in skin infections and inflammatory diseases, Infect. Disord. Drug Targets. 8 (2008) 144–155. doi:10.2174/1871526510808030144.

[44] J.Y. Lee, J.K. Park, E.Y. Lee, E.B. Lee, Y.W. Song, Circulating exosomes from patients with systemic lupus erythematosus induce a proinflammatory immune response, Arthritis Res. Ther. 18 (2016) 264. doi:10.1186/s13075-016-1159-y.

[45] N.P. Bretz, J. Ridinger, A.-K. Rupp, K. Rimbach, S. Keller, C. Rupp, F. Marmé, L. Umansky, V. Umansky, T. Eigenbrod, M. Sammar, P. Altevogt, Body Fluid Exosomes Promote Secretion of Inflammatory Cytokines in Monocytic Cells via Toll-like Receptor Signaling *, J. Biol. Chem. 288 (2013) 36691–36702. doi:10.1074/jbc.M113.512806.

[46] M.S. Abdul-Cader, A. Amarasinghe, M.F. Abdul-Careem, Activation of toll-like receptor signaling pathways leading to nitric oxide-mediated antiviral responses, Arch. Virol. 161 (2016) 2075–2086. doi:10.1007/s00705-016-2904-x.

[47] C. Bogdan, Nitric oxide and the immune response, Nat. Immunol. 2 (2001) 907–916. doi:10.1038/ni1001-907.

[48] K.A. Lucas, G.M. Pitari, S. Kazerounian, I. Ruiz-Stewart, J. Park, S. Schulz, K.P. Chepenik, S.A. Waldman, Guanylyl cyclases and signaling by cyclic GMP, Pharmacol. Rev. 52 (2000) 375–414.

[49] S. Gnipp, E. Mergia, M. Puschkarow, A. Bufe, D. Koesling, M. Peters, Nitric oxide dependent signaling via cyclic GMP in dendritic cells regulates migration and T-cell polarization, Sci. Rep. 8 (2018) 10969. doi:10.1038/s41598-018-29287-9.

[50] H. Li, Y. Liao, L. Gao, T. Zhuang, Z. Huang, H. Zhu, J. Ge, Coronary Serum Exosomes Derived from Patients with Myocardial Ischemia Regulate Angiogenesis through the miR-939-mediated Nitric Oxide Signaling Pathway, Theranostics. 8 (2018) 2079–2093. doi:10.7150/thno.21895.

[51] H. Hattori, K. Takaoka, M. Ueta, M. Oshitani, J. Tamaoka, K. Noguchi, H. Kishimoto, Senescent RAW264.7 cells exhibit increased production of nitric oxide and release inducible nitric oxide synthase in exosomes, Mol Med Rep. 24 (2021) 681. doi:10.3892/mmr.2021.12320.

[52] X.-H. Tan, D. Fang, Y.-D. Xu, T.-G. Nan, W.-P. Song, Y.-Y. Gu, S.-J. Gu, Y.-M. Yuan, Z.-C. Xin, L.-Q. Zhou, R.-L. Guan, X.-S. Li, Skimmed Bovine Milk-Derived Extracellular Vesicles Isolated via "Salting-Out": Characterizations and Potential Functions as Nanocarriers , Front. Nutr. . 8 (2021). https://www.frontiersin.org/article/10.3389/fnut.2021.769223.

[53] S.R. L., F. Maria, CD36, a Scavenger Receptor Involved in Immunity, Metabolism, Angiogenesis, and Behavior, Sci. Signal. 2 (2009) re3–re3. doi:10.1126/scisignal.272re3.

[54] I.N. Baranova, R. Kurlander, A. V Bocharov, T.G. Vishnyakova, Z. Chen, A.T. Remaley, G. Csako, A.P. Patterson, T.L. Eggerman, Role of human CD36 in bacterial recognition, phagocytosis, and pathogen-induced JNK-mediated signaling, J. Immunol. 181 (2008) 7147–7156. doi:10.4049/jimmunol.181.10.7147.

[55] M. Triantafilou, F.G.J. Gamper, R.M. Haston, M.A. Mouratis, S. Morath, T. Hartung, K. Triantafilou, Membrane Sorting of Toll-like Receptor (TLR)-2/6 and TLR2/1 Heterodimers at the Cell Surface Determines Heterotypic Associations with CD36 and Intracellular Targeting * , J. Biol. Chem. 281 (2006) 31002–31011. doi:10.1074/jbc.M602794200.

[56] E. Uribe-Querol, C. Rosales, Phagocytosis: Our Current Understanding of a Universal Biological Process , Front. Immunol. . 11 (2020). https://www.frontiersin.org/article/10.3389/fimmu.2020.01066.

[57] R. Kalluri, The biology and function of exosomes in cancer, J. Clin. Invest. 126 (2016) 1208–1215. doi:10.1172/JCI81135.

[58] R.F. Collins, N. Touret, H. Kuwata, N.N. Tandon, S. Grinstein, W.S. Trimble, Uptake of Oxidized Low Density Lipoprotein by CD36 Occurs by an Actin-dependent Pathway Distinct from Macropinocytosis *, J. Biol. Chem. 284 (2009)

30288–30297. doi:10.1074/jbc.M109.045104.

[59] N. Singhto, R. Kanlaya, A. Nilnumkhum, V. Thongboonkerd, Roles of Macrophage Exosomes in Immune Response to Calcium Oxalate Monohydrate Crystals, Front. Immunol. 9 (2018) 316. doi:10.3389/fimmu.2018.00316.

[60] G. Sugano, I. Bernard-Pierrot, M. Laé, C. Battail, Y. Allory, N. Stransky, S. Krumeich, M.-L. Lepage, P. Maille, M.-H. Donnadieu, C.C. Abbou, S. Benhamou, T. Lebret, X. Sastre-Garau, S. Amigorena, F. Radvanyi, C. Théry, Milk fat globule—epidermal growth factor—factor VIII (MFGE8)/lactadherin promotes bladder tumor development, Oncogene. 30 (2011) 642–653. doi:10.1038/onc.2010.446.

[61] S.K. Dasgupta, P. Thiagarajan, The Role of Lactadherin in the Phagocytosis of Phosphatidylserine-Expressing Sickle Red Blood Cell by Macrophages., Blood. 106 (2005) 3773. doi:10.1182/blood.V106.11.3773.3773.

[62] Y.-J. Zhou, J. Gao, H.-M. Yang, X.-L. Yuan, T.-X. Chen, Z.-J. He, The role of the lactadherin in promoting intestinal DCs development in vivo and vitro, Clin. Dev. Immunol. 2010 (2010) 357541. doi:10.1155/2010/357541.

[63] T.A. Reinhardt, J.D. Lippolis, B.J. Nonnecke, R.E. Sacco, Bovine milk exosome proteome, J. Proteomics. 75 (2012) 1486–1492. doi:https://doi.org/10.1016/j.jprot.2011.11.017.

[64] Q. Zhou, M. Li, X. Wang, Q. Li, T. Wang, Q. Zhu, X. Zhou, X. Wang, X. Gao, X. Li, Immune-related microRNAs are abundant in breast milk exosomes, Int. J. Biol. Sci. 8 (2012) 118–123. doi:10.7150/ijbs.8.118.

[65] D.E. Murphy, O.G. de Jong, M. Brouwer, M.J. Wood, G. Lavieu, R.M. Schiffelers, P. Vader, Extracellular vesicle-based therapeutics: natural versus engineered targeting and trafficking, Exp. Mol. Med. 51 (2019) 1–12. doi:10.1038/s12276-019-0223-5.

[66] Y.M. Park, M. Febbraio, R.L. Silverstein, CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima, J. Clin. Invest. 119 (2009) 136–145. doi:10.1172/JCI35535.

[67] D.P. Ramakrishnan, R.A. Hajj-Ali, Y. Chen, R.L. Silverstein, Extracellular Vesicles Activate a CD36-Dependent Signaling Pathway to Inhibit Microvascular Endothelial Cell Migration and Tube Formation, Arterioscler. Thromb. Vasc. Biol. 36 (2016) 534–544. doi:10.1161/ATVBAHA.115.307085.

[68] I. Wortzel, R. Seger, The ERK Cascade: Distinct Functions within Various Subcellular Organelles, Genes Cancer. 2 (2011) 195–209. doi:10.1177/1947601911407328.

[69] K.J. Svensson, H.C. Christianson, A. Wittrup, E. Bourseau-Guilmain, E. Lindqvist, L.M. Svensson, M. Mörgelin, M. Belting, Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1, J. Biol. Chem. 288 (2013) 17713–17724. doi:10.1074/jbc.M112.445403.

[70] J. Ma, L. Chen, X. Zhu, Q. Li, L. Hu, H. Li, Mesenchymal stem cell-derived exosomal miR-21a-5p promotes M2 macrophage polarization and reduces macrophage infiltration to attenuate atherosclerosis, Acta Biochim. Biophys. Sin. (Shanghai). 53 (2021) 1227–1236. doi:10.1093/abbs/gmab102.

[71] Y. Hao, W. Purtha, C. Cortesio, H. Rui, Y. Gu, H. Chen, E.A. Sickmier, P. Manzanillo, X. Huang, Crystal structures of human procathepsin H, PLoS One. 13 (2018) e0200374–e0200374. doi:10.1371/journal.pone.0200374.

[72] T. Jakoš, A. Pišlar, A. Jewett, J. Kos, Cysteine Cathepsins in Tumor-Associated Immune Cells , Front. Immunol. . 10 (2019). https://www.frontiersin.org/article/10.3389/fimmu.2019.02037.

[73] M.Z.I. Pranjol, N.J. Gutowski, M. Hannemann, J.L. Whatmore, Cathepsin D non-proteolytically induces proliferation and migration in human omental microvascular endothelial cells via activation of the ERK1/2 and PI3K/AKT pathways, Biochim. Biophys. Acta - Mol. Cell Res. 1865 (2018) 25–33. doi:https://doi.org/10.1016/j.bbamcr.2017.10.005.

[74] C.F. Tan, H.S. Teo, J.E. Park, B. Dutta, S.W. Tse, M.K.-S. Leow, W. Wahli, S.K. Sze, Exploring Extracellular Vesicles Biogenesis in Hypothalamic Cells through a Heavy Isotope Pulse/Trace Proteomic Approach, Cells. 9 (2020) 1320. doi:10.3390/cells9051320.

[75] G.S. Getz, C.A. Reardon, Apoproteins E, A-I, and SAA in Macrophage Pathobiology Related to Atherogenesis , Front. Pharmacol. . 10 (2019). https://www.frontiersin.org/article/10.3389/fphar.2019.00536.

[76] D. Baitsch, H.H. Bock, T. Engel, R. Telgmann, C. Müller-Tidow, G. Varga, M. Bot, J. Herz, H. Robenek, A. von Eckardstein, J.-R. Nofer, Apolipoprotein E Induces Antiinflammatory Phenotype in Macrophages, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 1160–1168. doi:10.1161/ATVBAHA.111.222745.

[77] P. Zheng, Q. Luo, W. Wang, J. Li, T. Wang, P. Wang, L. Chen, P. Zhang, H. Chen, Y. Liu, P. Dong, G. Xie, Y. Ma, L. Jiang, X. Yuan, L. Shen, Tumor-associated macrophages-derived exosomes promote the migration of gastric cancer cells by transfer of functional Apolipoprotein E, Cell Death Dis. 9 (2018) 434. doi:10.1038/s41419-018-0465-5.

[78] H. Wang, S. Du, J. Cai, J. Wang, X. Shen, Apolipoprotein E2 Promotes the Migration and Invasion of Pancreatic Cancer Cells via Activation of the ERK1/2 Signaling Pathway, Cancer Manag. Res. 12 (2020) 13161–13171. doi:10.2147/CMAR.S284115.

[79] X. Weng, H. Zhao, Q. Guan, G. Shi, S. Feng, M.E. Gleave, C.C.Y. Nguan, C. Du, Clusterin regulates macrophage expansion, polarization and phagocytic activity in response to inflammation in the kidneys, Immunol. Cell Biol. 99 (2021) 274–287. doi:https://doi.org/10.1111/imcb.12405.

[80] P. Cunin, C. Beauvillain, C. Miot, J.-F. Augusto, L. Preisser, S. Blanchard, P. Pignon, M. Scotet, E. Garo, I. Fremaux, A. Chevailler, J.-F. Subra, P. Blanco, M.R. Wilson, P. Jeannin, Y. Delneste, Clusterin facilitates apoptotic cell clearance and prevents apoptotic cell-induced autoimmune responses, Cell Death Dis. 7 (2016) e2215–e2215. doi:10.1038/cddis.2016.113.

[81] M. Cintio, G. Polacchini, E. Scarsella, T. Montanari, B. Stefanon, M. Colitti, MicroRNA Milk Exosomes: From Cellular Regulator to Genomic Marker, Anim. an Open Access J. from MDPI. 10 (2020) 1126. doi:10.3390/ani10071126.

[82] O.M. Delmonte, J.R.E. Bergerson, T. Kawai, H.S. Kuehn, D.H. McDermott, I. Cortese, M.T. Zimmermann, A.K. Dobbs, M. Bosticardo, D. Fink, S. Majumdar, B. Palterer, F. Pala, N.R. Dsouza, M. Pouzolles, N. Taylor, K.R. Calvo, S.R. Daley, D. Velez, A. Agharahimi, K. Myint-Hpu, L.K. Dropulic, J.J. Lyons, S.M. Holland, A.F. Freeman, R. Ghosh, M.B. Similuk, J.E. Niemela, J. Stoddard, D.B. Kuhns, R. Urrutia, S.D. Rosenzweig, M.A. Walkiewicz, P.M. Murphy, L.D. Notarangelo, SASH3 variants cause a novel form of X-linked combined immunodeficiency with immune dysregulation, Blood. 138 (2021) 1019–1033. doi:https://doi.org/10.1182/blood.2020008629.

[83] B. Gao, W.-I. Jeong, Z. Tian, Liver: An organ with predominant innate immunity, Hepatology. 47 (2008) 729–736. doi:https://doi.org/10.1002/hep.22034.

[84] A.T. Jan, S. Rahman, S. Khan, S.A. Tasduq, I. Choi, Biology, Pathophysiological Role, and Clinical Implications of Exosomes: A Critical Appraisal, Cells. 8 (2019)

99. doi:10.3390/cells8020099.

[85] X. Wang, X. Zhao, D. Huang, X. Pan, Y. Qi, Y. Yang, H. Zhao, G. Cheng, Proteomic analysis and cross species comparison of casein fractions from the milk of dairy animals, Sci. Rep. 7 (2017) 43020. doi:10.1038/srep43020.

[86] B. Lönnerdal, Nutritional and physiologic significance of human milk proteins, Am. J. Clin. Nutr. 77 (2003) 1537S-1543S. doi:10.1093/ajcn/77.6.1537S.

[87] M.A. Aminzadeh, M. Fournier, A. Akhmerov, K.C. Jones-Ungerleider, J.B. Valle, E. Marbán, Casein-enhanced uptake and disease-modifying bioactivity of ingested extracellular vesicles, J. Extracell. Vesicles. 10 (2021) e12045. doi:https://doi.org/10.1002/jev2.12045.

[88] S. Malagobadan, N.H. Nagoor, Anoikis, in: P. Boffetta, P.B.T.-E. of C. (Third E. Hainaut (Eds.), Academic Press, Oxford, 2019: pp. 75–84. doi:https://doi.org/10.1016/B978-0-12-801238-3.65021-3.

[89] R. Escate, T. Padro, L. Badimon, LDL accelerates monocyte to macrophage differentiation: Effects on adhesion and anoikis, Atherosclerosis. 246 (2016) 177–186. doi:https://doi.org/10.1016/j.atherosclerosis.2016.01.002.

[90] S. Aparicio-Siegmund, C. Garbers, The biology of interleukin-27 reveals unique pro- and anti-inflammatory functions in immunity, Cytokine Growth Factor Rev. 26 (2015) 579–586. doi:https://doi.org/10.1016/j.cytogfr.2015.07.008.

[91] T.T. Wheeler, A.J. Hodgkinson, C.G. Prosser, S.R. Davis, Immune Components of Colostrum and Milk—A Historical Perspective, J. Mammary Gland Biol. Neoplasia. 12 (2007) 237–247. doi:10.1007/s10911-007-9051-7.

[92] M.J. Sadler, N. Smith, Beta-casein proteins and infant growth and development, Infant J. 9 (2013) 173–176.

[93] M. Samuel, D. Chisanga, M. Liem, S. Keerthikumar, S. Anand, C.-S. Ang, C.G. Adda, E. Versteegen, M. Jois, S. Mathivanan, Bovine milk-derived exosomes from colostrum are enriched with proteins implicated in immune response and growth, Sci. Rep. 7 (2017) 5933. doi:10.1038/s41598-017-06288-8.

[94] S. Vordenbäumen, A. Braukmann, K. Petermann, A. Scharf, E. Bleck, A. von Mikecz, J. Jose, M. Schneider, Casein α s1 is expressed by human monocytes and upregulates the production of GM-CSF via p38 MAPK, J. Immunol. 186 (2011) 592–601.

[95] S. Vordenbäumen, A. Braukmann, I. Altendorfer, E. Bleck, J. Jose, M. Schneider, Human casein alpha s1 (CSN1S1) skews in vitro differentiation of monocytes

towards macrophages, BMC Immunol. 14 (2013) 46. doi:10.1186/1471-2172-14-46.

[96] H.A. Arnett, J.L. Viney, Immune modulation by butyrophilins, Nat. Rev. Immunol. 14 (2014) 559–569. doi:10.1038/nri3715.

[97] Y. Wang, W. Yang, Q. Pu, Y. Yang, S. Ye, Q. Ma, J. Ren, Z. Cao, G. Zhong, X. Zhang, L. Liu, W. Zhu, The effects and mechanisms of SLC34A2 in tumorigenesis and progression of human non-small cell lung cancer, J. Biomed. Sci. 22 (2015) 52. doi:10.1186/s12929-015-0158-7.

[98] W. Yang, Y. Wang, Q. Pu, S. Ye, Q. Ma, J. Ren, G. Zhong, L. Liu, W. Zhu, Elevated expression of SLC34A2 inhibits the viability and invasion of A549 cells, Mol. Med. Rep. 10 (2014) 1205–1214. doi:10.3892/mmr.2014.2376.

[99] M.F. Brinchmann, D.M. Patel, M.H. Iversen, The Role of Galectins as Modulators of Metabolism and Inflammation, Mediators Inflamm. 2018 (2018) 9186940. doi:10.1155/2018/9186940.

[100] Y. Lu, M. Zhang, P. Zhao, M. Jia, B. Liu, Q. Jia, J. Guo, L. Dou, J. Li, Modified citrus pectin inhibits galectin-3 function to reduce atherosclerotic lesions in apoE-deficient mice, Mol Med Rep. 16 (2017) 647–653. doi:10.3892/mmr.2017.6646.

Figures

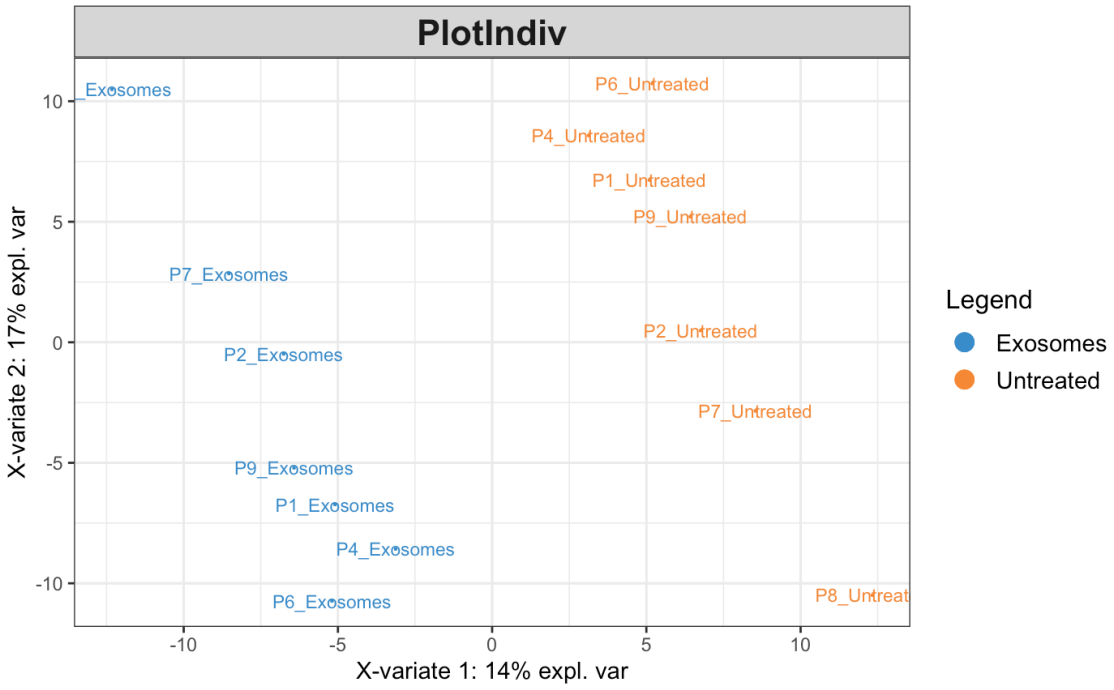


Fig. 1. Sparse partial least squares discriminant analysis (sPLS-DA) for paired data individual plot (PlotIndiv). The individual plot shows the similarities and relationship (clustering) between samples of the porcine milk exosomes (blue) and the untreated control (orange) treatment groups.

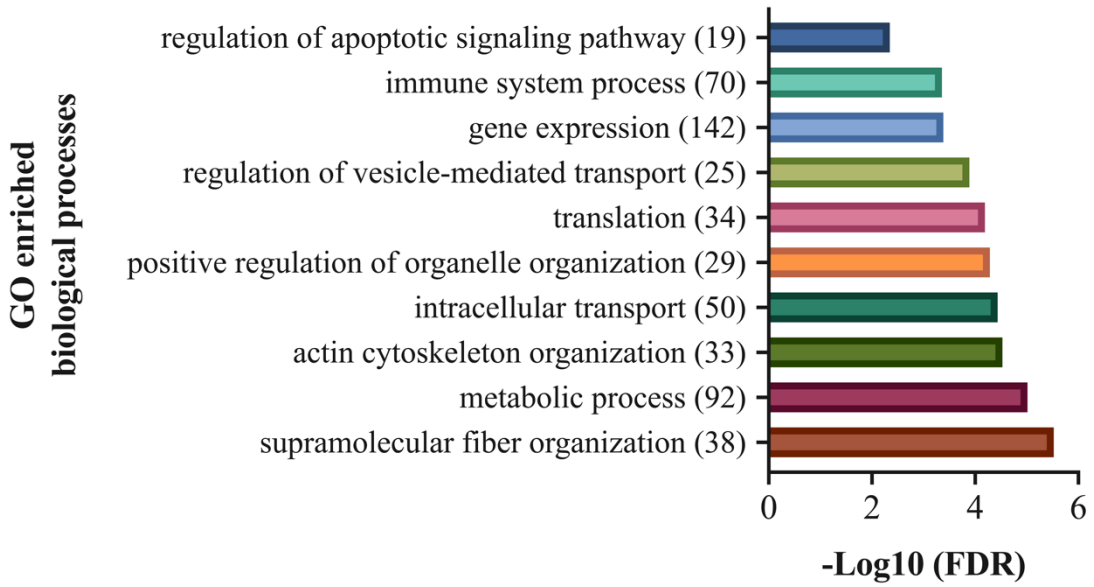


Fig. 2. Global enriched (P<0.05) Gene ontology (GO) terms within the biological processes (BP) category that has annotated all 384 discriminant proteins (DP). GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

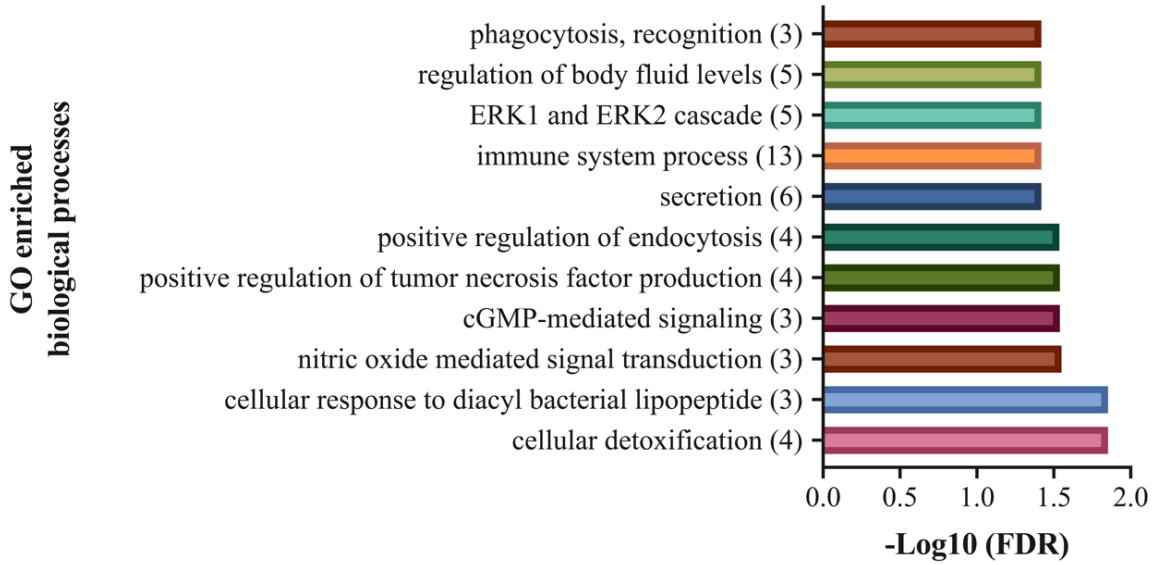


Fig. 3. Enriched (P<0.05) Gene ontology (GO) terms within the biological processes (BP) category that have annotated the 54 discriminant proteins (DP) with the highest abundance in the porcine milk exosomes group. GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

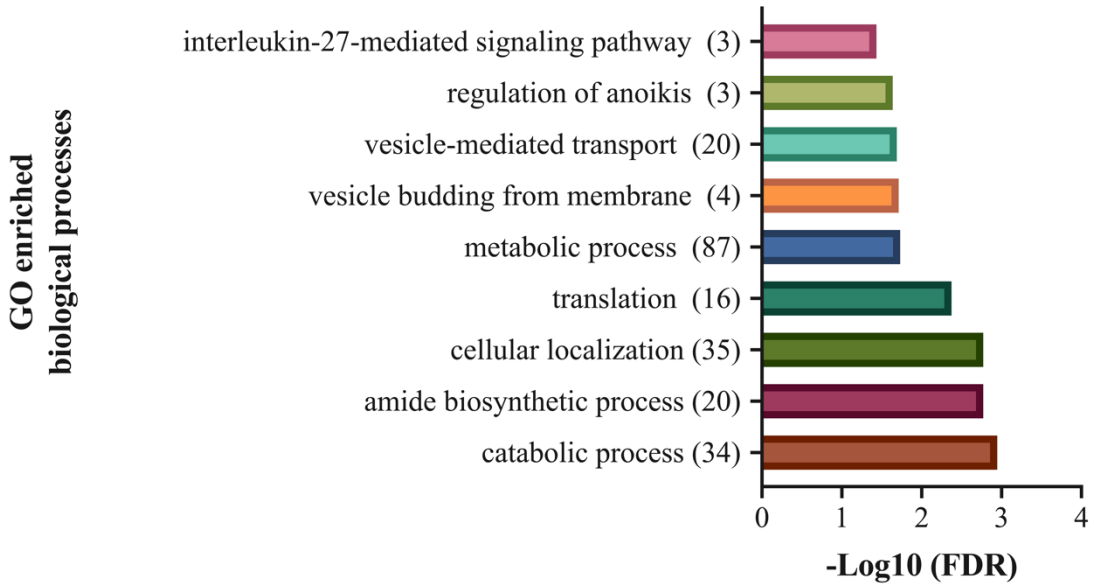


Fig. 4. Enriched (P<0.05) Gene ontology (GO) terms within the biological processes (BP) category that have annotated the 142 discriminant proteins (DP) with the highest abundance in the control group (no milk exosomes). GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

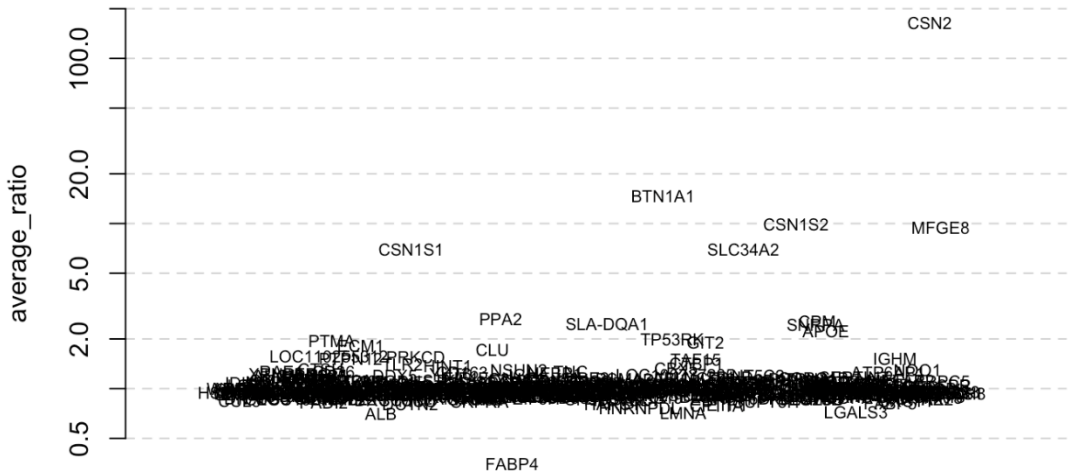


Fig. 5. Average log fold-change (logFC) of protein raw abundances of the 384 discriminant proteins (DP) selected by sPLS-DA. The y axis shows the average logFC (ratio) of the raw abundances from all the seven different biological replicates (samples) after porcine milk exosomes treatment. Proteins are represented with their corresponding Gene ID.

Supplementary material

Table S1. List of some of the main enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by all 384 discriminant proteins (DP) selected the sPLS-DA.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0097435	supramolecular fiber organization	0,00000	5,52	ABI1 ACTN1 ACTN2 ANXA2 APOE ARPC1B ARPC2 ARPC5 ARPC5L ARRB1 ASC LOC100522011 CD36 LOC100511343 CLU CORO1B CUL3 DIAPH1 ITGB1 KPNB1 LOC100513346 PAM16 LOC100737113 MAPRE1 MCU MECP2 MYADM NCBP1 NCK2 PFDN2 PRKCD RDX RPS3 S100A10 SERPINH1 SH3KBP1 WASF2 WDR1
GO:0008152	metabolic process	0,00001	5,01	FABP4 A-FABP AARS1 ABCE1 ABI1 ACADL ACLY ACL ACP1 ACSS2 AKR1A1 ANXA2 ANXA4 AP3B1 APEH APOE ARRB1 ASC LOC100522011 ATP1B1 ATP2A2 ATP6V1A ATP6V1B2 BAG5 CKB BANF1 BRCC3 BTK CAND1 CASP1 CASP6 CBFB CBX5 CCT4 CD36 LOC100511343 CD47 CDK2 RAB5B CLU CNN2 CPM CPT1A CSN2 CTBP1 CTRC EFHD2 CTSH CTSS CUL3 CYB5R3 DARS1 DAZAP1 DDB1 DDOST DDX17 DDX5 DEK DLAT DNAJB11 DNASE1L1 DPP3 ECHS1 ECM1 EEF1A EEF1A1 EFTUD2 EIF2S1 EIF3E EIF3F EIF3H EIF4E EIF4G1 EPRS1 ERP44 ESD ETF1 F13A1 FDPS FGB G3BP1 GAR1 GARS1 GFPT1 GGT1 GLRX3 GZMA GZMB HADHA HARS1 HINT1 HK2 HMGB2 HNMT HNRNPU HSD17B4 IDH2 IDH3A IDI1 LOC100625049 ILVBL INPP5D ITGB1 SRSF6 L3MBTL1 LAMP2 LCP2 LDHA LMNA LNPEP ZGLP1 LOC100526209 LOC110260088 LONP1 LSM3 LYPLA1 MAN2B1 MAP2K2 MECP2 METAP2 MTREX MVP MYADM MYBBP1A NAA15 NAAA NAMPT PBEF1 NCBP1

				NCF4 NCK2 NDUFA13 NDUFS6 NIBAN1 NIT2 NME2 NSUN2 OXCT1 PADI2 PCK2 PCYOX1 PDCD5 PDHB PDXK PFDN2 PKM PLD4 PMPCB PNP PPA2 PPP2R2A PRDX2 PREP PRKCD PRMT1 PRPF19 PRPF40A PSMA3 PSMA5 RAPSN PSMC3 PSMC5 PSMD1 PTBP3 PTPN12 PTPN6 PTPRJ PTRH2 PYGL PYGM QARS1 RAB7A RAE1 RALY RBBP7 RBM14 RBM4 RBM39 RBMX RDX RO60 RPA1 RPIA RPL10A RPL13A RPL18 RPL19 RPL27 RPL29 RPN1 RPS13 RPS19 RPS25 RPS3 RPS8 RPS9 RTCB RUVBL1 SAFB SARNP SART1 SASH3 SCARB2 SCPEP1 SDCBP SDHA SERPINH1 SF3B6 SKP1 SLA-DQB*G01 SLA-DQB1 SLADQB SLA-DQB SMC3 SMU1 SND1 SPN SPR SRI SRSF1 STAT1 STK24 STK4 STX4 SUB1 SUPT5H TAF15 TBXAS1 TCIRG1 TCOF1 THBS1 THOC2 TLR2 TNC TOP2B TP53RK TRIM28 TUFM UBA1 UBE2M UFL1 UGDH UGGT1 UGP2 UMPS USP7 VPS35 WBP11 WDR61 XPNPEP1 YWHAQ
GO:0030036	actin cytoskeleton organization	0,00003	4,53	ABI1 ACTN1 ACTN2 ARPC1B ARPC2 ARPC5 ARPC5L ARRB1 ASC LOC100522011 CAP1 CNN2 CORO1B CUL3 DIAPH1 DOCK2 FLII ITGB1 LOC100513346 PAM16 MCU MYADM NCBP1 NCK2 PFN1 PRKCD RDX S100A10 SDCBP SH3KBP1 TLN1 WASF2 WDR1
GO:0046907	intracellular transport	0,00004	4,44	ACTN2 ANXA2 AP3B1 ATP2A2 CD36 LOC100511343 COPA COPB2 CUL3 ERGIC1 HNRNPU HSPA4 IPO5 KIF5B KPNB1 LAMP1 LAMP2 LMNA PAM16 LOC100513346 Mx1 MX2 MX1 NCBP1 NDUFA13 NSUN2 PDCD5 PMPCB PRKCD RAB10 RAB7A RAE1 RANBP1 RDX SCARB2 SEC23B SEC24C SNX2 SRP68 SRPRA STOM STX4 TAP1 THOC2 TRIM28 USO1 USP7 VPS35 YKT6 YWHAQ

GO:0010638	positive regulation of organelle organization	0,00005	4,29	ACTN2 ANXA2 ARPC1B ARPC2 ARPC5 ARPC5L ARRB1 ASC LOC100522011 CCT4 CORO1B CTBP1 CUL3 G3BP1 LMNA MAPRE1 MCU MECP2 NCK2 PDCD5 PFDN2 RPS3 S100A10 SDCBP SURF4 TRIM28 VPS35 WDR1 WDR61
GO:0006412	translation	0,00006	4,19	AARS1 DARS1 EEF1A EEF1A1 EIF2S1 EIF3E EIF3F EIF3H EIF4E EIF4G1 EPRS1 ETF1 GARS1 HARS1 LOC110260088 NCBP1 NCK2 NIBAN1 QARS1 RPL10A RPL13A RPL18 RPL19 RPL27 RPL29 RPS13 RPS19 RPS3 RPS8 RPS9 TCOF1 THBS1 TUFM
GO:0060627	regulation of vesicle-mediated transport	0,00013	3,89	ACTN2 ANXA2 APOE ARRB1 ASC LOC100522011 ATP2A2 ATP6AP1 CD36 LOC100511343 CD47 CLU CNN2 DNAJC5 DOCK2 FGB LAMP1 LGALS3 LYPLA1 MFGE8 RAB7A RDX SDCBP STX4 USP7
GO:0010467	gene expression	0,00041	3,38	FABP4 A-FABP AARS1 ANXA4 AP3B1 APOE ARRB1 ASC LOC100522011 ATP1B1 BTK CASP1 CBFB CBX5 CD36 LOC100511343 CD47 CDK2 RAB5B CLU CNN2 CTBP1 CTSH CTSS CUL3 DARS1 DAZAP1 DDX17 DDX5 ECM1 EEF1A EEF1A1 EFTUD2 EIF2S1 EIF3E EIF3F EIF3H EIF4E EIF4G1 EPRS1 ETF1 FGB G3BP1 GARS1 GFPT1 GLRX3 HARS1 HINT1 HMGB2 HNRNPU INPP5D SRSF6 L3MBTL1 LMNA ZGLP1 LOC100526209 LOC110260088 LSM3 MECP2 METAP2 MTREX MYADM MYBBP1A NAA15 NAMPT PBEF1 NCBP1 NCK2 NDUFA13 NIBAN1 NSUN2 PADI2 PDCD5 PLD4 PMPCB PNP PRDX2 PRPF19 PRPF40A RAPSN PSMC3 PSMC5 PTBP3 PTPN6 PTRH2 PYGM QARS1 RAE1 RBBP7 RBM14 RBM4 RBM39 RBMX RDX RO60 RPL10A RPL13A RPL18 RPL19 RPL27 RPL29 RPS13 RPS19 RPS25 RPS3 RPS8 RPS9 RTCB SAFB SARNP SART1 SASH3 SERPINH1 SF3B6 SLA-DQB*G01 SLA-DQB1 SLADQB SLA-DQB SMU1 SND1 SPN SRI SRSF1 STAT1 STX4 SUB1 SUPT5H TAF15 TCIRG1

				TCOF1 THBS1 THOC2 TLR2 TNC TRIM28 TUFM UFL1 USP7 VPS35 WBP11 WDR61 YWHAQ
GO:0002376	immune system process	0,00044	3,35	ABI1 ACTN1 ANXA2 AP3B1 APOE ASC LOC100522011 ATP6AP1 BTK CASP1 CBFB CD36 LOC100511343 CD47 CLU CNN2 CTSH CTSS DOCK2 ECM1 EPRS1 FGB G3BP1 GGT1 HMGB2 IFIT3 INPP5D ITGAL ITGB1 KIF5B LAMP1 LCP2 LGALS3 MCU Mx1 MX2 MX1 NCK2 PADI2 PLD4 PNP PRDX2 PRKCD PRMT1 PTPN6 PTPRJ RAB10 RBM14 RBM4 RO60 RPS3 SART1 SASH3 SH3KBP1 SLA-DQB*G01 SLA-DQB1 SLADQB SLA-DQB SPN STAT1 STK4 STX4 TAP1 TCIRG1 THBS1 TLR2 TRIM28 UFL1 WASF2 WDR1 WDR61
GO:2001233	regulation of apoptotic signaling pathway	0,00449	2,35	ASC LOC100522011 CKB BAG5 CLU CTSH FGB HMGB2 LGALS3 LMNA NCK2 NDUFA13 PRDX2 PRKCD QARS1 RPS3 STK4 STX4 THBS1

Table S2. List of some of the main enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins (DP) selected by the sPLS-DA with the highest abundance in the milk exosomes group.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:1990748	cellular detoxification	0,01	1,85	AKR1A1 CD36 LOC100511343 ESD
GO:0071726	cellular response to diacyl bacterial lipopeptide	0,01	1,85	CD36 LOC100511343 TLR2
GO:0007263	nitric oxide mediated signal transduction	0,03	1,55	APOE CD36 LOC100511343
GO:0019934	cGMP-mediated signaling	0,03	1,54	APOE CD36 LOC100511343
GO:0032760	positive regulation of tumor necrosis factor production	0,03	1,54	CD36 LOC100511343 CLU SASH3
GO:0045807	positive regulation of endocytosis	0,03	1,54	APOE CD36 LOC100511343 CLU
GO:0071706	secretion	0,04	1,42	APOE ATP6AP1 CSN2 CSN3 TLR2 YKT6
GO:0002376	immune system process	0,04	1,42	APOE ATP6AP1 CD36 LOC100511343 CLU CTSH ECM1 PLD4 PRMT1 RBM14 RBM4 SASH3 TLR2
GO:0070371	ERK1 and ERK2 cascade	0,04	1,42	APOE ATP6AP1 CD36 LOC100511343 CTSH
GO:0050878	regulation of body fluid levels	0,04	1,42	APOE CD36 LOC100511343 CSN2 CSN3

GO:0006910	phagocytosis, recognition	0,04	1,42	CD36 LOC100511343 MFGE8

Table S3. List of some of the main enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins (DP) selected by the sPLS-DA with the highest abundance in the control group.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0009056	catabolic process	0,001	2,95	ACADL ANXA2 ASC LOC100522011 ATP2A2 CTSS CUL3 EIF3E EIF3H EIF4G1 GGT1 GZMA HADHA HK2 HSD17B4 ILVBL ITGB1 LAMP2 LSM3 NCBP1 NDUFA13 OXCT1 PCYOX1 PNP PSMC5 PSMD1 SCARB2 SND1 SUPT5H TCIRG1 UFL1 USP7 VPS35
GO:0043604	amide biosynthetic process	0,002	2,77	AARS1 ACLY ACL DARS1 EEF1A EEF1A1 EIF2S1 EIF3E EIF3H EIF4G1 ETF1 GARS1 GGT1 HARS1 LOC110260088 NCBP1 PDHB QARS1 RPS19 RPS9
GO:0051641	cellular localization	0,002	2,77	ALB ANXA2 ATP1B1 ATP2A2 CCT4 COPA COPB2 CUL3 DCTN2 DIAPH1 ERGIC1 HK2 ITGAL ITGB1 KIF5B KPNB1 LAMP1 LAMP2 LGALS3 MX2 MX1 NCBP1 NDUFA13 PTPN6 RAB10 RPA1 S100A10 SCARB2 SEC23B SNX2 SRPRA STOM TCIRG1 USP7 VPS35
GO:0006412	translation	0,004	2,37	AARS1 DARS1 EEF1A EEF1A1 EIF2S1 EIF3E EIF3H EIF4G1 ETF1 GARS1 HARS1 LOC110260088 NCBP1 QARS1 RPS19 RPS9
GO:0008152	metabolic process	0,019	1,73	FABP4 A-FABP AARS1 ABCE1 ACADL ACLY ACL ANXA2 ANXA4 ASC LOC100522011 ATP1B1 ATP2A2 ATP6V1A ATP6V1B2 BANF1 CAND1 CBFB CCT4 CD47 CNN2 CPT1A CTSS CUL3 CYB5R3 DARS1 DDOST DLAT EEF1A EEF1A1

				EFTUD2 EIF2S1 EIF3E EIF3H EIF4G1 ERP44 ETF1 GARS1 GFPT1 GGT1 GLRX3 GZMA GZMB HADHA HARS1 HK2 HSD17B4 IDI1 LOC100625049 ILVBL INPP5D ITGB1 LAMP2 LOC110260088 LSM3 MVP MYBBP1A NAA15 PBEF1 NAMPT NCBP1 NDUFA13 NME2 OXCT1 PADI2 PCK2 PCYOX1 PDHB PNP PRPF40A PSMC5 PSMD1 PTPN6 PTPRJ PTRH2 QARS1 RBMX RPA1 RPN1 RPS19 RPS9 RTCB RUVBL1 SCARB2 SF3B6 SND1 STAT1 SUPT5H TCIRG1 TOP2B UFL1 UMPS USP7 VPS35
GO:0006900	vesicle budding from membrane	0,019	1,71	ANXA2 CUL3 S100A10 SEC23B
GO:0016192	vesicle-mediated transport	0,021	1,69	ANXA2 ASC LOC100522011 ATP2A2 CD47 CNN2 COPA COPB2 CUL3 ERGIC1 ITGB1 LAMP1 LGALS3 RAB10 S100A10 SCARB2 SEC23B SNX2 USP7 VPS35
GO:2000209	regulation of anoikis	0,023	1,64	ITGB1 MYBBP1A PTRH2
GO:0070106	interleukin-27-mediated signaling pathway	0,037	1,43	MX1 MX2 STAT1

Manuscript draft 7: Citrus pectin (CP) modulates chicken peripheral blood mononuclear cells (PBMC) proteome *in vitro*

Citrus pectin (CP) modulates chicken peripheral blood mononuclear cells (PBMC) proteome *in vitro*

G. Ávila[a], M.Bonnet[b], , D. Viala[b,c], S. Dejean[d], G. Grilli[a], C. Lecchi[a], F. Ceciliani[b]*

[a]Department of Veterinary and Animal Sciences, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy

[b]INRAE, Université Clermont Auvergne, Vetagro Sup, UMRH, 63122, Saint-Genès-Champanelle, France

[c]INRAE, Metabolomic and Proteomic Exploration Facility (PFEM), F-63122 Saint-Genès-Champanelle, France

[d]Institut de Mathématiques de Toulouse, Université de Toulouse, CNRS, UPS, UMR 5219,31062 Toulouse, France

[*]Corresponding author: Gabriela Ávila Morales

E-mail address: gabriela.avila@unimi.it

Department of Veterinary and Animal Science, Università Degli Studi di Milano, Via dell'Università 6, 26900, Lodi, Italy

Tel: 0039 02 50334531

ABSTRACT

Citrus pectin (CP) is a dietary fiber used in animal nutrition with antioxidative, anticancer and anti-inflammatory properties. In chicken, CP dampens the inflammatory reaction by downregulating monocytes' chemotaxis and phagocytosis. No information on the molecular mechanisms underlying the immunomodulatory effects on chicken mononuclear cells is available so far. To cover this gap, this study aimed to assess the effects of CP on chicken peripheral blood mononuclear cells (PBMC) proteome. Cells were purified from 7 different peripheral blood pools of healthy chickens and incubated with 0.5 mg/mL of CP or medium as a control for 20 h at 41°C in a humified atmosphere with 5% CO_2. Proteins were extracted, concentrated with the FASP column and analyzed by nano-LC-MS/MS. A total of 1503 quantifiable proteins were identified, and the supervised multivariate statistical analysis, sparse variant partial least squares – discriminant analysis (sPLS-DA) for paired data, was applied to identify discriminant proteins that contributed to a clear separation between CP and control groups. A total of 373 discriminant proteins from both components were selected and a Gene Ontology (GO) analysis was performed using ProteINSIDE. The main enriched GO terms in the biological process (BP) category were related to metabolic processes, actin cytoskeleton organization, translation and cell migration. To further elucidate the specific role of the discriminant proteins identified by the sPLS-DA for each group, 50 proteins with the highest abundance in CP and 137 in the control group were selected and mined again with ProteINSIDE. Discriminant proteins with higher abundance in CP were mainly involved in actin cytoskeleton organization, cell morphogenesis, intracellular signal transduction, cell communication and negative regulation of cell migration, while the ones with higher abundance in the control group they were mainly related to different metabolic processes, translation and gene expression. Remarkably, MARCKSL1, protein known to restrict cell movement was upregulated in CP treated cells, while other proteins involved in cytoskeleton rearrangement, cell migration and phagocytosis, such as MARCKS, LGALS3 and LGALS8, were downregulated in CP treated cells. In conclusion, these results provide a proteomics background to the anti-inflammatory activity of CP, demonstrating that the *in vitro* downregulation of phagocytosis and chemotaxis is related to changes in proteins related to the cytoskeleton.

***Keywords**:* citrus pectin, chicken PBMC proteome, anti-inflammatory, cell migration, MARCKSL1, LGALS3

1. Introduction

Citrus pectin (CP) is a dietary fiber widely used in both human and animal nutrition (Langhout and Schutte, 1996; Leclere et al., 2013), as it has been shown that exerts beneficial immunomodulatory and anti-inflammatory effects (Chen et al., 2006; Salman et al., 2008; Sahasrabudhe et al., 2018; Beukema et al., 2020). In human peripheral blood mononuclear cells (PBMC), CP inhibited the *in vitro* production of pro-inflammatory cytokines (IL-1β), but upregulated anti-inflammatory cytokines (IL-1ra and IL-10) (Salman et al., 2008). In human macrophages, a similar scenario was observed as CP inhibited Toll-like receptors 1 and 2 (TLR1 and TLR2) pro-inflammatory pathways by directly blocking TLR2, and potentially reducing NFκB activation, downregulating pro-inflammatory cytokines. CP has shown also to block directly other surface immune receptors such galectin-3, which is highly expressed in monocytes and macrophages (Sahasrabudhe et al., 2018; Beukema et al., 2020).

In chickens, CP has an anti-inflammatory activity on monocytes by suppressing their chemotaxis and phagocytosis, *in vitro* (Ávila et al., 2021). However, the molecular mechanisms underlying these anti-inflammatory effects are still unknown, and no further studies on CP molecular impact on other subsets of chicken mononuclear cells, which include lymphocytes, monocytes and thrombocytes have been reported yet (Désert et al., 2016).

Proteomics analysis is the large-scale analysis of the protein profile in a given biological system, widely applied to animal science (Almeida et al., 2021), allowing a better understanding of both physiological and pathological phenotypes (Pardanani et al., 2002; Diz et al., 2012). The knowledge of chicken PBMC proteomics is limited to a few studies focused on infectious diseases (Korte et al., 2013; Deng et al., 2014; Sekelova et al., 2017) or the characterization of specific immunological pathways (Deeg et al., 2020). Therefore, proteomics might be a convenient methodology to elucidate the molecular impact of nutrition on animals' immunity. The present study aims at evaluating the *in vitro* capacity of CP in modulating chicken PBMC proteome through untargeted proteomics, using nano-LC-MS/MS spectrometry.

2. Materials and methods

2.1. Purification of chicken PBMC from blood

Chicken PBMC were isolated from peripheral blood through double discontinuous gradient centrifugation, as previously described for chicken (Ávila et al., 2021). Briefly, 50 mL of seven different peripheral blood pools (biological replicates) from 42 days-old hybrid healthy broilers (ROSS 308) were collected during routine slaughtering procedures at a local slaughterhouse in sterile flasks containing 0.2% EDTA. The blood was then mixed with methylcellulose 1% (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) and centrifuged at 40 x g without brakes for 30 min at 4°C. Then, the supernatant diluted (1:1) with sterile PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) and layered over a double discontinuous Ficoll-Percoll gradient (specific gravity 1.077 g/mL of Ficoll over 1.119 g/mL of Percoll; Sigma-Aldrich) in 15 mL conical centrifuge tubes, and centrifuged at 200g without brakes for 30 min at 4°C. The PBMC ring was collected at the Ficoll/supernatant interface and washed with PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA to remove the contaminating thrombocytes. Red blood cells were eliminated using the Red Blood Cell Lysis Buffer (Roche Diagnostics GmbH, Mannheim, Germany). Finally, the PBMC were counted, their viability assessed with trypan blue exclusion and resuspended at the desired concentration in complete medium (RPMI 1640 Medium + 25 mM HEPES + L-Glutamine, supplemented with 1% of Non-essential Amino Acid Solution 100X and 1% Penicillin Streptomycin Solution 100X and 1% FBS (Sigma-Aldrich)).

2.2. Chicken PBMC stimulation with CP

Firstly, pectin esterified (55-70%) potassium salt from citrus fruit (Sigma-Aldrich) was prepared as previously described for chicken monocytes (Ávila et al., 2021) with some minor modifications. Briefly, only a working dilution of 0.5 mg/mL CP was prepared with the complete medium (1% FBS) when needed for each experiment and it was set as the working concentration for the stimulation of chicken PBMC. Secondly, a total of $5x10^6$ chicken PBMC (1.5 mL) were seeded in triplicates in Corning® tissue-culture treated culture 60 mm dishes (Corning Inc., Costar, Kennebunk, ME, USA) and incubated with 0.5 mg/mL of CP (500 µL) for 20 h at 41°C in a humified atmosphere with 5% CO_2. Complete medium (1% FBS) was then added to each dish to reach a final volume of 3 mL.

2.3. Chicken PBMC collection and protein extraction

After incubation time, the cells' supernatant (3mL), containing the cells in suspension (lymphocytes), was collected into two tubes of 1.5 mL. Cells were centrifuged at 500 x g for 7 min at 4°C to obtain the lymphocytes pellet. The lymphocytes pellet was then washed twice with 500 µL sterile-PBS without Ca^{2+} and Mg^{2+} (room temperature) and again centrifuged twice at 500 x g for 7 min at 4°C to remove the rest of the medium and of FBS that could interfere with the proteomic analysis. In the meantime, three washes with 2 mL of sterile-PBS without Ca^{2+} and Mg^{2+} (room temperature) were performed in the 60 mm dishes to wash the adherent cells (monocytes) and remove dead cells and the rest of the contaminant medium and FBS. When both, the lymphocytes pellet and the adhered monocytes were washed, 500 µL of Igepal buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA and EGTA, 100 mM NaF, 4 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 2 mM Na_3VO_4, 1% Triton X100, 0.5% IGEPAL CA-630 (Sigma-Aldrich), 1 tablet for 50 mL of the total volume of Protease Inhibitor Cocktail (Roche)) was added to the lymphocytes pellet, the cells were resuspended and then collected and added directly to the 60 mm dishes containing the monocytes. The dishes were then carefully mixed to make sure all of the surfaces were covered with Igepal buffer and incubated for 1h at 4°C with regular shaking to lyse the cells. After incubation, the cells were scraped using sterile Corning Cell scrapers (blade L 1.8 cm, handle L 25 cm; Corning Inc.), the cell lysate was collected in 1.5 mL tubes and sonicated for 10 min in an ultrasonic bath (Branson 2200, Danbury, CT, USA) to complete the cells' lysis and homogenization. Finally, the tubes with the cell lysate were centrifuged for 10 min at 6000 x g at 4°C to remove the cell debris, and the supernatant containing the extracted proteins of all 3 technical replicates for each biological replicate was pooled in 1.5 mL tubes, aliquoted and stored at -80 °C for further analyses.

2.4. Sample preparation for proteomic analysis

Total protein concentration was first determined with the Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's instructions. For peptide preparation, extracted proteins were digested and concentrated using the Filter Aided Sample Preparation (FASP) method, following the manufacturer's instructions with some minor modifications. Briefly, fifty µg of proteins lysate were put into Millipore 10 kDa MWCO filters and 100 µl of Urea 8 M in 0.1 M Tris-HCl pH 8.5 were added for solubilization. The detergent together with other contaminant components within the protein lysate were removed by repeated filtration by

centrifugation. After discarding the flow-through, proteins were reduced with 100 µl DTT (10 mM) for 46 min and centrifuged at 14000 rpm for 10 min. Then, 200 µl of 25 mM iodoacetamide solution were added to the filters and incubated for 20 min at room temperature for protein alkylation. After performing several washes of the filters with Urea 8 M and 50 mM of ammonium bicarbonate, proteins were digested with 200 µl of 0.25 mg/mL trypsin (50:1 ratio of protein:enzyme) and mixed at 600 rpm in a thermomixer for 1 min. Filters were then transferred to new collection tubes, incubated in a wet chamber at 37 °C overnight. Finally, peptides were eluted by adding 200 µl of 50 mM of ammonium bicarbonate and the filtrate lyophilized in a SpeedVac and then resuspended in 100 µl of 1% formic acid.

2.5.Nano-LC-MS/MS analysis

For each sample, 50µg of proteins were used according to the FASP preparation. The final volume was adjusted exactly to 300 µL with a recovery solution (H_2O/ACN/TFA – 94.95/5/0.05). After passing through the ultrasonic bath (10 min), the entire supernatant was transferred to an HPLC vial before LC-MS/MS analysis. Peptides mixtures were analysed by nano-LC-MS/MS (ThermoFisher Scientific) using an Ultimate 3000 system coupled to a QExactive HF-X mass spectrometer (MS) with a nanoelectrospray ion source. Five µL of hydrolyzate was first preconcentrated and desalted at a flow rate of 30 µl/mn on a C18 pre-column 5 cm length x 100 µm (Acclaim PepMap 100 C18, 5µm, 100A nanoViper) equilibrated with Trifluoroacetic Acid 0.05% in water. After 6 min, the concentration column was switched online with a nanodebit analytical C18 column (Acclaim PepMap 100 - 75 µm inner diameter × 25 cm length; C18 - 3 µm -100Å - SN 20106770) operating at 400 nL/min equilibrated with 96 % solvent A (99.9 % H2O, 0.1 % formic acid). The peptides were then separated according to their hydrophobicity thanks to a gradient of solvent B (99.9 % acetonitrile, 0.1 % formic acid) of 4 to 25% in 60 minutes. For MS analysis, eluted peptides were electro sprayed in positive-ion mode at 1.6 kV through a Nano electrospray ion source heated to 250°C. The mass spectrometer operated in data-dependent mode: the parent ion is selected in the orbitrap cell (FTMS) at a resolution of 60,000 and each MS analysis is succeeded by 18 MS/MS with analysis of the MS/MS fragments at a resolution of 15,000).

2.6.Processing of raw mass spectrometry data

At the end of the LC-MS/MS analysis, for raw data processing, MS/MS ion search was carried out with Mascot v2.5.1 (http://www.matrixscience.com) against the chicken database (i.e. ref_Gallus_gallus 210114-27,536 sequences). The following parameters were used during the request: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da, a maximum of two missed cleavage sites of trypsin, carbamidomethylation (C), oxidation (M) and deamidation (NQ) set as variable modifications. Protein identification was validated when at least two peptides originating from one protein showed statistically significant identity above Mascot scores with a False Discovery Rate of 1%. Ions score is -10 log(P), where P is the probability that an observed match is a random event. For the chicken proteome, the Mascot score was 31 with a False Discovery Rate (FDR) of 1%. The adjusted p-value was 0.02357. Finally, for label-free protein quantification analysis, LC-Progenesis QI software (version 4.2, Nonlinear Dynamics, Newcastle upon Tyne, UK) was used with the same identification parameters described above with the phenotypic data among all matrices. All unique validated peptides of an identified protein were included and the total cumulative abundance was calculated by summing the abundances of all peptides allocated to the respective protein. LC-Progenesis analysis yielded a total of 1503 unique quantifiable proteins, having at least two peptides and two unique peptides.

2.7.Statistical analyses

2.7.1. Univariate analysis

To identify the differential abundant proteins a univariate statistical approach was performed on all 1503 quantified proteins, using an R package for proteomic analysis (available on GitHub with the DOI https://doi.org/10.5281/zenodo.2539329) as previously described (Bazile et al., 2019). Briefly, data normality distribution was assessed by the Shapiro-Wilk-Test and then either a paired Student's t-test, for normally distributed data, or a paired Wilcoxon test for not normally distributed data, was applied to determine the p-value. Finally, the obtained p-values were corrected using different adjustment methods (e.g. Benjamini & Hochberg or FDR, Hochberg and Bonferroni). For all of these analyses, R version 3.6.3 was used.

2.7.2. Multivariate analysis

As no differentially abundant proteins were obtained after performing the p-value corrections, an alternative supervised multilevel sparse variant partial least square

discriminant analysis (sPLS-DA) was applied, using the mixOmics package in R. The multilevel sPLS-DA enables the selection of the most predictive or discriminative proteins in the data of the two components (PC1 and PC2) to classify or cluster the samples between the treatment groups (Lê Cao et al., 2011).

2.8. Bioinformatic analysis of discriminant proteins selected by sPLS-DA

First, before performing the bioinformatic and functional annotation analyses, the accession numbers from all 1503 quantified proteins were converted into Gene ID using UniProt retrieve/ID mapping online tool. For undefined proteins, the Gene ID of human orthologs of chicken proteins was assigned. Second, only for the selected discriminant proteins a gene ontology (GO) enrichment analysis, focused on the Biological Processes (BP) was performed using ProteINSIDE online tool version 2.0 (available at https://umrh-bioinfo.clermont.inrae.fr/ProteINSIDE_2/index.php?page=upload.php), as previously described (Kaspric et al., 2015), and using *Gallus gallus* as reference specie. Only significant (FDR < 0.05) GO BP terms were considered as enriched and were used for further interpretation and figure creation. The enriched GO BP terms were summarized and their enrichments were expressed as -log10 (FDR) for visualization on horizontal bar graphs plotted using GraphPad Prism 9.1.2 for Mac OS X, GraphPad Software (San Diego, California, USA). Finally, the average fold-change (FC) of the raw abundances of all the discriminant proteins identified with sPLS-DA from all the seven 7 biological replicates was calculated and plotted using R version 3.6.3. In the plot, proteins with greater fold-changes in their abundances showed bigger font sizes for better visualization.

3. Results

3.1. The proteome of chicken PBMC and identification of the molecular signature of CP treatment

A total of 1503 proteins, with at least two unique peptides, were identified and quantified in chicken PBMC (data not shown). A classical univariate statistical analysis (paired t-test or Wilcoxon test) was applied and no differentially abundant proteins were detected between the CP and control groups after adjusting the p-value (FDR and Bonferroni). However, to retrieve meaningful biological information, the multivariate sPLS-DA analysis was applied to identify the molecular signature of CP treatment. The sPLS-DA model selected a total of 373 discriminant proteins (approximately 25% of the total

proteins identified) from both components (data not shown), which contributed to a good clustering between the CP and control groups (Fig. 1). From the 373 discriminant proteins, only the proteins from the sPLS-DA component 1 that strongly correlated together (data not shown) for each treatment group were selected to perform separate GO BP enrichment analyses. From these proteins, a total of 50 proteins had the highest abundance in the CP group, while 137 in the control group (data not shown).

3.2. GO enrichment analyses of total discriminant proteins identified by s-PLS-DA

To identify the global function of all the 373 discriminant proteins a GO enrichment analysis was performed. These 373 proteins were annotated by 121 enriched (FDR < 0.05) GO terms within the BP category. The main enriched BP were related to cellular amide metabolic process (GO:0043603), vesicle-mediated transport (GO:0016192), actin cytoskeleton organization (GO:0030036), peptide metabolic process (GO:0006518), organonitrogen compound biosynthesis (GO:1901566), actin filament organization (GO:0007015), translation (GO:0006412), cell migration (GO:0016477) and regulation of leukocyte chemotaxis (GO:0002688) (Fig. 2). Among the most annotated BP were: organonitrogen compound biosynthesis (49), vesicle-mediated transport (47), cellular amide metabolic processes (39) and cell migration (36) (Supplementary Table S1).

3.3.GO enrichment analyses of discriminant proteins with the highest abundance in the CP group

To further elucidate the differential effects of the CP and control treatments and the specific role of the discriminant proteins with the highest abundance in each group separate GO enrichment analyses were performed. The 50 proteins with the highest abundance in CP were annotated by 81 enriched (FDR < 0.05) GO terms within the BP category. These proteins were mainly involved in BP such as actin cytoskeleton organization (GO:0030036), regulation of cell morphogenesis (GO:0022604), actin filament-based process (GO:0030029), positive regulation of intracellular signal transduction (GO:1902533), integrin-mediated signalling pathway (GO:0007229), tumour necrosis factor-mediated signalling pathway (GO:0033209), regulation of phosphatidylinositol 3-kinase signalling (GO:0014066), negative regulation of cell migration (GO:0030336), positive regulation of cell communication (GO:0010647) and cytokine-mediated signalling pathway (GO:0019221). From these enriched BP, the ones with the highest number of annotated proteins were: actin cytoskeleton, actin filament-

based process and positive regulation of cell communication with nine proteins, and positive regulation of intracellular transduction with eight proteins (Fig. 3). APOA1 and SLC9A3R1 proteins were mainly annotated by almost all of these enriched BP, and TLN1 on those related to the actin cytoskeleton and actin-filament organization and cell morphogenesis (Supplementary Table S2).

3.4. GO enrichment analyses of discriminant proteins with the highest abundance in the control group

From the 373 discriminant proteins that contributed to a good clustering between the two treatments groups, a higher number (137) was found to be more abundant in the control group. These proteins were annotated by a total of 55 enriched (FDR < 0.05) BP GO terms. These enriched GO terms were mainly related to cellular amide metabolic process (GO:0043603), peptide metabolic process (GO:0006518), organonitrogen compound biosynthetic process (GO:1901566), translation (GO:0006412), catabolic process (GO:0009056), cellular homeostasis (GO:0019725), carbohydrate metabolic process (GO:0005975) and post-transcriptional regulation of gene expression (GO:0010608) (Fig. 4). The enriched BP with the highest number of annotated proteins were related to different metabolic processes (> 20) and translation (16) (Supplementary Table S3).

3.5. Average fold-change (FC) of the abundances of the discriminant proteins identified with sPLS-DA

Lastly, to have a clearer overview of the changes in abundances of all the 373 discriminant proteins between the CP and control groups, the average fold-change of the raw abundances of the proteins from the cells treated with CP was calculated (Fig. 5). The protein with the greatest change in abundance (FC > 1.6) between the CP and control was ALB. Other proteins with higher abundances in the CP group were found, including SCD and IL4I1 (FC ≥1.4); and FLNA, TPM2, ISG12(2), PDLIM1, LPP, HPS5, TXNDC5 and MARCKSL1 (FC > 1.2). On the other hand, proteins such as MARCKS, LGALS8, LGALS3, SQLE, CYP51A1, FABP7, FAM3C, TFRC, AHNAK2 showed the lowest abundance in the CP group (FC ≤ 0.8).

4. Discussion

The present study describes the proteome of mononuclear cells after stimulation with CP. The immunomodulatory and anti-inflammatory activity of CP on chicken monocytes has been previously reported *in vitro* (Ávila et al., 2021). However, the exact molecular mechanisms underlying such effects were not elucidated. We present herein for the first time the molecular background of CP immunomodulatory activity by demonstrating the capacity of CP in modulating chicken PBMC proteome. Moreover, we revealed the molecular signature of CP treatment using a supervised multivariate analysis (sPLS-DA). Our main findings were that CP caused an enrichment in BP related and fundamental for the cells' immune response such as actin cytoskeleton organization, cell migration, intracellular signal transduction and cell communication. Moreover, proteins such as MARCKSL1 and SLC9A3R1, known to restrict cell movement and migration, were among the discriminant proteins with the highest abundance in the CP group, while MARCKS, LGALS3 and LGALS8 were all involved with cell migration, phagocytosis and other actin cytoskeleton organization-based processes were found with the lowest abundance in the CP group.

4.1. Global biological processes enriched by the discriminant proteins selected by sPLS-DA

In the first part of the study, an sPLS-DA model was applied to identify the molecular signature of CP treatment. The sPLS-DA method allows the selection of the most discriminative features, or in this case proteins, in the data to classify the samples (Lê Cao et al., 2011). In other words, in our study, the sPLS-DA selected 373 discriminant proteins that enabled an adequate clustering of the samples from the CP and control groups, and therefore clear discrimination between them.

To have a global idea of the function of these discriminant proteins a GO enrichment analysis was performed, and it revealed that the proteins were involved in very general BP processes related to metabolisms such as cellular amide and peptide metabolic process, organonitrogen compound biosynthetic process, and translation. Moreover, other more specific and immune-related processes such as actin cytoskeleton and actin filament organization, vesicle-mediated transport, cell migration and regulation of leukocyte chemotaxis, were also enriched

4.2. Biological processes enriched by discriminant proteins with the highest abundance in CP group on chicken PBMC

In the second part of the study, to further elucidate the specific function of the discriminant proteins identified for the CP and control group, separate GO enrichment analyses were performed, using the proteins with the highest abundance in each group. The 50 discriminants proteins with the highest abundance in the CP group were mainly related to BP such as actin cytoskeleton organization, regulation of cell morphogenesis, positive regulation of intracellular signal transduction, positive regulation of cell communication, negative regulation of cell migration, among other signalling pathways; being the first four BP the most annotated ones.

The ability of cells to rapidly change their morphology in response to activation is an important feature of immune cells and their effector functions. The actin cytoskeleton plays a critical role in providing the cells the mechanical structure to support their changes in shape and membrane (Rivero et al., 1996). Indeed, the actin cytoskeleton is one of the immediate targets of signal transduction during phagocytosis by driving the formation of phagosomes to engulf the foreign particles, while in chemotaxis the cytoskeleton allows the cells to acquire the proper morphology and orientation of the cellular contents towards the chemoattractant (May and Machesky, 2001; Affolter and Weijer, 2005). Furthermore, the actin cytoskeleton has also been shown to be necessary for immune cells crosstalk or communication, by playing a crucial role in the formation and maintenance of the immunological synapse structure (Roy and Burkhardt, 2018).

Talin-1 (TLN1) and Na(+)/H(+) exchange regulatory cofactor (NHERF1/SLC9A3R1) were two of the proteins that were annotated for the actin cytoskeleton organization and actin filament-based process GO BP terms. TLN1is a cytoskeletal protein with actin-binding functions that are involved in phagocytosis and phagosome formation, by providing a physical link between the actin cytoskeleton and integrin receptors and thus regulating integrin-dependent engulfment (Lim et al., 2007). Moreover, TLN1 is also involved in the integrin-mediated signalling pathway related to phagocytosis (Torres-Gomez et al., 2020). SLC9A3R1 is a scaffold protein that stabilizes protein complexes at the plasma membrane by connecting signalling pathways and structural proteins to the actin cytoskeleton (Bretscher et al., 2000). Interestingly, besides being annotated in the BP terms of actin cytoskeleton organization and cell morphogenesis, this protein was also found annotated in negative regulation of cell migration. Our results are consistent with what has been previously reported on lung cancer cells, where overexpression of the SLC9A3R1 protein inhibited the cells' migration (YANG et al., 2017). This finding is of special interest, as in our previous study CP inhibited chicken monocytes chemotaxis *in*

vitro (Ávila et al., 2021). Similar effects were described for other types of pectins, such as ginseng pectin (Fan et al., 2018).

Enrichment of other immune and inflammation-related pathways such as tumour necrosis, regulation of phosphatidylinositol 3-kinase and cytokine-mediated signalling pathways was observed in the CP group, further suggesting immunomodulatory activities of CP. Apolipoprotein A-I (APOA1) was one of the proteins that were annotated in almost all the enriched GO BP terms, including the tumour necrosis signalling pathway and cytokine-mediated signalling pathway. APOA1 is the major structural protein of high-density lipoprotein (HDL) and is widely known for its atheroprotective and anti-inflammatory properties (Bursill et al., 2010). APOA1 inhibits macrophages chemotaxis and monocytes recruitment by modulating the reorganization of the actin component of the cytoskeleton (Iqbal et al., 2016) and has been shown to suppress the TLR-mediated secretion of the pro-inflammatory cytokines IL-6 and TNF in monocytes and macrophages (De Nardo et al., 2014). Lastly, the present finding is also consistent with a previous study on mice fed with pectin oligosaccharides that increased APOA1 levels in plasma (Zhu et al., 2017). Thus, these results suggest that CP modulated the abundance of some chicken PBMC proteins and therefore could alter the cells' immune response, mainly through changes in their actin cytoskeleton organization and signal transduction pathways often critical for regulation of several immune cell processes such as phagocytosis, cell migration, cell communication and inflammation.

4.3. Biological processes enriched by discriminant proteins with the highest abundance in the control group

As performed for the CP group, the 137 discriminant proteins with the highest abundance in the control group were mined using ProteINSIDE to determine the most enriched GO BP terms. In contrast with the enriched BP found in the CP group, the enriched BP in the control group were very general GO terms, related mainly to different metabolic processes, translation, cellular homeostasis and post-transcriptional regulation of gene expression. One of the issues of finding GO BP terms that are not specific is that the functional information that can be retrieved is quite limited. Indeed, none of the 55 enriched BP found in this treatment group was related to specific immune response GO terms. However, these results made evident that after CP treatment there was a functional switch from very general BP to others highly related to different processes of the immune response.

4.4. Discriminant proteins with greater changes in abundance after CP treatment

To further elucidate the effects of CP on chicken PBMC proteome and determine its molecular signature, we focused on identifying key proteins that presented greater changes in their raw abundances when treated with CP, considering their fold-change, and that could be involved in the most enriched BP detected with the GO analysis.

The protein with the highest abundance after CP treatment was albumin (ALB), the most abundant circulating protein in plasma that transports fatty acids, cholesterol, metals and other components by binding them (Park et al., 2014). Albumin is also known to be a major antioxidant protein by trapping free radicals, negative acute-phase protein and to be involved in negative regulation of apoptosis in endothelial cells, suggesting that CP might exert protective effects in chicken PBMC against oxidative stress and apoptosis (Zoellner et al., 1996; Roche et al., 2008; Jain et al., 2011). Other proteins with higher abundances in the CP group and involved with immune response were also detected such as serum amyloid A protein (HPS5), an acute-phase response protein in chicken (Marques et al., 2017), L-amino-acid oxidase (IL4I1), a negative regulator of T cell activation/proliferation and regulator of other adaptive immune responses (Castellano and Molinier-Frenkel, 2017), the putative ISG12(2) protein and the uncharacterized protein TXNDC5, both related with negative regulation of apoptosis processes (Horna-Terrón et al., 2014; Qi et al., 2015). Remarkably, the analysis of the discriminant proteins with greater changes in abundance after CP treatment confirm a rearrangement of the cytoskeleton as shown by the upregulation in the CP group of Myristoylated alanine-rich C kinase substrate-like 1 (MARCKSL1) protein. MARCKSL1 is a membrane-bound protein part of the MARCKS family with known actin-binding functions involved in regulating actin cytoskeleton homeostasis and coordinating other membrane-cytoskeletal signalling events such as cell migration and phagocytosis (Kim et al., 2016). Depending on its phosphorylation state, is known to enhance (unphosphorylated) or restrict cell migration (phosphorylated) of neurons and cancer cells, by inducing actin bundles formation and stabilization, which reduce actin plasticity (Björkblom et al., 2012). MARCKSL1 is highly expressed in macrophages and its role in inflammation has been widely documented, as it has been shown to affect macrophage transmigration (Chun et al., 2009). These results are consistent with the suppression of chicken monocytes' chemotaxis that was observed in our previous *in vitro* study (Ávila et al., 2021).

Finally, there were also identified with a higher abundance in the CP group: Filamin-A (FLNA), tropomyosin beta chain (TPM2), the uncharacterized protein (PDLIM1) and lipoma-preferred partner homolog (LPP), which have been also associated with actin cytoskeleton organization and stabilization (Gunning et al., 2008; Ono et al., 2015; Welter et al., 2020), cell migration (Savinko et al., 2018), maintaining cell shape and cell adhesion (Petit et al., 2000; Ngan et al., 2018). The fatty acid- desaturase domain-containing (SCD) protein, involved with fatty acid biosynthesis, which might be necessary for some humoral immune responses was also identified (Zhou et al., 2021).

On the contrary, proteins such as MARCKS, LGALS3 and LGALS8 showed a lower abundance in the CP group. Specifically, MARCKS has been shown to promote cell migration of human neutrophils (Eckert et al., 2010) and of murine macrophages *in vitro* (Green et al., 2012). Moreover, downregulating MARCKS inhibits murine macrophages phagocytosis (Carballo et al., 1999) and macrophages pro-inflammatory cytokines production (Lee et al., 2015), confirming its involvement in modulating inflammation.

Galectin-3 (LGALS3) and galectin-8 (LGALS8) are ubiquitous carbohydrate-binding proteins (Brinchmann et al., 2018), participating in many cellular processes, including inflammation, immune responses, cell migration, autophagy and cell signalling. In chickens, 5 members have been identified (Kaltner et al., 2011): among them, LGALS3 and LGALS. LGALS3 is known to be highly expressed in monocytes and macrophages, and to be a strong regulator of cell-extracellular matrix (ECM) and cell-cell interactions, cell migration and phagocytosis (Lu et al., 2017). The detection of a lower abundance of LGALS3 in the CP group is remarkable, as mouse LGALS3 was downregulated by modified citrus pectin (MCP) - a derivative of citrus pectin (Kolatsi-Joannou et al., 2011), thus reducing disease severity and inflammation. Moreover, MCP directly inhibits LGALS3, causing a suppression in the migration of human endothelial cells *in vitro* and cancer cells *in vivo* (Nangia-Makker et al., 2002), and in the adhesion of monocytes and macrophages *in vitro* and *in vivo,* by tightly binding LGALS3 through its carbohydrate recognition domain (Lu et al., 2017). LGALS8 is involved in cytoskeleton reorganization processes, cell adhesion and cell migration, as well as in autophagy and cytokines and chemokines expression (Gentilini et al., 2017; Johannes et al., 2018). In LGALS8 knockout mice, the expression of several pro-inflammatory cytokines and chemokines (TNF-α, IL-1β, MCP-1 and IL-6) was reduced (Shatz-Azoulay et al., 2020), confirming the critical role of CP in potentially lowering inflammation through the inhibition of galectins.

Finally, proteins involved in the positive regulation of T and B cells proliferation (TFRC) (Ned et al., 2003) and human mononuclear cells migration (AHNAK2) were also identified (Zheng et al., 2021), supporting the CP immune-modulatory function.

5. Conclusions

In conclusion, the results of this study demonstrate for the first time that CP does modulate chicken PBMC proteome. Specifically, when performing the GO BP analysis of the sPLS-DA-selected discriminant proteins with higher abundance in the CP group, an enrichment in biological processes mainly related to actin cytoskeleton organization, cell morphogenesis and negative regulation of cell migration was observed. Therefore, these results confirm the suppressive effects observed in our previous *in vitro* study on chicken monocytes' phagocytosis and chemotaxis and provide novel information on the immunomodulatory activity of CP on chicken PBMC. By elucidating the molecular mechanisms underlying such immunomodulatory effects, we might have a better understanding of CP's practical biological significance *in vivo* and hence on how to include it as a dietary fibre in novel nutrition strategies to modulate the animals' immune and health status. Moreover, this study demonstrates the suitability of the supervised multivariate statistical analysis sPLS-DA to retrieve meaningful functional information from complex proteomics data, converting it into a noteworthy option for the analysis of data coming from other OMIC technologies. Finally, further functional annotation analyses such as molecular pathway enrichment and protein-protein interaction analyses, and integration of proteomics data with other OMIC technologies should be performed to increase and deepen the knowledge on the overall impact of CP on chicken immunity.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the paper's content.

Ethics statement

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The procedures for the blood collection were carried out during routine slaughtering procedures.

Acknowledgements

The authors acknowledge A. Delavaud (INRAE, Herbivore Research Unit) for his technical assistance in protein extraction, quantification, and concentration for mass spectrometry analyses, and also Arnaud Cougoul, Jeremy Tournayre and Céline Boby (INRAE, Herbivore Research Unit) for their assistance in the statistical and bioinformatic analyses, respectively.

Funding

This study was supported by the European Union's Horizon 2020 research and innovation programme H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative Training Networks (European Joint Doctorate) [Grant agreement nº: 765423, 2017] – MANNA.

References

Affolter, M., and C.J. Weijer. 2005. Signaling to Cytoskeletal Dynamics during Chemotaxis. Dev. Cell 9:19–34. doi:https://doi.org/10.1016/j.devcel.2005.06.003.

Almeida, A.M., S.A. Ali, F. Ceciliani, P.D. Eckersall, L.E. Hernández-Castellano, R. Han, J.J. Hodnik, S. Jaswal, J.D. Lippolis, M. McLaughlin, I. Miller, A.K. Mohanty, V. Mrljak, J.E. Nally, P. Nanni, J.E. Plowman, M.D. Poleti, D.M. Ribeiro, P. Rodrigues, B. Roschitzki, R. Schlapbach, J. Starič, Y. Yang, and M. Zachut. 2021. Domestic animal proteomics in the 21st century: A global retrospective and viewpoint analysis.. J. Proteomics 241:104220. doi:10.1016/j.jprot.2021.104220.

Ávila, G., D. De Leonardis, G. Grilli, C. Lecchi, and F. Ceciliani. 2021. Anti-inflammatory activity of citrus pectin on chicken monocytes' immune response. Vet. Immunol. Immunopathol. 237:110269. doi:https://doi.org/10.1016/j.vetimm.2021.110269.

Bazile, J., B. Picard, C. Chambon, A. Valais, and M. Bonnet. 2019. Pathways and biomarkers of marbling and carcass fat deposition in bovine revealed by a combination of gel-based and gel-free proteomic analyses. Meat Sci. 156:146–155. doi:https://doi.org/10.1016/j.meatsci.2019.05.018.

Beukema, M., M.M. Faas, and P. de Vos. 2020. The effects of different dietary fiber pectin structures on the gastrointestinal immune barrier: impact via gut microbiota and direct effects on immune cells. Exp. Mol. Med. 52:1364–1376. doi:10.1038/s12276-020-0449-2.

Björkblom, B., A. Padzik, H. Mohammad, N. Westerlund, E. Komulainen, P. Hollos, L. Parviainen, A.C. Papageorgiou, K. Iljin, O. Kallioniemi, M. Kallajoki, M.J. Courtney, M. Mågård, P. James, and E.T. Coffey. 2012. c-Jun N-terminal kinase phosphorylation of MARCKSL1 determines actin stability and migration in neurons and in cancer cells. Mol. Cell. Biol. 32:3513–3526. doi:10.1128/MCB.00713-12.

Bretscher, A., D. Chambers, R. Nguyen, and D. Reczek. 2000. ERM-Merlin and EBP50 Protein Families in Plasma Membrane Organization and Function. Annu. Rev. Cell Dev. Biol. 16:113–143. doi:10.1146/annurev.cellbio.16.1.113.

Brinchmann, M.F., D.M. Patel, and M.H. Iversen. 2018. The Role of Galectins as Modulators of Metabolism and Inflammation. Mediators Inflamm. 2018:9186940. doi:10.1155/2018/9186940.

Bursill, C.A., M.L. Castro, D.T. Beattie, S. Nakhla, E. van der Vorst, A.K. Heather, P.J. Barter, and K.-A. Rye. 2010. High-Density Lipoproteins Suppress Chemokines and Chemokine Receptors In Vitro and In Vivo. Arterioscler. Thromb. Vasc. Biol. 30:1773–1778. doi:10.1161/ATVBAHA.110.211342.

Carballo, E., D.M. Pitterle, D.J. Stumpo, R.T. Sperling, and P.J. Blackshear. 1999. Phagocytic and macropinocytic activity in MARCKS-deficient macrophages and fibroblasts. Am. J. Physiol. Physiol. 277:C163–C173. doi:10.1152/ajpcell.1999.277.1.C163.

Castellano, F., and V. Molinier-Frenkel. 2017. An Overview of l-Amino Acid Oxidase Functions from Bacteria to Mammals: Focus on the Immunoregulatory Phenylalanine Oxidase IL4I1. Molecules 22:2151. doi:10.3390/molecules22122151.

Chen, C.-H., M.-T. Sheu, T.-F. Chen, Y.-C. Wang, W.-C. Hou, D.-Z. Liu, T.-C. Chung, and Y.-C. Liang. 2006. Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways. Biochem. Pharmacol. 72:1001–1009. doi:https://doi.org/10.1016/j.bcp.2006.07.001.

Chun, K.-R., E.M. Bae, J.-K. Kim, K. Suk, and W.-H. Lee. 2009. Suppression of the lipopolysaccharide-induced expression of MARCKS-related protein (MRP) affects transmigration in activated RAW264.7 cells. Cell. Immunol. 256:92–98. doi:https://doi.org/10.1016/j.cellimm.2009.01.011.

Deeg, C.A., R.L. Degroote, I.M. Giese, S. Hirmer, B. Amann, M. Weigand, C. Wiedemann, and S.M. Hauck. 2020. CD11d is a novel antigen on chicken leukocytes.. J. Proteomics 225:103876. doi:10.1016/j.jprot.2020.103876.

Deng, X., Y. Cong, R. Yin, G. Yang, C. Ding, S. Yu, X. Liu, C. Wang, and Z. Ding. 2014. Proteomic analysis of chicken peripheral blood mononuclear cells after infection by Newcastle disease virus. J Vet Sci 15:511–517.

Désert, C., E. Merlot, T. Zerjal, B. Bed'hom, S. Härtle, A. Le Cam, P.-F. Roux, E. Baeza, F. Gondret, M.J. Duclos, and S. Lagarrigue. 2016. Transcriptomes of whole blood and PBMC in chickens. Comp. Biochem. Physiol. Part D Genomics Proteomics 20:1–9. doi:https://doi.org/10.1016/j.cbd.2016.06.008.

Diz, A.P., M. MARTÍNEZ-FERNÁNDEZ, and E. ROLÁN-ALVAREZ. 2012. Proteomics in evolutionary ecology: linking the genotype with the phenotype. Mol. Ecol. 21:1060–1080.

Eckert, R.E., L.E. Neuder, J. Park, K.B. Adler, and S.L. Jones. 2010. Myristoylated alanine-rich C-kinase substrate (MARCKS) protein regulation of human neutrophil migration. Am. J. Respir. Cell Mol. Biol. 42:586–594.

Fan, Y., L. Sun, S. Yang, C. He, G. Tai, and Y. Zhou. 2018. The roles and mechanisms of homogalacturonan and rhamnogalacturonan I pectins on the inhibition of cell migration. Int. J. Biol. Macromol. 106:207–217. doi:https://doi.org/10.1016/j.ijbiomac.2017.08.004.

Gentilini, L.D., F.M. Jaworski, C. Tiraboschi, I.G. Pérez, M.L. Kotler, A. Chauchereau, D.J. Laderach, and D. Compagno. 2017. Stable and high expression of Galectin-8 tightly controls metastatic progression of prostate cancer. Oncotarget 8:44654–44668. doi:10.18632/oncotarget.17963.

Green, T.D., J. Park, Q. Yin, S. Fang, A.L. Crews, S.L. Jones, and K.B. Adler. 2012. Directed migration of mouse macrophages in vitro involves myristoylated alanine-rich C-kinase substrate (MARCKS) protein. J. Leukoc. Biol. 92:633–639. doi:https://doi.org/10.1189/jlb.1211604.

Gunning, P., G. O'neill, and E. Hardeman. 2008. Tropomyosin-Based Regulation of the Actin Cytoskeleton in Time and Space. Physiol. Rev. 88:1–35. doi:10.1152/physrev.00001.2007.

Horna-Terrón, E., A. Pradilla-Dieste, C. Sánchez-de-Diego, and J. Osada. 2014. TXNDC5, a newly discovered disulfide isomerase with a key role in cell physiology and pathology. Int. J. Mol. Sci. 15:23501–23518.

Iqbal, A.J., T.J. Barrett, L. Taylor, E. McNeill, A. Manmadhan, C. Recio, A. Carmineri, M.H. Brodermann, G.E. White, D. Cooper, J.A. DiDonato, M. Zamanian-Daryoush, S.L. Hazen, K.M. Channon, D.R. Greaves, and E.A. Fisher. 2016. Acute exposure

to apolipoprotein A1 inhibits macrophage chemotaxis in vitro and monocyte recruitment in vivo. Elife 5:e15190. doi:10.7554/eLife.15190.

Jain, S., V. Gautam, and S. Naseem. 2011. Acute-phase proteins: As diagnostic tool. J. Pharm. Bioallied Sci. 3:118–127. doi:10.4103/0975-7406.76489.

Johannes, L., R. Jacob, and H. Leffler. 2018. Galectins at a glance. J. Cell Sci. 131:jcs208884. doi:10.1242/jcs.208884.

Kaltner, H., D. Kübler, L. López-Merino, M. Lohr, J.C. Manning, M. Lensch, J. Seidler, W.D. Lehmann, S. André, and D. Solís. 2011. Toward comprehensive analysis of the galectin network in chicken: unique diversity of galectin-3 and comparison of its localization profile in organs of adult animals to the other four members of this lectin family. Anat. Rec. Adv. Integr. Anat. Evol. Biol. 294:427–444.

Kaspric, N., B. Picard, M. Reichstadt, J. Tournayre, and M. Bonnet. 2015. ProteINSIDE to Easily Investigate Proteomics Data from Ruminants: Application to Mine Proteome of Adipose and Muscle Tissues in Bovine Foetuses. PLoS One 10:e0128086–e0128086. doi:10.1371/journal.pone.0128086.

Kim, B.-R., S.-H. Lee, M.-S. Park, S.-H. Seo, Y.-M. Park, Y.-J. Kwon, and S.-B. Rho. 2016. MARCKSL1 exhibits anti-angiogenic effects through suppression of VEGFR-2-dependent Akt/PDK-1/mTOR phosphorylation. Oncol Rep 35:1041–1048. doi:10.3892/or.2015.4408.

Kolatsi-Joannou, M., K.L. Price, P.J. Winyard, and D.A. Long. 2011. Modified Citrus Pectin Reduces Galectin-3 Expression and Disease Severity in Experimental Acute Kidney Injury. PLoS One 6:e18683.

Korte, J., T. Fröhlich, M. Kohn, B. Kaspers, G.J. Arnold, and S. Härtle. 2013. 2D DIGE analysis of the bursa of Fabricius reveals characteristic proteome profiles for different stages of chicken B-cell development.. Proteomics 13:119–33. doi:10.1002/pmic.201200177.

Langhout, D.J., and J.B. Schutte. 1996. Nutritional Implications of Pectins in Chicks in Relation to Esterification and Origin of Pectins. Poult. Sci. 75:1236–1242. doi:10.3382/ps.0751236.

Lê Cao, K.-A., S. Boitard, and P. Besse. 2011. Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems. BMC Bioinformatics 12:253. doi:10.1186/1471-2105-12-253.

Leclere, L., P. Van Cutsem, and C. Michiels. 2013a. Anti-cancer activities of pH- or heat-modified pectin. Front. Pharmacol. 4 OCT:1–8. doi:10.3389/fphar.2013.00128.

Leclere, L., P. Van Cutsem, and C. Michiels. 2013b. Anti-cancer activities of pH- or heat-modified pectin . Front. Pharmacol. 4:128.

Lee, S.-M., K. Suk, and W.-H. Lee. 2015. Myristoylated alanine-rich C kinase substrate (MARCKS) regulates the expression of proinflammatory cytokines in macrophages through activation of p38/JNK MAPK and NF-κB. Cell. Immunol. 296:115–121. doi:https://doi.org/10.1016/j.cellimm.2015.04.004.

Lim, J., A. Wiedemann, G. Tzircotis, S.J. Monkley, D.R. Critchley, and E. Caron. 2007. An essential role for talin during alpha(M)beta(2)-mediated phagocytosis. Mol. Biol. Cell 18:976–985. doi:10.1091/mbc.e06-09-0813.

Lu, Y., M. Zhang, P. Zhao, M. Jia, B. Liu, Q. Jia, J. Guo, L. Dou, and J. Li. 2017. Modified citrus pectin inhibits galectin-3 function to reduce atherosclerotic lesions in apoE-deficient mice. Mol Med Rep 16:647–653. doi:10.3892/mmr.2017.6646.

Marques, A.T., L. Nordio, C. Lecchi, G. Grilli, C. Giudice, and F. Ceciliani. 2017. Widespread extrahepatic expression of acute-phase proteins in healthy chicken (Gallus gallus) tissues.. Vet. Immunol. Immunopathol. 190:10–17. doi:10.1016/j.vetimm.2017.06.006.

May, R.C., and L.M. Machesky. 2001. Phagocytosis and the actin cytoskeleton. J. Cell Sci. 114:1061–1077. doi:10.1242/jcs.114.6.1061.

Nangia-Makker, P., V. Hogan, Y. Honjo, S. Baccarini, L. Tait, R. Bresalier, and A. Raz. 2002. Inhibition of Human Cancer Cell Growth and Metastasis in Nude Mice by Oral Intake of Modified Citrus Pectin. JNCI J. Natl. Cancer Inst. 94:1854–1862. doi:10.1093/jnci/94.24.1854.

De Nardo, D., L.I. Labzin, H. Kono, R. Seki, S. V Schmidt, M. Beyer, D. Xu, S. Zimmer, C. Lahrmann, F.A. Schildberg, J. Vogelhuber, M. Kraut, T. Ulas, A. Kerksiek, W. Krebs, N. Bode, A. Grebe, M.L. Fitzgerald, N.J. Hernandez, B.R.G. Williams, P. Knolle, M. Kneilling, M. Röcken, D. Lütjohann, S.D. Wright, J.L. Schultze, and E. Latz. 2014. High-density lipoprotein mediates anti-inflammatory reprogramming of macrophages via the transcriptional regulator ATF3. Nat. Immunol. 15:152–160. doi:10.1038/ni.2784.

Ned, R.M., W. Swat, and N.C. Andrews. 2003. Transferrin receptor 1 is differentially required in lymphocyte development. Blood 102:3711–3718. doi:10.1182/blood-2003-04-1086.

Ngan, E., A. Kiepas, C.M. Brown, and P.M. Siegel. 2018. Emerging roles for LPP in metastatic cancer progression. J. Cell Commun. Signal. 12:143–156.

doi:10.1007/s12079-017-0415-5.

Ono, R., T. Kaisho, and T. Tanaka. 2015. PDLIM1 inhibits NF-κB-mediated inflammatory signaling by sequestering the p65 subunit of NF-κB in the cytoplasm. Sci. Rep. 5:18327. doi:10.1038/srep18327.

Pardanani, A., E.D. Wieben, T.C. Spelsberg, and A. Tefferi. 2002. Primer on medical genomics part IV: expression proteomics. Pages 1185–1196 in Mayo Clinic Proceedings. Elsevier.

Park, K.T., C.-H. Yun, C.-S. Bae, and T. Ahn. 2014. Decreased level of albumin in peripheral blood mononuclear cells of streptozotocin-induced diabetic rats. J. Vet. Med. Sci. 76:1087–1092. doi:10.1292/jvms.13-0631.

Petit, M.M.R., J. Fradelizi, R.M. Golsteyn, T.A.Y. Ayoubi, B. Menichi, D. Louvard, W.J.M. Van de Ven, and E. Friederich. 2000. LPP, an Actin Cytoskeleton Protein Related to Zyxin, Harbors a Nuclear Export Signal and Transcriptional Activation Capacity. Mol. Biol. Cell 11:117–129. doi:10.1091/mbc.11.1.117.

Qi, Y., Y. Li, Y. Zhang, L. Zhang, Z. Wang, X. Zhang, L. Gui, and J. Huang. 2015. IFI6 Inhibits Apoptosis via Mitochondrial-Dependent Pathway in Dengue Virus 2 Infected Vascular Endothelial Cells. PLoS One 10:e0132743–e0132743. doi:10.1371/journal.pone.0132743.

Rivero, F., B. Koppel, B. Peracino, S. Bozzaro, F. Siegert, C.J. Weijer, M. Schleicher, R. Albrecht, and A.A. Noegel. 1996. The role of the cortical cytoskeleton: F-actin crosslinking proteins protect against osmotic stress, ensure cell size, cell shape and motility, and contribute to phagocytosis and development. J. Cell Sci. 109:2679–2691. doi:10.1242/jcs.109.11.2679.

Roche, M., P. Rondeau, N.R. Singh, E. Tarnus, and E. Bourdon. 2008. The antioxidant properties of serum albumin. FEBS Lett. 582:1783–1787.

Roy, N.H., and J.K. Burkhardt. 2018. The Actin Cytoskeleton: A Mechanical Intermediate for Signal Integration at the Immunological Synapse . Front. Cell Dev. Biol. 6.

Sahasrabudhe, N.M., M. Beukema, L. Tian, B. Troost, J. Scholte, E. Bruininx, G. Bruggeman, M. van den Berg, A. Scheurink, H.A. Schols, M.M. Faas, and P. de Vos. 2018. Dietary Fiber Pectin Directly Blocks Toll-Like Receptor 2–1 and Prevents Doxorubicin-Induced Ileitis . Front. Immunol. 9.

Salman, H., M. Bergman, M. Djaldetti, J. Orlin, and H. Bessler. 2008. Citrus pectin affects cytokine production by human peripheral blood mononuclear cells. Biomed.

Pharmacother. 62:579–582. doi:https://doi.org/10.1016/j.biopha.2008.07.058.

Savinko, T., C. Guenther, L.M. Uotila, M. Llort Asens, S. Yao, S. Tojkander, and S.C. Fagerholm. 2018. Filamin A Is Required for Optimal T Cell Integrin-Mediated Force Transmission, Flow Adhesion, and T Cell Trafficking. J. Immunol. 200:3109 LP – 3116. doi:10.4049/jimmunol.1700913.

Sekelova, Z., O. Polansky, H. Stepanova, R. Fedr, M. Faldynova, I. Rychlik, and L. Vlasatikova. 2017. Different roles of CD4, CD8 and γδ T-lymphocytes in naive and vaccinated chickens during Salmonella Enteritidis infection.. Proteomics 17. doi:10.1002/pmic.201700073.

Shatz-Azoulay, H., Y. Vinik, R. Isaac, U. Kohler, S. Lev, and Y. Zick. 2020. The Animal Lectin Galectin-8 Promotes Cytokine Expression and Metastatic Tumor Growth in Mice. Sci. Rep. 10:7375. doi:10.1038/s41598-020-64371-z.

Torres-Gomez, A., C. Cabañas, and E.M. Lafuente. 2020. Phagocytic Integrins: Activation and Signaling. Front. Immunol. 11:738. doi:10.3389/fimmu.2020.00738.

Welter, H., C. Herrmann, T. Fröhlich, F. Flenkenthaler, K. Eubler, H. Schorle, D. Nettersheim, A. Mayerhofer, and A. Müller-Taubenberger. 2020. Filamin A Orchestrates Cytoskeletal Structure, Cell Migration and Stem Cell Characteristics in Human Seminoma TCam-2 Cells. Cells 9. doi:10.3390/cells9122563.

YANG, F., Y. GU, Z. ZHAO, J. HUANG, W.E.N.G. JIANG, and S. CHENG. 2017. NHERF1 Suppresses Lung Cancer Cell Migration by Regulation of Epithelial–Mesenchymal Transition. Anticancer Res. 37:4405 LP – 4414.

Zheng, L., S. Li, X. Zheng, R. Guo, and W. Qu. 2021. AHNAK2 is a novel prognostic marker and correlates with immune infiltration in papillary thyroid cancer: Evidence from integrated analysis. Int. Immunopharmacol. 90:107185. doi:https://doi.org/10.1016/j.intimp.2020.107185.

Zhou, X., X. Zhu, and H. Zeng. 2021. Fatty acid metabolism in adaptive immunity. FEBS J.

Zhu, R.-G., Y.-D. Sun, Y.-T. Hou, J.-G. Fan, G. Chen, and T.-P. Li. 2017. Pectin penta-oligogalacturonide reduces cholesterol accumulation by promoting bile acid biosynthesis and excretion in high-cholesterol-fed mice. Chem. Biol. Interact. 272:153–159. doi:https://doi.org/10.1016/j.cbi.2017.05.018.

Zoellner, H., M. Hofler, R. Beckmann, P. Hufnagl, E. Vanyek, E. Bielek, J. Wojta, A. Fabry, S. Lockie, and B.R. Binder. 1996. Serum albumin is a specific inhibitor of apoptosis in human endothelial cells. J. Cell Sci. 109:2571–2580.

doi:10.1242/jcs.109.10.2571.

Figures

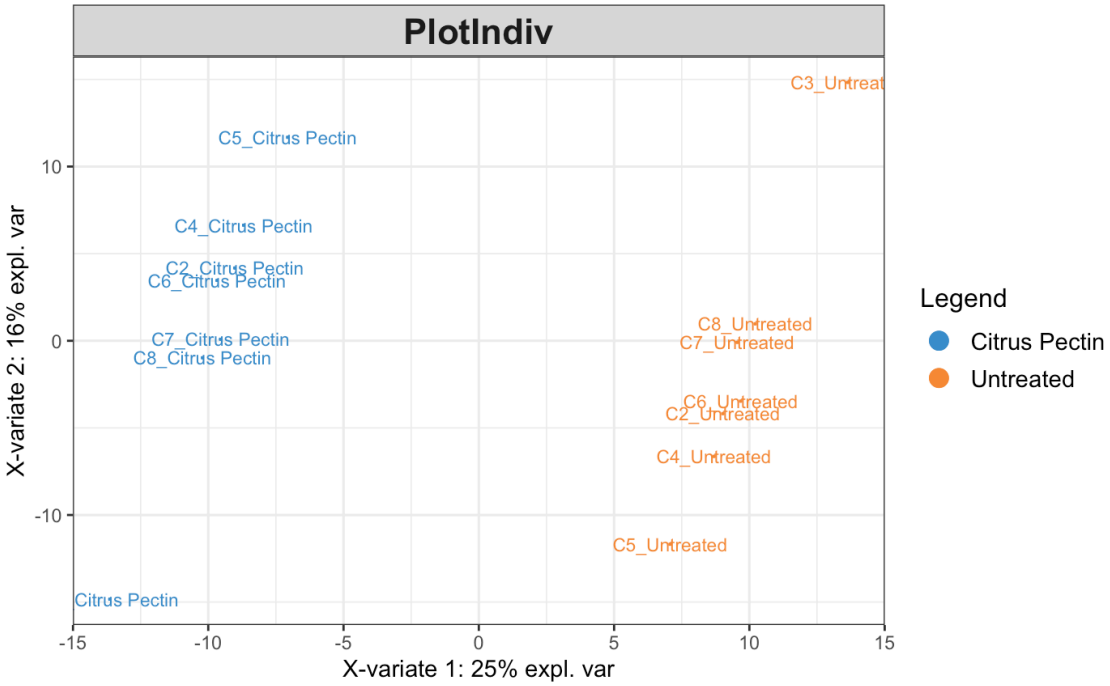


Fig. 1. Sparse partial least squares discriminant analysis (sPLS-DA) for paired data individual plot. The individual ploy shows the similarities and relationship (clustering) between samples of the citrus pectin (CP) (blue) and control (orange) treatment groups.

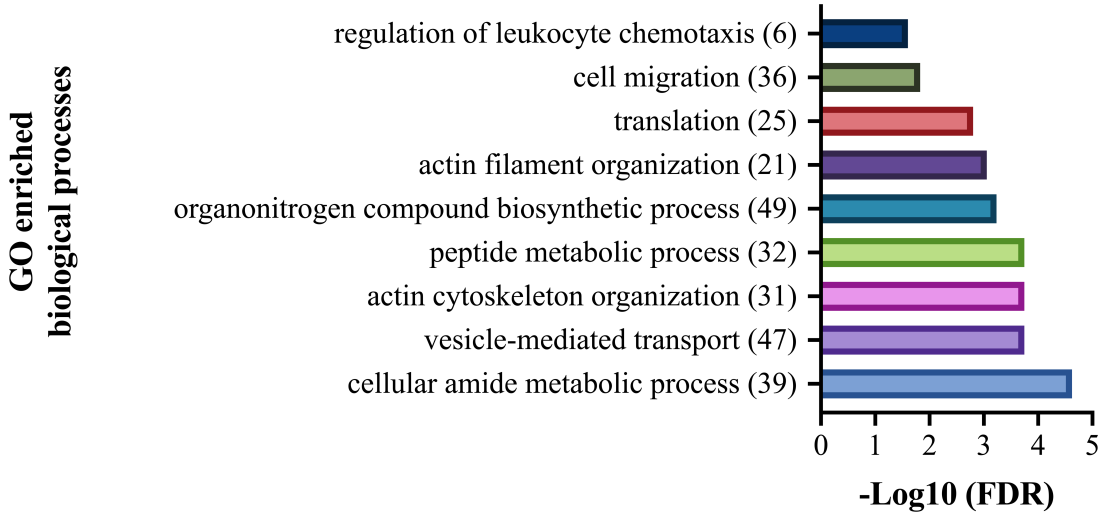


Fig. 2. Global enriched (P<0.05) Gene ontology (GO) terms within the biological processes (BP) category that has annotated all 373 discriminant proteins. GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

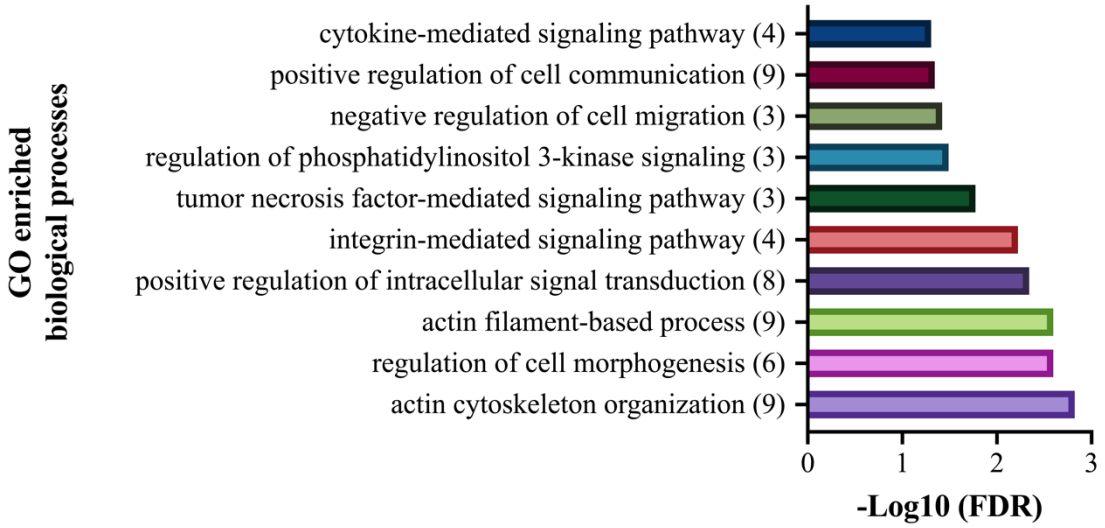


Fig. 3. Enriched (P<0.05) Gene ontology (GO) terms within the biological processes category that have annotated the 50 discriminant proteins with the highest abundance in the citrus pectin (CP) group. GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

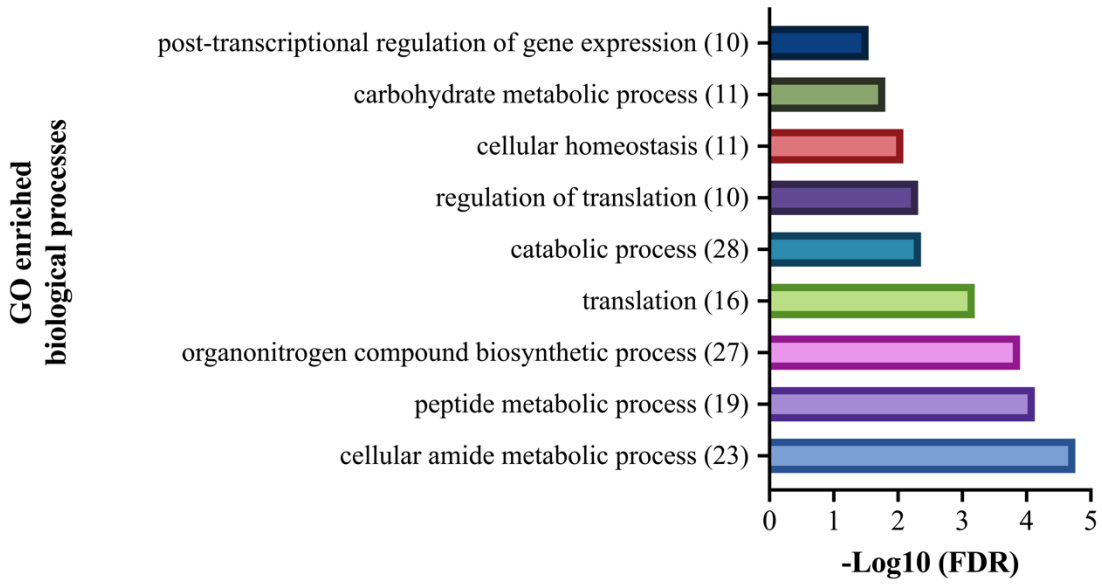


Fig. 4. Enriched (P<0.05) Gene ontology (GO) terms within the biological processes category that have annotated the 137 discriminant proteins with the highest abundance in the control group (no CP). GO terms enrichments are expressed as -log10 (FDR) for visualization on graphs. The total number of proteins annotated by GO BP terms is shown in brackets.

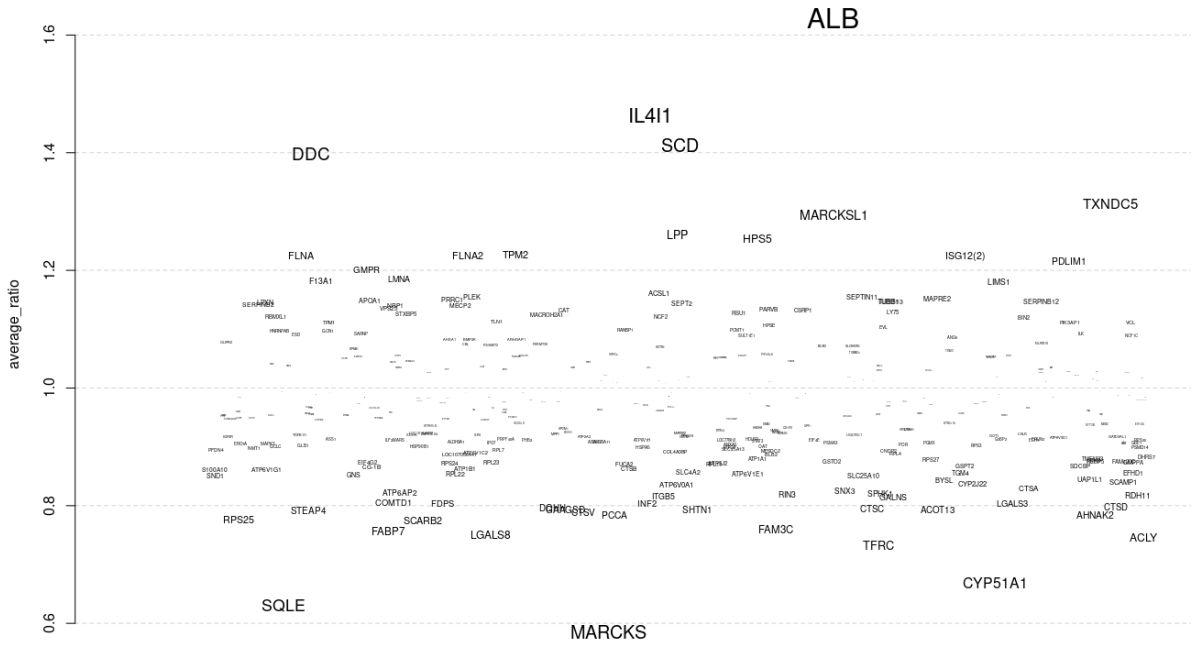


Fig. 5. Average fold-change of protein raw abundances of the 373 discriminant proteins selected by sPLS-DA. The y (vertical?) axis shows the average fold-change (ratio) of the raw abundances from all the seven different biological replicates (samples) after citrus pectin (CP) treatment. Proteins (Gene ID) that showed greater fold-changes are represented with bigger font sizes.

Supplementary material

Supplementary Table S1. List of global enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by all 373 discriminant proteins selected the sPLS-DA.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0043603	cellular amide metabolic process	0,00002	4,63	ACLY ACSS1A ASAH1 ASNS ASS1 ATP6AP2 CALR CLIC2 COL4A3BP EIF3D EIF3I EIF3L EIF4E EIF4G1 EIF4G2 EIF5 GAPDH GARS GCLC GCN1 GSPT2 GSS GSTM2 LTA4H MARS PKM RPL11 RPL22 RPL23 RPL4 RPS15 RPS20 RPS24 RPS27 RPS3 RTN4 SPHK1 TARDBP UPF1
GO:0016192	vesicle-mediated transport	0,00018	3,75	AP2A2 AP2M1 AP3M1 APOA1 ARF1 ARF4 ARHGAP1 ATP2A2 ATP6V1H BMP2K CALR CBL COPB2 EHD3 EPN2 EZR GRB2 HIP1 HSPA8 ITGB1 LAMTOR1 LGALS3 LYN NCF2 PIP4K2A PLEK PLSCR1 PRKCB PTPRC RAB11A RAP1B RIN3 S100A10 SCAMP1 SCARB2 SEC31B SEPTIN2 SEPT2 SNX3 SPHK1 STXBP5 TLN1 TMED7 VAMP3 VPS25 VPS26A
GO:0030036	actin cytoskeleton organization	0,00018	3,75	ACTC1 ACTN1 ACTR2 ACTR3 APOA1 ARPC2 ARPC5 CALR CORO7 CSRP1 DSTN EVL EZR FLNA FLNB GRB2 INF2 ITGB1 ITGB5 LCP1 LOC107056441 MARCKS NRP1 PARVB PLEK S100A10 SDCBP SH3KBP1 SLC9A3R1 TLN1 TPM1
GO:0006518	peptide metabolic process	0,00018	3,75	ATP6AP2 CALR CLIC2 EIF3D EIF3I EIF3L EIF4E EIF4G1 EIF4G2 EIF5 GAPDH GARS GCLC GCN1 GSPT2 GSS GSTM2 LTA4H MARS PKM RPL11 RPL22 RPL23 RPL4 RPS15 RPS20 RPS24 RPS27 RPS3 RTN4 TARDBP UPF1

GO:1901566	organonitrogen compound biosynthetic process	0,00058	3,23	ACLY ACSS1A ADSS2 ADSS ALG12 APOA1 ASAH1 ASNS ASS1 CALR CHP1 DBI EIF3D EIF3I EIF3L EIF4E EIF4G1 EIF4G2 EIF5 GAPDH GARS GCLC GCN1 GOT1 GOT2 GSPT2 GSS IMPDH2 MARS NMT1 OAT OGT PGM3 PKM RPL11 RPL22 RPL23 RPL4 RPS15 RPS20 RPS24 RPS27 RPS3 SLC25A13 SPHK1 STT3B TARDBP UMPS UPF1
GO:0007015	actin filament organization	0,00088	3,05	ACTC1 ACTN1 ACTR2 ACTR3 APOA1 ARPC2 ARPC5 CORO7 DSTN EVL EZR GRB2 ITGB5 LCP1 LOC107056441 MARCKS NRP1 PLEK S100A10 SH3KBP1 TPM1
GO:0006412	translation	0,00157	2,80	CALR EIF3D EIF3I EIF3L EIF4E EIF4G1 EIF4G2 EIF5 GAPDH GARS GCN1 GSPT2 MARS PKM RPL11 RPL22 RPL23 RPL4 RPS15 RPS20 RPS24 RPS27 RPS3 TARDBP UPF1
GO:0016477	cell migration	0,01487	1,83	AIMP1 APOA1 ARF4 ARPC5 C1QBP CALR CORO7 EVL GLIPR2 ILK ITGB1 LCP1 LGALS3 LGALS8 LGMN LOC107056441 LYN MAPRE2 MPP1 NACA NRP1 PHB2 PRPF40A PTPRC RAB11A RAP2C RIN3 RTN4 SDCBP SH3KBP1 SHTN1 SLC9A3R1 SPHK1 STAT3 STRAP VCL
GO:0002688	regulation of leukocyte chemotaxis	0,02495	1,60	C1QBP CALR LGMN LYN MPP1 RIN3

Supplementary Table S2. List of enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins selected by the sPLS-DA with the highest abundance in citrus pectin (CP) group.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0030036	actin cytoskeleton organization	0,0015	2,82	ACTN1 APOA1 CSRP1 EVL PARVB PLEK SLC9A3R1 TLN1 TPM1
GO:0022604	regulation of cell morphogenesis	0,0025	2,60	APOA1 ILK LIMS1 PARVB SLC9A3R1 TPM1
GO:0030029	actin filament-based process	0,0025	2,60	ACTN1 APOA1 CSRP1 EVL PARVB PLEK SLC9A3R1 TLN1 TPM1
GO:1902533	positive regulation of intracellular signal transduction	0,0046	2,34	APOA1 CAT GLIPR2 HPSE ILK LIMS1 MAPRE2 PIK3AP1 SLC9A3R1
GO:0007229	integrin-mediated signaling pathway	0,0060	2,22	APOA1 ILK PLEK TLN1
GO:0033209	tumor necrosis factor-mediated signaling pathway	0,0168	1,77	APOA1 ILK LIMS1
GO:0014066	regulation of phosphatidylinositol 3-kinase signaling	0,0325	1,49	CAT PIK3AP1 SLC9A3R1
GO:0030336	negative regulation of cell migration	0,0378	1,42	EVL SLC9A3R1 TPM1
GO:0010647	positive regulation of cell communication	0,0453	1,34	APOA1 CAT GLIPR2 HPSE ILK LIMS1 MAPRE2 PIK3AP1 SLC9A3R1
GO:0019221	cytokine-mediated signaling pathway	0,0497	1,30	ACSL1 APOA1 ILK LIMS1

Supplementary Table S3. List of enriched Gene Ontology (GO) terms within the biological processes (BP) annotated by the discriminant proteins selected by the sPLS-DA with the highest abundance in the control group.

GO Term BP	GO Term Description	P-value (FDR)	-log10(FDR)	Annotated Proteins (Gene Name)
GO:0043603	cellular amide metabolic process	0,0000	4,76	ACLY ASAH1 ASS1 ATP6AP2 CALR COL4A3BP EIF3D EIF4E EIF4G1 EIF4G2 GCLC GSPT2 MARS RPL11 RPL22 RPL4 RPS20 RPS24 RPS27 RPS3 RTN4 STAT3 UPF1
GO:0006518	peptide metabolic process	0,0001	4,13	ATP6AP2 CALR EIF3D EIF4E EIF4G1 EIF4G2 GCLC GSPT2 MARS RPL11 RPL22 RPL4 RPS20 RPS24 RPS27 RPS3 RTN4 STAT3 UPF1
GO:1901566	organonitrogen compound biosynthetic process	0,0001	3,90	ACLY ASAH1 ASS1 CALR EIF3D EIF4E EIF4G1 EIF4G2 GCLC GOT1 GOT2 GSPT2 MARS NMT1 OAT PGM3 RPL11 RPL22 RPL4 RPS20 RPS24 RPS27 RPS3 SLC25A13 STAT3 UMPS UPF1
GO:0006412	translation	0,0006	3,20	CALR EIF3D EIF4E EIF4G1 EIF4G2 GSPT2 MARS RPL11 RPL22 RPL4 RPS20 RPS24 RPS27 RPS3 STAT3 UPF1
GO:0009056	catabolic process	0,0044	2,36	ASAH1 ATP2A2 ATP6V0A1 CTSA CTSB EIF4G1 EIF4G2 ERLIN2 FUCA2 GCLC GOT1 GOT2 HSP90B1 ITGB1 LGALS8 OAT PHB2 PRDX6 PSMA3 PSMD11 PSMD14 RPL11 SCARB2 SDCBP SNX3 STAT3 UPF1 XPO1
GO:0006417	regulation of translation	0,0049	2,31	CALR EIF3D EIF4E EIF4G1 EIF4G2 GSPT2 RPL22 RPS3 STAT3 UPF1
GO:0019725	cellular homeostasis	0,0082	2,08	ATP1A1 ATP1B1 ATP2A2 ATP6AP2 ATP6V0D1 ATP6V1G1 ERO1A GCLC RTN4 STEAP4 UPF1

GO:0005975	carbohydrate metabolic process	0,0158	1,80	FAM3C FUCA2 GAAGSD GBE1 GCLC GLB1 GOT1 NANS PGM3 SCARB2 STAT3
GO:0010608	posttranscriptional regulation of gene expression	0,02861	1,54	CALR EIF3D EIF4E EIF4G1 EIF4G2 GSPT2 RPL22 RPS3 STAT3 UPF1

miRNAomics: *In vitro* impact of CLA, milk exosomes and CP on bovine, porcine and chicken miRNA profile

A miRNAomics analysis was also performed as an attempt to further elucidate the molecular impact of CLA, porcine milk exosomes and CP on bovine, porcine and chicken immunity, respectively. Specifically, with the present study we aim at evaluating the *in vitro* capacity of these molecules in modulating the animals' PBMC miRNAome. In this last section of the chapter, a first glance to the results of the bioinformatic analysis from the miRNA seq data is presented.

Material and Methods

Bovine, porcine and chicken PBMC isolation and stimulation with nutrition molecules

Firstly, PBMC from all three species were isolated and stimulated with the correspondent treatments as previously mentioned in the proteomics manuscripts (see Manuscripts 5,6 and 7 in pages 159, 212 and 255), with some minor modifications. Briefly, a total of 2 x 10^6 bovine, porcine and chicken PBMC (200 µL) were seeded in triplicates in sterile 6 well plates and incubated with 50 µM of CLA (50:50) mixture, 4 x 10^8 exosomes porcine milk exosomes (in a ratio of 200 exosomes/cell), and 0.5 mg/mL of CP, respectively. Complete medium (1% FBS) was then added to each well to reach a final volume of 2 mL. Bovine, porcine and chicken PBMC treated only with complete medium (1% FBS) (untreated cells) were used as controls. In addition, bovine PBMC incubated with 0.014% ethanol (vehicle) were also used as control as this is the same concentration of ethanol used for the solubilization of the two CLA isomers.

Bovine, porcine and chicken PBMC collection, miRNA extraction and miRNA Sequencing

The cells' supernatant (2 mL) that contain the cells in suspension (lymphocytes), was collected into tubes of 2 mL. Cells were centrifuged at 500 x g for 7 min at 4°C to pellet the lymphocytes, which were then washed with 500 µL of cold sterile-PBS without Ca^{2+} and Mg^{2+} and again centrifuged at 500 x g for 7 min at 4°C to remove the rest of medium and of FBS that could interfere with downstream analyses. In the meantime, three washes with 2 mL of cold sterile-PBS without Ca^{2+} and Mg^{2+} were performed in the 6 well plates to wash the adhered monocytes, and remove dead cells and rest of contaminant medium and FBS. When both, the lymphocytes and monocytes were washed, 1 mL of Qiazol

Lysis Reagent (Qiagen, Milan, Italy) was added directly to the 6 well plates containing the monocytes and cells were incubated for 5 min at room temperature. During the incubation with Qiazol, monocytes were scraped using sterile Corning Cell scrapers (blade L 1.8 cm, handle L 25 cm; Corning Inc.), and then the cell lysate was added into the 2 mL tubes containing the lymphocytes. The tubes were then vortexed quickly for further disruption and homogenization, and immediately stored at –80 °C for further RNA extraction. Finally, total RNA enriched with small RNA (miRNA) was extracted using miRNeasy Mini Kit from Qiagen, following the manufacturer's instructions. The quantity and quality of the extracted total RNA were assessed using a NanoDrop ND-1000 UV–vis spectrophotometer and Agilent Bioanalyzer 2100, respectively.

Libraries were prepared at the Center for Genomic Regulation (CRG) in Barcelona, Spain, using the NEBNext® Small RNA Library Prep Set for Illumina, following the manufacturer's protocol. Libraries were also sequenced at the CRG, using a HiSeq 2500 sequence analyzer (Illumina, San Diego, CA, USA), and 50 bp single reads were generated.

Bioinformatic analysis

miRNA seq data preparation, quantification and differential expression analysis. First, a quality control analysis of the reads was performed using the CLC Genomics Workbench software 20.0.4 (CLC Bio, Aarhus, Denmark). The adapter and low-quality sequences were trimmed from the raw sequences. Then, mature miRNA were annotated and quantified using miRbase v22, selecting the appropriate specie (*Bos taurus, Sus scrofa* and *Gallus gallus*) as reference. Finally, the differential expression analysis was performed between all the treatment groups by the Wald test (CLC Bio).The differentially expressed (DE) miRNA were selected as those with a p-value <0.05 and |log(FC)|>2.

Functional enrichment analysis: Gene Ontology (GO) analysis. First, the prediction of potential target genes of each DE miRNA was performed using targetscan (http://www.targetscan.org/). Second, the identified target genes were used to perform a Gene Ontology (GO) functional enrichment analysis. The three main GO categories (biological processes (BP), molecular function (MF), cellular compartment (CC)) were analyzed using the list of potential target genes as described previously (Cánovas et al., 2012). The R package WebgestaltR was used to perform an overrepresentation test using

the human annotation for GO terms using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database. A multiple testing correction based on a false-discovery ratio 5% (FDR 5%) was adopted to identify the enriched significant GO terms. Due to the absence of a *Gallus gallus* specific database, the enrichment analysis for GO terms was performed using the human database on WebgestaltR based on the gene symbols for each predicted target of the DE miRNA.

Results

Identification of differentially expressed (DE) miRNA

Differential expression analyses were first performed for each treatment group comparisons used in the cow, pig and chicken studies. Only miRNA with p-value <0.05 and |log(FC)|>2 were considered as DE.

In the cow study, 3 treatments groups were compared with each other: untreated vs CLA (UNT_CL), ethanol vs CLA (VEH_CL) and ethanol vs untreated (VEH_UNT). A total of 7 DE miRNA were identified in the UNT_CL comparison group, 5 in the VEH_CL, and 4 in the UNT_CL.
In the pig study only two treatments groups were compared: exosomes vs untreated (EXO_UNTR), and a total of 5 DE miRNA were identified.
Finally, in the chicken study only two treatments groups were compared: untreated vs citrus pectin (UNTR_CP), and a total of 3 DE miRNA were identified.

Gene Ontology (GO) enrichment analysis for potential target genes of DE miRNA

The DE miRNA identified in each treatment group comparison were first used for performing a target gene prediction analysis. Then, a GO functional analysis was carried out using those predicted target genes to elucidate which biological process (BP), molecular functions (MF) and cellular compartment (CC) associated with them were enriched.

In the cow study, the GO analyses for each group comparison: (1) UNT_CL, (2) VEH_CL and (3) VEH_UNT were performed. Table 1 shows the top 5 significant enriched GO terms for each GO category (BP, MF and CC) and for each comparison.

Table 1. Top five significant enriched gene ontology (GO) terms for the three main GO categories (biological processes (BP), molecular function (MF), cellular compartment (CC)) associated with the target genes for the DE miRNA identified in each comparison (untreated vs CLA (UNT_CL), ethanol vs CLA (VEH_CL) and untreated vs ethanol (VEH_UNT)).

GO Term	p-value	FDR	GO category	Comparison group
Regulation of anatomical structure morphogenesis	4.00E-15	1.87E-12	BP	UNT_CL
Tube morphogenesis	2.56E-13	5.98E-11	BP	UNT_CL
Actin filament-based process	3.42E-12	5.33E-10	BP	UNT_CL
Cellular component morphogenesis	1.07E-11	1.25E-09	BP	UNT_CL
Cardiovascular system development	3.59E-11	3.36E-09	BP	UNT_CL
Regulatory region nucleic acid binding	0	0	MF	UNT_CL
DNA-binding transcription factor activity, RNA polymerase II-specific	0	0	MF	UNT_CL
Sequence-specific double-stranded DNA binding	1.11E-16	6.18E-15	MF	UNT_CL
Transcription factor binding	2.21E-11	9.22E-10	MF	UNT_CL
Kinase binding	8.07E-11	2.70E-09	MF	UNT_CL
Synapse part	1.67E-05	0.0019	CC	UNT_CL
Cell junction	8.00E-05	0.0045	CC	UNT_CL
Transport vesicle	0.0002	0.0085	CC	UNT_CL
Endosome	0.0004	0.0085	CC	UNT_CL
Golgi apparatus part	0.0004	0.0085	CC	UNT_CL
Animal organ morphogenesis	1.17E-12	4.68E-10	BP	VEH_CL
Cellular component morphogenesis	2.00E-12	4.68E-10	BP	VEH_CL
Regulation of anatomical structure morphogenesis	6.68E-12	1.04E-09	BP	VEH_CL
Neuron development	3.37E-11	3.47E-09	BP	VEH_CL
Tube morphogenesis	3.71E-11	3.47E-09	BP	VEH_CL

DNA-binding transcription factor activity, RNA polymerase II-specific	0	0	MF	VEH_CL
Regulatory region nucleic acid binding	1.85E-13	1.54E-11	MF	VEH_CL
Sequence-specific double-stranded DNA binding	1.21E-12	6.73E-11	MF	VEH_CL
Kinase binding	7.14E-08	2.98E-06	MF	VEH_CL
Protein domain specific binding	2.14E-06	7.14E-05	MF	VEH_CL
Glutamatergic synapse	1.46E-06	0.0001	CC	VEH_CL
Synapse part	2.06E-06	0.0001	CC	VEH_CL
Cell junction	1.28E-05	0.0005	CC	VEH_CL
Cytoplasmic vesicle part	5.78E-05	0.0016	CC	VEH_CL
Endosome	9.91E-05	0.00227	CC	VEH_CL
Regulation of anatomical structure morphogenesis	2.44E-11	7.94E-09	BP	VEH_UNT
Negative regulation of transcription by RNA polymerase II	3.39E-11	7.94E-09	BP	VEH_UNT
Actin filament-based process	8.36E-10	1.30E-07	BP	VEH_UNT
Regulation of protein localization	1.36E-09	1.59E-07	BP	VEH_UNT
Tube morphogenesis	2.18E-09	2.04E-07	BP	VEH_UNT
DNA-binding transcription factor activity, RNA polymerase II-specific	0	0	MF	VEH_UNT
Sequence-specific double-stranded DNA binding	8.88E-16	6.18E-14	MF	VEH_UNT
Regulatory region nucleic acid binding	1.11E-15	6.18E-14	MF	VEH_UNT
Transcription factor binding	3.19E-09	1.33E-07	MF	VEH_UNT
Kinase binding	2.71E-07	9.04E-06	MF	VEH_UNT
Glutamatergic synapse	0.0001	0.0105	CC	VEH_UNT
Cell junction	0.0001	0.0105	CC	VEH_UNT
SNARE complex	0.0002	0.0107	CC	VEH_UNT
Transcription factor complex	0.0006	0.0169	CC	VEH_UNT
Transcriptional repressor complex	0.0007	0.0169	CC	VEH_UNT

In the pig study, a GO analysis for the EXO_UNTR comparison was performed. Table 2 shows the top 5 significant enriched GO terms for each GO category (BP, MF and CC).

Table 2. Top five significant enriched gene ontology (GO) terms for the three main GO categories (biological processes (BP), molecular function (MF) and cellular compartment (CC)) associated with the target genes for the DE miRNA identified for the comparison between exosomes and untreated group (EXO_UNTR).

GO Term	p-value	FDR	GO category	Comparison group
Ras protein signal transduction	1.85E-13	1.57E-10	BP	EXO_UNTR
Positive regulation of neurogenesis	2.87E-11	1.22E-08	BP	EXO_UNTR
Cell-substrate adhesion	6.81E-11	1.93E-08	BP	EXO_UNTR
Regulation of neuron projection development	9.41E-11	2.00E-08	BP	EXO_UNTR
Regulation of cell morphogenesis	1.46E-10	2.48E-08	BP	EXO_UNTR
Synaptic membrane	1.10E-08	1.90E-06	CC	EXO_UNTR
Glutamatergic synapse	1.68E-07	1.44E-05	CC	EXO_UNTR
Early endosome	2.54E-06	0.00014575	CC	EXO_UNTR
Presynapse	3.77E-06	0.000162	CC	EXO_UNTR
Neuron to neuron synapse	5.45E-06	0.00018732	CC	EXO_UNTR
Metal ion transmembrane transporter activity	5.88E-08	1.66E-05	MF	EXO_UNTR
Transmembrane receptor protein kinase activity	6.06E-07	8.55E-05	MF	EXO_UNTR
Beta-catenin binding	3.90E-06	0.00032615	MF	EXO_UNTR
Guanyl-nucleotide exchange factor activity	5.65E-06	0.00032615	MF	EXO_UNTR
Protein serine/threonine kinase activity	5.78E-06	0.00032615	MF	EXO_UNTR

Finally, in the chicken study, a GO analysis for the UNTR_CP comparison was performed. Table 3 shows the top 5 significant enriched GO terms for each GO category (BP, MF and CC).

Table 3. Top five significant enriched gene ontology (GO) terms for the three main GO categories (biological processes (BP), molecular function (MF), cellular compartment

(CC)) associated with the target genes for the DE miRNA identified between untreated and Citrus Pectin (UNTR_CP) groups.

GO Term	p-value	FDR	GO category	Comparison group
Axon development	3.3E-12	2.8E-09	BP	UNTR_CP
Cell-cell adhesion via plasma-membrane adhesion molecules	8.7E-11	3.7E-08	BP	UNTR_CP
Dendrite development	4.8E-09	1.4E-06	BP	UNTR_CP
Regulation of cell morphogenesis	8.1E-09	1.6E-06	BP	UNTR_CP
Cell-cell signaling by wnt	9.3E-09	1.6E-06	BP	UNTR_CP
SMAD binding	9.0E-08	2.5E-05	MF	UNTR_CP
DNA-binding transcription repressor activity, RNA polymerase II-specific	1.9E-05	2.7E-03	MF	UNTR_CP
Transcription corepressor activity	5.4E-05	5.1E-03	MF	UNTR_CP
Protein serine/threonine/tyrosine kinase activity	8.3E-05	5.9E-03	MF	UNTR_CP
PDZ domain binding	1.9E-04	7.9E-03	MF	UNTR_CP
Synaptic membrane	2.6E-10	4.4E-08	CC	UNTR_CP
Glutamatergic synapse	3.7E-08	3.1E-06	CC	UNTR_CP
Neuron spine	2.9E-07	1.3E-05	CC	UNTR_CP
Neuron to neuron synapse	3.0E-07	1.3E-05	CC	UNTR_CP
Axon part	5.6E-07	1.9E-05	CC	UNTR_CP

Chapter 9. General discussion and conclusions

The findings of this thesis provide for the first time an overview of the *in vitro* activity of CLA, milk exosomes and n-3 PUFA, and CP on bovine, porcine and chicken immune response, respectively. Moreover, to elucidate the overall molecular impact of these molecules on both, mammalian and avian immune defense, a system biology approach was implemented by performing proteomics and miRNAomics analyses.

Livestock species such as cattle, pigs and chickens are the main source of animal protein worldwide (Gilbert et al., 2018). The increase in the world population and demand for their dietary products has made necessary to improve the animals' productivity and product quality, which often could jeopardize their health and welfare (Scholten et al., 2013). Specifically, the rise in infectious diseases, and the indiscriminate use of antibiotics have affected the animals' productivity and health (Hafez and Attia, 2020). One way to overcome these challenges is by improving the animals' immunity, and nutrition has been demonstrated to be a valuable and effective tool to do so (Field et al., 2002; Wu et al., 2019; Vlasova and Saif, 2021).

The overall characterization of the effects of commonly used and novel dietary molecules on mammalian and avian immunity could be of great help for a better understanding of the molecules' practical biological significance *in vivo*, and thus on how to include them efficiently in animal nutrition. Therefore, in the present thesis, through *in vitro* functional analyses it was first investigated the impact of CLA, porcine milk exosomes and n-3 PUFA, and CP, on bovine, porcine and chicken monocytes' immune activities (e.g. chemotaxis, ROS production, phagocytosis, and killing capability), respectively. Second, proteomics and miRNAomics analyses were performed to further elucidate the molecular mechanisms underlying the effects observed *in vitro*.

CLA has been routinely used in dairy cows' nutrition due to its beneficial *in vivo* effects (Selberg et al., 2004; de Veth et al., 2009; Galamb et al., 2017). A mixture of the two main CLA isomers in equal proportions (50:50) (c9, t11 and t10, c12) is used in most of the animal studies reporting CLA benefits, and in the commercially available CLA supplements. Indeed, synergic and enhanced effects (50:50) are often observed when this mixture is utilized (Pariza et al., 2001; Song et al., 2005; Renner et al., 2012).

As there is limited information on the effects of CLA on bovine immunity, in this thesis the effects of the two main CLA isomers, both individually and in the mixture (50:50),

on porcine monocytes' immune activities were investigated. The main findings of this study were that only the 50:50 CLA mixture, when used at a concentration of 50 μM, reduced the apoptosis of monocytes, and increased their ROS production under a pro-inflammatory environment (PMA-challenge). The anti-apoptotic effect of CLA has already been reported in bovine aortic endothelial cells (Lai et al., 2005). Additionally, in previous studies, CLA has already been demonstrated to increase the production of ROS in several cell types and species, supporting the results observed in this study (Stachowska et al., 2008; Dipasquale et al., 2018). It is thought that CLA induces ROS in a PPARγ-dependent manner (Stachowska et al., 2008). Altogether, these results suggest a potential anti-apoptotic, and immunomodulatory activity of the CLA mixture (50:50) on bovine monocytes. The results also make evident that the CLA (50:50) mixture, at least in our *in vitro* model, is more effective than the CLA isomers individually, possibly due to their synergic effects.

To further elucidate the overall *in vitro* impact of CLA (50:50) mixture on bovine mononuclear cells (PBMC) immune response, and specifically identify the underlying molecular mechanisms of its' immunomodulatory activity, an untargeted proteomics analysis was performed. The proteomic results revealed for the first time the capacity of the CLA (50:50) mixture in modulating bovine PBMC proteome and proposed the molecular background of CLA anti-apoptotic and immunomodulatory activities. The main findings were that CLA (50:50) mixture caused an enrichment in biological processes related to negative regulation of proteolysis and endopeptidases activity, and in immune-related processes such as acute-phase response, complement activation, response to oxidative stress and ROS metabolism.

Proteolysis is essential for many cell functions, including apoptosis (Combaret et al., 2016). Indeed, the proteolytic cleavage of specific cellular proteins is a recognized central biochemical feature of apoptosis (Elmore, 2007). Besides the caspases, which are the main proteolytic executioner enzymes of apoptosis, other proteases such as serine endopeptidases are known to modulate apoptosis (Utz and Anderson, 2000; Moffitt et al., 2007; O'Connell and Stenson-Cox, 2007). Remarkably, proteins with serine endopeptidases inhibitor activities, and known for their anti-apoptotic effects (e.g. SERPINA3-7 and ITIH4) were found annotated in those biological processes, suggesting a potential anti-apoptotic role of CLA (50:50) mixture (Hwang et al., 2002, 2009; Herrera-Mendez et al., 2009; Chen et al., 2021). Moreover, proteins with anti-oxidant effects (e.g. HMOX1 and HMOX2), and involved in the host innate immune defense (C3,

C4A, agp and A2M) were found annotated in the other enriched biological immune-related processes. Thus, these results suggest a potential role of CLA (50:50) mixture in modulating bovine immune defense and in exerting potential cytoprotective effects against oxidative stress, by increasing the abundance of proteins with anti-oxidant capacity. In conclusion, these results support the anti-apoptotic and immunomodulatory effects of the CLA (50:50) mixture observed in our previous *in vitro* study on bovine monocytes.

Exosomes are important mediators of intercellular communication and they have been shown to modulate both innate and adaptive immune responses (Raposo et al., 1996; Dalvi et al., 2017). Milk exosomes seem to play an important role in the transmission of immunity from the mother to the offspring, by transferring immunoregulatory molecules (e.g. miRNA), and in the development of the newborn immune system and growth (Zhou et al., 2012). Indeed, milk exosomes, containing functional miRNA, have shown to be taken up by immune cells such as macrophages (Lässer et al., 2011; Arntz et al., 2015; Izumi et al., 2015). Specifically, porcine milk exosomes have enhanced porcine intestinal epithelial cells proliferation *in vitro* and *in vivo* in mice (Chen et al., 2016), but their effects on porcine monocytes' immune response have been so far unexplored. Therefore, this study investigated the *in vitro* uptake of porcine milk exosomes by porcine monocytes and their influence on the monocyte immune functions. This study reported for the first time the *in vitro* uptake of porcine milk exosomes, and the ability of these exosomes in modulating porcine monocytes' chemotaxis, phagocytosis and ROS production. Specifically, porcine milk exosomes decreased the cells' chemotaxis and phagocytosis, while increased their ROS production under both, resting (non-inflammatory) and pro-inflammatory conditions. The immunosuppressive and anti-inflammatory roles of milk exosomes and their miRNA cargo have been already demonstrated in previous studies (Ahn et al., 2021; Melnik et al., 2021). Specifically, studies in other models reported inhibitory effects of exosomes on human and murine macrophages migration (Nguyen et al., 2018); and of milk exosomes on murine macrophages phagocytosis (Zhu et al., 2014). On the other hand, exosomes from different sources have also been shown to boost some immune functions such as ROS production in other cell models (Cañas et al., 2017; Hervera et al., 2018). Altogether, these results suggest that porcine milk exosomes can exert pleiotropic functions on porcine monocytes, as both immunosuppressive and/ or immune-enhancing effects were observed. Finally, our results also suggest a potentially

critical role of porcine milk exosomes in the sow-to-piglet transmission of regulatory molecules and immunomodulation.

The proteomics study of porcine PBMC demonstrated for the first time that porcine milk exosomes can modulate porcine PBMC proteome *in vitro* and it provided a molecular signature of their immunomodulatory activity. Our main findings were that porcine milk exosomes caused an enrichment in biological processes related to different immune system processes, such as response to diacyl bacterial lipopeptide, nitric oxide-mediated signal transduction, positive regulation of tumor necrosis factor and phagocytosis, suggesting an immunomodulatory role of porcine milk exosomes. An enrichment in processes related to exosome internalization or uptake by immune cells, including phagocytosis and endocytosis was also observed. Moreover, proteins like TLR2 and CD36 were found to be annotated in those enriched biological processes. It has been previously demonstrated that exosomes can modulate the innate immune response through the interaction with TLR (Guo et al., 2020). In fact, in a previous study, exosomes also upregulated the expression of TLR2 in astrocytes, triggering the cells' inflammatory responses (Pan et al., 2021). The production of TNF and NO are two processes known to be dependent as well on the TLR signalling pathway (Lai and Gallo, 2008), which suggest that the immunomodulation caused by milk exosomes could be mediated mainly in a TLR2 dependent manner. CD36, besides being a co-receptor of TLR2 is a member of the class B scavenger receptor family (Triantafilou et al., 2006). It is known to be involved in phagocytosis, endocytosis, migration and ROS production (Baranova et al., 2008; Collins et al., 2009; Park et al., 2009; Silverstein and Febbraio, 2009). Remarkably, a previous study, reported that extracellular vesicles (EV), including exosomes, inhibited endothelial cells migration and increased ROS production via the CD36-mediated pathway (Ramakrishnan et al., 2016). Lastly, the abundance of LGALS3, which is a strong regulator of cell migration and phagocytosis, was found to be decreased in the milk exosomes group (Lu et al., 2017; Brinchmann et al., 2018). Altogether, these results support our previous *in vitro* study, where porcine milk exosomes caused an increase in the ROS production and an inhibition of the chemotaxis and phagocytosis of porcine monocytes. In conclusion, this study demonstrated that porcine milk exosomes might exert immunomodulation on porcine PBMC by upregulating innate immune processes that are mainly mediated in a TLR2 dependent manner, and processes that allow their uptake by immune cells. In addition, these results also support the pleiotropic

immunomodulatory functions of porcine milk exosomes, as they increased the abundance of proteins with both, immune-enhancing and dampening properties.

The immunomodulatory and anti-inflammatory effects of the n-3 PUFA, EPA and DHA, have been widely reported in both human and animal studies (Agazzi et al., 2004; Bronzo et al., 2010; Calder, 2017; Al-Khalaifah, 2020). However, no information on their *in vitro* effects on porcine monocytes' defensive functions is available yet. Thus, in this last study, the *in vitro* effects of n-3 PUFA (EPA and DHA) on porcine monocytes' immune functions were investigated. The *in vitro* functional analyses demonstrated for the first time the capacity of DHA and EPA in modulating two main porcine monocytes' inflammatory functions, namely chemotaxis and intracellular ROS production. The main findings were that the cells' chemotaxis was suppressed by DHA, and their intracellular ROS production was increased by EPA under both, resting (non-inflammatory) and pro-inflammatory conditions (PMA-challenge). The ability of DHA and EPA in suppressing immune cells migration has been widely documented in other models such as in human neutrophils and monocytes (Schmidt et al., 1992), being indeed one of the key anti-inflammatory effects of these n-3 PUFA (Calder, 2017). Although, the exact mechanisms by which DHA and EPA inhibit chemotaxis are not fully clear yet, its inhibition has been attributed to a reduction in the expression of receptors for chemoattractants, the production of some chemoattractants and the expression of adhesion molecule genes (Calder, 2015). Additionally, the production of SPM and reduction in the cells' membrane fluidity after their incorporation have also been proposed as inhibitory mechanisms (Sipka et al., 1996; Serhan et al., 2000). PUFA are also considered a substantial source of ROS (Schönfeld and Wojtczak, 2008). Indeed, previous studies demonstrated that EPA could increase the *in vitro* production of intracellular and extracellular ROS in rat neutrophils; and also in LPS-stimulated murine macrophages, it increased the total ROS production (Ambrozova et al., 2010; Paschoal et al., 2013). The exact mechanisms underlying these effects are still not completely clarified, but it is thought they could up-regulate ROS production via NADPH oxidase (Paschoal et al., 2013). In conclusion, these results suggest that long-chain n-3 PUFA can exert anti-inflammatory, inflammation resolving, and/or immune-enhancing effects in pigs, and that EPA and DHA exert differential immunomodulatory effects that might also vary between species and cell types. These properties make them quite attractive to be used as dietary integrators to improve the overall health and immune status of the animals. Finally, proteomics and miRNAomics analyses were not carried out for this study. Thus, our study does not

provide evidence on the exact molecular mechanisms underlying such immunomodulatory effects. However, it is recognized that their elucidation using a system biology approach, including miRNAomics and proteomic analyses, could help to have an integral characterization of the potential impact of these molecules on pigs' immunity.

Citrus pectin is a dietary fibre that has been widely used in animal and human nutrition (Langhout and Schutte, 1996; Leclere et al., 2013), as it has been shown to exert beneficial immunomodulatory and anti-inflammatory effects (Chen et al., 2006a; Salman et al., 2008; Sahasrabudhe et al., 2018; Beukema et al., 2020). However, scarce information on the effects of CP on chicken immunity is available so far. Therefore, this thesis aimed at covering this gap, by evaluating for the first time the *in vitro* effects of CP on two main inflammatory functions of chicken monocytes, namely chemotaxis and phagocytosis. This study reported for the first time that CP (0.5 mg/mL) suppresses both, chicken monocytes chemotactic and phagocytic capacity *in vitro*, suggesting a potential anti-inflammatory activity of CP. These results are consistent with those reported in previous studies using different cell models, where CP and ginseng pectin inhibited the migration of cancer cells and murine fibroblasts, respectively (Fan et al., 2018; do Prado et al., 2019). It is thought that CP, among other pectins, exert suppressive and anti-inflammatory effects by changing the cells' morphology and organization of actin filaments, and/or by inhibiting PRR found in monocytes and macrophages such as TLR and galectin-3 (Nangia-Makker et al., 2002; Fan et al., 2018; Sahasrabudhe et al., 2018). Altogether, these results indicate that CP may play an essential role in the chickens' immunity, specifically in dampening inflammation. However, is important to highlight that the potential anti-inflammatory functions of CP could be beneficial for animals that are under a pro-inflammatory status, but an excess in CP content in their diets could induce an immunosuppressive status, increasing the risk of developing opportunistic diseases. Finally, having this knowledge is of great value as it would allow the design of adequate nutrition strategies to enhance the animals' health.

An untargeted proteomic analysis was also carried out on chicken PBMC. This study described for the first the proteome of chicken mononuclear cells after stimulation with CP and revealed that CP does modulate chicken PBMC protein profile. Our main findings were that CP caused an enrichment in biological processes related to, and fundamental for the cells' immune response such as actin cytoskeleton organization, negative regulation of cell migration, intracellular signal transduction and cell communication.

The actin cytoskeleton is critical for providing the cells with the mechanical structure to support their changes in shape and membrane (Rivero et al., 1996). Upon activation, the immune cells need to change their morphology to carry out their defensive functions such as phagocytosis and chemotaxis. Indeed, during phagocytosis, the actin cytoskeleton drives the formation of phagosomes to engulf foreign particles, while in chemotaxis it allows the cells to acquire the proper morphology and orientation of the cellular contents towards the chemoattractant (May and Machesky, 2001; Affolter and Weijer, 2005). The actin cytoskeleton is also necessary for immune cells communication (Roy and Burkhardt, 2018). Moreover, proteins such as MARCKSL1 and SLC9A3R1, known to restrict cell movement and migration, were among the discriminant proteins with the highest abundance in the CP group. On the contrary, proteins such as MARCKS, LGALS3 and LGALS8, all involved with cell migration, phagocytosis and other actin cytoskeleton organization-based processes were found with the lowest abundance in the CP group. These results support the suppressive effects observed in our previous *in vitro* study on chicken monocytes' phagocytosis and chemotaxis. In conclusion, this study provides a molecular background to the anti-inflammatory activity of CP, demonstrating that the *in vitro* downregulation of phagocytosis and chemotaxis is mainly caused by changes in the abundance of proteins related to the cytoskeleton.

The miRNAomics analyses concerning the bovine, porcine and chicken studies, in porcine only considering the impact of milk exosomes, have been carried out and finalized. Indeed, a first glance to the results of the GO functional enrichment analyses was presented herein. Those results mostly showed the identification of the main enriched biological processes, molecular functions and cellular compartments associated with the target genes of the identified DE miRNA. However, further bioinformatic analyses and research are necessary, and must be performed for an appropriate and more in depth biological interpretation of these results.

To conclude, this thesis tried to cover, at least in part, the gap in knowledge concerning the *in vitro* effects of CLA, porcine milk exosomes and n-3 PUFA, and CP on bovine, porcine and chicken immune response, respectively. The results of this thesis will hopefully provide data that would increase the knowledge of the potential immunomodulatory effects of these dietary molecules in *in vivo* systems. Specifically, by providing both, phenotypical and molecular data coming from *in vitro* studies, we aspire to provide an integral and holistic view of these molecules' practical biological

significance. Consequently, this information could be useful for the design of targeted and balanced nutrition strategies to improve the animals' immunity, health and welfare. The future perspectives of this thesis include the finalization of the bioinformatic analyses from the miRNAomics data of all three species, and the integration of the results coming from both OMIC approaches with the *in vitro* data. It is expected that by doing this, relevant functional information would be retrieved, including novel molecular networks and pathways, which could allow a better understanding and prediction of the immunomodulatory effects of these molecules. Finally, it is important to highlight that these results should be considered cautiously, as they just represent the first step into the complete elucidation of the molecule's impact on mammalian and avian immunity. Therefore, to have a systems biology perspective, it would be greatly valuable and necessary to further integrate the data not only coming from proteomics and miRNAomics, but also from *in vivo* studies.

References

Ablondi, M., M. Malacarne, C. Cipolat-Gotet, J.-T. van Kaam, A. Sabbioni, and A. Summer. 2021. Genome-wide scan reveals genetic divergence in Italian Holstein cows bred within PDO cheese production chains. Sci. Rep. 11:12601. doi:10.1038/s41598-021-92168-1.

Admyre, C., S.M. Johansson, K.R. Qazi, J.-J. Filén, R. Lahesmaa, M. Norman, E.P.A. Neve, A. Scheynius, and S. Gabrielsson. 2007. Exosomes with Immune Modulatory Features Are Present in Human Breast Milk. J. Immunol. 179:1969 LP – 1978.

Aebersold, R., and M. Mann. 2016. Mass-spectrometric exploration of proteome structure and function. Nature 537:347–355. doi:10.1038/nature19949.

Affolter, M., and C.J. Weijer. 2005. Signaling to Cytoskeletal Dynamics during Chemotaxis. Dev. Cell 9:19–34. doi:https://doi.org/10.1016/j.devcel.2005.06.003.

Agazzi, A., D. Cattaneo, V. Dell'Orto, P. Moroni, L. Bonizzi, D. Pasotto, V. Bronzo, and G. Savoini. 2004. Effect of administration of fish oil on aspects of cell-mediated immune response in periparturient dairy goats. Small Rumin. Res. 55:77–83.

Ahn, G., Y.-H. Kim, and J.-Y. Ahn. 2021. Multifaceted effects of milk-exosomes (Mi-Exo) as a modulator of scar-free wound healing. Nanoscale Adv. 3:528–537. doi:10.1039/D0NA00665C.

Al-Khalaifah, H. 2020. Modulatory Effect of Dietary Polyunsaturated Fatty Acids on

Immunity, Represented by Phagocytic Activity. Front. Vet. Sci. 7:569939. doi:10.3389/fvets.2020.569939.

Alqazlan, N., M. Emam, É. Nagy, B. Bridle, M. Sargolzaei, and S. Sharif. 2021. Transcriptomics of chicken cecal tonsils and intestine after infection with low pathogenic avian influenza virus H9N2. Sci. Rep. 11:20462. doi:10.1038/s41598-021-99182-3.

Ambrozova, G., M. Pekarova, and A. Lojek. 2010. Effect of polyunsaturated fatty acids on the reactive oxygen and nitrogen species production by raw 264.7 macrophages. Eur. J. Nutr. 49:133–139. doi:10.1007/s00394-009-0057-3.

Anderson, J.W., P. Baird, R.H. Davis Jr, S. Ferreri, M. Knudtson, A. Koraym, V. Waters, and C.L. Williams. 2009. Health benefits of dietary fiber. Nutr. Rev. 67:188–205. doi:10.1111/j.1753-4887.2009.00189.x.

Arndt, C., A. Feldmann, M. Von Bonin, M. Cartellieri, E.M. Ewen, S. Koristka, I. Michalk, S. Stamova, N. Berndt, A. Gocht, M. Bornhäuser, G. Ehninger, M. Schmitz, and M. Bachmann. 2014. Costimulation improves the killing capability of T cells redirected to tumor cells expressing low levels of CD33: Description of a novel modular targeting system. Leukemia 28:59–69. doi:10.1038/leu.2013.243.

Arntz, O.J., B.C.H. Pieters, M.C. Oliveira, M.G.A. Broeren, M.B. Bennink, M. de Vries, P.L.E.M. van Lent, M.I. Koenders, W.B. van den Berg, P.M. van der Kraan, and F.A.J. van de Loo. 2015. Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models. Mol. Nutr. Food Res. 59:1701–1712. doi:https://doi.org/10.1002/mnfr.201500222.

Ascanius, S.R., M.S. Hansen, M.S. Ostenfeld, and J.T. Rasmussen. 2021. Milk-Derived Extracellular Vesicles Suppress Inflammatory Cytokine Expression and Nuclear Factor-κB Activation in Lipopolysaccharide-Stimulated Macrophages. Dairy 2:165–178.

Auray, G., M.R. Facci, J. van Kessel, R. Buchanan, L.A. Babiuk, and V. Gerdts. 2010. Differential activation and maturation of two porcine DC populations following TLR ligand stimulation. Mol. Immunol. 47:2103–2111. doi:https://doi.org/10.1016/j.molimm.2010.03.016.

Ávila, G., D. De Leonardis, G. Grilli, C. Lecchi, and F. Ceciliani. 2021. Anti-inflammatory activity of citrus pectin on chicken monocytes' immune response. Vet. Immunol. Immunopathol. 237:110269. doi:https://doi.org/10.1016/j.vetimm.2021.110269.

Badimon, L., G. Vilahur, and T. Padro. 2017. Systems biology approaches to understand the effects of nutrition and promote health. Br. J. Clin. Pharmacol. 83:38–45. doi:https://doi.org/10.1111/bcp.12965.

Baranova, I.N., R. Kurlander, A. V Bocharov, T.G. Vishnyakova, Z. Chen, A.T. Remaley, G. Csako, A.P. Patterson, and T.L. Eggerman. 2008. Role of human CD36 in bacterial recognition, phagocytosis, and pathogen-induced JNK-mediated signaling. J. Immunol. 181:7147–7156. doi:10.4049/jimmunol.181.10.7147.

Becker, A., B.K. Thakur, J.M. Weiss, H.S. Kim, H. Peinado, and D. Lyden. 2016. Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis. Cancer Cell 30:836–848. doi:10.1016/j.ccell.2016.10.009.

Bedouhène, S., F. Moulti-Mati, M. Hurtado-Nedelec, P. My-Chan Dang, and J. El-Benna. 2017. Luminol-amplified chemiluminescence detects mainly superoxide anion produced by human neutrophils. Am J Blood Res 7:41–48.

Befus, A.D., N. Johnston, G.A. Leslie, and J. Bienenstock. 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches.. J. Immunol. 125:2626–2632.

Bennett, C.E., R. Thomas, M. Williams, J. Zalasiewicz, M. Edgeworth, H. Miller, B. Coles, A. Foster, E.J. Burton, and U. Marume. 2022. The broiler chicken as a signal of a human reconfigured biosphere. R. Soc. Open Sci. 5:180325. doi:10.1098/rsos.180325.

Beukema, M., M.M. Faas, and P. de Vos. 2020. The effects of different dietary fiber pectin structures on the gastrointestinal immune barrier: impact via gut microbiota and direct effects on immune cells. Exp. Mol. Med. 52:1364–1376. doi:10.1038/s12276-020-0449-2.

Bevins, C.L., and N.H. Salzman. 2011. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat. Rev. Microbiol. 9:356–368. doi:10.1038/nrmicro2546.

Bhattacharya, A., J. Banu, M. Rahman, J. Causey, and G. Fernandes. 2006. Biological effects of conjugated linoleic acids in health and disease. J. Nutr. Biochem. 17:789–810. doi:https://doi.org/10.1016/j.jnutbio.2006.02.009.

Bobeck, E.A. 2020. NUTRITION AND HEALTH: COMPANION ANIMAL APPLICATIONS: Functional nutrition in livestock and companion animals to modulate the immune response. J. Anim. Sci. 98:skaa035. doi:10.1093/jas/skaa035.

Bonilla, F.A., and H.C. Oettgen. 2010. Adaptive immunity. J. Allergy Clin. Immunol.

125:S33–S40. doi:10.1016/j.jaci.2009.09.017.

Bonnardel, J., C. Da Silva, S. Henri, S. Tamoutounour, L. Chasson, F. Montañana-Sanchis, J.-P. Gorvel, and H. Lelouard. 2015. Innate and Adaptive Immune Functions of Peyer’s Patch Monocyte-Derived Cells. Cell Rep. 11:770–784. doi:10.1016/j.celrep.2015.03.067.

Borràs, E., and E. Sabidó. 2017. What is targeted proteomics? A concise revision of targeted acquisition and targeted data analysis in mass spectrometry. Proteomics 17:1700180. doi:https://doi.org/10.1002/pmic.201700180.

Brand, K. 1985. Glutamine and glucose metabolism during thymocyte proliferation. Pathways of glutamine and glutamate metabolism. Biochem. J. 228:353–361. doi:10.1042/bj2280353.

Brinchmann, M.F., D.M. Patel, and M.H. Iversen. 2018. The Role of Galectins as Modulators of Metabolism and Inflammation. Mediators Inflamm. 2018:9186940. doi:10.1155/2018/9186940.

Bronzo, V., M. Puricelli, A. Agazzi, G. Invernizzi, M. Ferroni, P. Moroni, and G. Savoini. 2010. Effects of protected fish oil in the diet of periparturient dairy goats on phenotypic variation in blood and milk leukocytes. Animal 4:1510–1517. doi:https://doi.org/10.1017/S1751731110000522.

Brucklacher-Waldert, V., E.J. Carr, M.A. Linterman, and M. Veldhoen. 2014. Cellular Plasticity of CD4+ T Cells in the Intestine . Front. Immunol. 5.

Calder, P.C. 2007. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot. Essent. Fat. Acids 77:327–335. doi:10.1016/j.plefa.2007.10.015.

Calder, P.C. 2012. Long-chain fatty acids and inflammation. Proc. Nutr. Soc. 71:284–289. doi:DOI: 10.1017/S0029665112000067.

Calder, P.C. 2013. Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology?. Br. J. Clin. Pharmacol. 75:645–662. doi:10.1111/j.1365-2125.2012.04374.x.

Calder, P.C. 2015. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1851:469–484. doi:https://doi.org/10.1016/j.bbalip.2014.08.010.

Calder, P.C. 2017. Omega-3 fatty acids and inflammatory processes: from molecules to man. Biochem. Soc. Trans. 45:1105–1115. doi:10.1042/BST20160474.

Calder, P.C., and S. Kew. 2002. The immune system: a target for functional foods?. Br. J. Nutr. 88:S165. doi:10.1079/BJN2002682.

Calder, P.C., and P. Yaqoob. 2009. Understanding Omega-3 Polyunsaturated Fatty Acids. Postgrad. Med. 121:148–157. doi:10.3810/pgm.2009.11.2083.

Campbell, P.A., B.P. Canono, and D.A. Drevets. 1994. Measurement of bacterial ingestion and killing by macrophages. Curr. Protoc. Immunol. 12:14–16.

Cañas, J.A., B. Sastre, C. Mazzeo, M. Fernández-Nieto, J.M. Rodrigo-Muñoz, A. González-Guerra, M. Izquierdo, P. Barranco, S. Quirce, J. Sastre, and V. del Pozo. 2017. Exosomes from eosinophils autoregulate and promote eosinophil functions. J. Leukoc. Biol. 101:1191–1199. doi:10.1189/jlb.3AB0516-233RR.

Cánovas, A., R.N. Pena, D. Gallardo, O. Ramírez, M. Amills, and R. Quintanilla. 2012. Segregation of Regulatory Polymorphisms with Effects on the Gluteus Medius Transcriptome in a Purebred Pig Population. PLoS One 7:e35583.

Casteleyn, C., M. Doom, E. Lambrechts, W. Van den Broeck, P. Simoens, and P. Cornillie. 2010. Locations of gut-associated lymphoid tissue in the 3-month-old chicken: a review. Avian Pathol. 39:143–150. doi:10.1080/03079451003786105.

De Caterina, R. 2011. n–3 Fatty Acids in Cardiovascular Disease. N. Engl. J. Med. 364:2439–2450. doi:10.1056/NEJMra1008153.

Ceciliani, F., G. Ávila Morales, G. De Matteis, F. Grandoni, R. Furioso Ferreira, P. Roccabianca, and C. Lecchi. 2021. Methods in isolation and characterization of bovine monocytes and macrophages. Methods 186:22–41. doi:https://doi.org/10.1016/j.ymeth.2020.06.017.

Ceciliani, F., V. Pocacqua, A. Miranda-Ribera, V. Bronzo, C. Lecchi, and P. Sartorelli. 2007. α1-Acid glycoprotein modulates apoptosis in bovine monocytes. Vet. Immunol. Immunopathol. 116:145–152. doi:10.1016/j.vetimm.2007.01.006.

Chang, H.Y., H.-N. Lee, W. Kim, and Y.-J. Surh. 2015. Docosahexaenoic acid induces M2 macrophage polarization through peroxisome proliferator-activated receptor γ activation. Life Sci. 120:39–47. doi:https://doi.org/10.1016/j.lfs.2014.10.014.

Chaplin, D.D. 2010. Overview of immune response. J Allergy Clin Immunol. 125:41. doi:10.1016/j.jaci.2009.12.980.Overview.

Chaput, N., and C. Théry. 2011. Exosomes: Immune properties and potential clinical implementations. Semin. Immunopathol. 33:419–440. doi:10.1007/s00281-010-0233-9.

Chase, C.C.L. 2018. Enteric Immunity: Happy Gut, Healthy Animal. Vet. Clin. North Am. - Food Anim. Pract. 34:1–18. doi:10.1016/j.cvfa.2017.10.006.

Chávez-galán, L., M.L. Olleros, D. Vesin, I. Garcia, and R.A. Harris. 2015. Much more

than M1 and M2 macrophages , there are also CD169 + and TCR + macrophages 6:1–15. doi:10.3389/fimmu.2015.00263.

Chen, C.-H., M.-T. Sheu, T.-F. Chen, Y.-C. Wang, W.-C. Hou, D.-Z. Liu, T.-C. Chung, and Y.-C. Liang. 2006a. Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways. Biochem. Pharmacol. 72:1001–1009. doi:https://doi.org/10.1016/j.bcp.2006.07.001.

Chen, C.H., M.T. Sheu, T.F. Chen, Y.C. Wang, W.C. Hou, D.Z. Liu, T.C. Chung, and Y.C. Liang. 2006b. Suppression of endotoxin-induced proinflammatory responses by citrus pectin through blocking LPS signaling pathways. Biochem. Pharmacol. 72:1001–1009. doi:10.1016/j.bcp.2006.07.001.

Chen, G.Y., and G. Nuñez. 2010. Sterile inflammation: sensing and reacting to damage. Nat. Rev. Immunol. 10:826–837. doi:10.1038/nri2873.

Chen, T., M.Y. Xie, J.J. Sun, R.S. Ye, X. Cheng, R.P. Sun, L.M. Wei, M. Li, D.L. Lin, Q.Y. Jiang, Q.Y. Xi, and Y.L. Zhang. 2016. Porcine milk-derived exosomes promote proliferation of intestinal epithelial cells. Sci. Rep. 6:1–12. doi:10.1038/srep33862.

Chen, X.-Y., P.-H. Feng, C.-L. Han, Y.-T. Jheng, C.-D. Wu, H.-C. Chou, Y.-Y. Chen, S.-M. Wu, K.-Y. Lee, H.-P. Kuo, K.F. Chung, T.-C. Hsiao, K.-Y. Chen, S.-C. Ho, T.-Y. Chang, and H.-C. Chuang. 2021. Alveolar epithelial inter-alpha-trypsin inhibitor heavy chain 4 deficiency associated with senescence-regulated apoptosis by air pollution. Environ. Pollut. 278:116863. doi:https://doi.org/10.1016/j.envpol.2021.116863.

Chew, B.P., and J.S. Park. 2004. Carotenoid action on the immune response. J. Nutr. 134:257S-261S.

Chiang, N., and C.N. Serhan. 2020. Specialized pro-resolving mediator network: an update on production and actions. Essays Biochem. 64:443–462. doi:10.1042/EBC20200018.

Chinnadurai, K. 2011. Conjugated Linoleic Acid: A Milk Fatty Acid with Unique Health Benefit Properties. A.T.E.-H. El-Shemy, ed. IntechOpen, Rijeka.

Churruca, I., A. Fernández-Quintela, and M.P. Portillo. 2009. Conjugated linoleic acid isomers: Differences in metabolism and biological effects. BioFactors 35:105–111. doi:https://doi.org/10.1002/biof.13.

Cohen, I.R. 2000. Discrimination and dialogue in the immune system. Pages 215–219 in Seminars in immunology. Elsevier.

Coker, M.O., and J.C. Madan. 2020. Chapter 3 - The microbiome and immune system

development. E.C.B.T.-T.D.M. Claud, ed. Academic Press.

Collins, R.F., N. Touret, H. Kuwata, N.N. Tandon, S. Grinstein, and W.S. Trimble. 2009. Uptake of Oxidized Low Density Lipoprotein by CD36 Occurs by an Actin-dependent Pathway Distinct from Macropinocytosis *. J. Biol. Chem. 284:30288–30297. doi:10.1074/jbc.M109.045104.

Collomb, M., A. Schmid, R. Sieber, D. Wechsler, and E.L. Ryhänen. 2006. Conjugated linoleic acids in milk fat: Variation and physiological effects. Int. Dairy J. 16:1347–1361. doi:10.1016/j.idairyj.2006.06.021.

Combaret, L., D. Taillandier, C. Polge, D. Béchet, and D. Attaix. 2016. Chapter 3 - Cellular Mechanisms of Protein Degradation Among Tissues. D.B.T.-T.M.N. of A.A. and P. Dardevet, ed. Academic Press, Boston.

Cominelli, F., K.O. Arseneau, and T.T. Pizarro. 2003. The mucosal inflammatory response. Cytokines and chemokines. Springer.

Corrêa-Oliveira, R., J.L. Fachi, A. Vieira, F.T. Sato, and M.A.R. Vinolo. 2016. Regulation of immune cell function by short-chain fatty acids. Clin. Transl. Immunol. 5:1–8. doi:10.1038/cti.2016.17.

Dalvi, P., B. Sun, N. Tang, and L. Pulliam. 2017. Immune activated monocyte exosomes alter microRNAs in brain endothelial cells and initiate an inflammatory response through the TLR4/MyD88 pathway. Sci. Rep. 7:1–12. doi:10.1038/s41598-017-10449-0.

Dänicke, S., J. Kowalczyk, L. Renner, J. Pappritz, U. Meyer, R. Kramer, E.-M. Weber, S. Döll, J. Rehage, and G. Jahreis. 2012. Effects of conjugated linoleic acids fed to dairy cows during early gestation on hematological, immunological, and metabolic characteristics of cows and their calves. J. Dairy Sci. 95:3938–3953. doi:https://doi.org/10.3168/jds.2011-4879.

Deeg, C.A., R.L. Degroote, I.M. Giese, S. Hirmer, B. Amann, M. Weigand, C. Wiedemann, and S.M. Hauck. 2020. CD11d is a novel antigen on chicken leukocytes.. J. Proteomics 225:103876. doi:10.1016/j.jprot.2020.103876.

Deng, X., Y. Cong, R. Yin, G. Yang, C. Ding, S. Yu, X. Liu, C. Wang, and Z. Ding. 2014. Proteomic analysis of chicken peripheral blood mononuclear cells after infection by Newcastle disease virus. J Vet Sci 15:511–517.

Denning, T.L., Y. Wang, S.R. Patel, I.R. Williams, and B. Pulendran. 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17–producing T cell responses. Nat. Immunol. 8:1086–1094.

doi:10.1038/ni1511.

Désert, C., E. Merlot, T. Zerjal, B. Bed'hom, S. Härtle, A. Le Cam, P.-F. Roux, E. Baeza, F. Gondret, M.J. Duclos, and S. Lagarrigue. 2016. Transcriptomes of whole blood and PBMC in chickens. Comp. Biochem. Physiol. Part D Genomics Proteomics 20:1–9. doi:https://doi.org/10.1016/j.cbd.2016.06.008.

Dill-McFarland, K.A., P.J. Weimer, J.D. Breaker, and G. Suen. 2019. Diet influences early microbiota development in dairy calves without long-term impacts on milk production. Appl. Environ. Microbiol. 85:e02141-18.

Dipasquale, D., L. Basiricò, P. Morera, R. Primi, A. Tröscher, and U. Bernabucci. 2018. Anti-inflammatory effects of conjugated linoleic acid isomers and essential fatty acids in bovine mammary epithelial cells. Animal 12:2108–2114. doi:10.1017/S1751731117003676.

Diz, A.P., M. MARTÍNEZ-FERNÁNDEZ, and E. ROLÁN-ALVAREZ. 2012. Proteomics in evolutionary ecology: linking the genotype with the phenotype. Mol. Ecol. 21:1060–1080.

Domenichiello, A.F., A.P. Kitson, and R.P. Bazinet. 2015. Is docosahexaenoic acid synthesis from α-linolenic acid sufficient to supply the adult brain?. Prog. Lipid Res. 59:54–66. doi:https://doi.org/10.1016/j.plipres.2015.04.002.

Dranoff, G. 2004. Cytokines in cancer pathogenesis and cancer therapy. Nat. Rev. Cancer 4:11.

Ebeid, T., Y. Eid, A. Saleh, and H. Abd El-Hamid. 2008. Ovarian follicular development, lipid peroxidation, antioxidative status and immune response in laying hens fed fish oil-supplemented diets to produce n-3-enriched eggs*. Animal 2:84–91. doi:https://doi.org/10.1017/S1751731107000882.

Elmore, S. 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35:495–516. doi:10.1080/01926230701320337.

Fagarasan, S., S. Kawamoto, O. Kanagawa, and K. Suzuki. 2010. Adaptive Immune Regulation in the Gut: T Cell–Dependent and T Cell–Independent IgA Synthesis. Annu. Rev. Immunol. 28:243–273. doi:10.1146/annurev-immunol-030409-101314.

Fan, Y., L. Sun, S. Yang, C. He, G. Tai, and Y. Zhou. 2018. The roles and mechanisms of homogalacturonan and rhamnogalacturonan I pectins on the inhibition of cell migration. Int. J. Biol. Macromol. 106:207–217. doi:https://doi.org/10.1016/j.ijbiomac.2017.08.004.

Feig, C., and M.E. Peter. 2007. How apoptosis got the immune system in shape.. Eur. J.

Immunol. 37 Suppl 1:S61-70. doi:10.1002/eji.200737462.

Feng, D., W.-L. Zhao, Y.-Y. Ye, X.-C. Bai, R.-Q. Liu, L.-F. Chang, Q. Zhou, and S.-F. Sui. 2010. Cellular Internalization of Exosomes Occurs Through Phagocytosis. Traffic 11:675–687. doi:https://doi.org/10.1111/j.1600-0854.2010.01041.x.

Ferreira Júnior, Á., J.P. dos Santos, I. de O. Sousa, I. Martin, E.G.L. Alves, and I.R. Rosado. 2018. Gallus gallus domesticus: sistema imunológico e seu potencial para geração de imunobiológicos. Ciência Rural 48.

Field, C.J., I.R. Johnson, and P.D. Schley. 2002. Nutrients and their role in host resistance to infection. J. Leukoc. Biol. 71:16–32. doi:https://doi.org/10.1189/jlb.71.1.16.

Fritz, J.H., R.L. Ferrero, D.J. Philpott, and S.E. Girardin. 2006. Nod-like proteins in immunity, inflammation and disease. Nat. Immunol. 7:1250–1257. doi:10.1038/ni1412.

Fu, Y., Y. Wang, H. Gao, D. Li, R. Jiang, L. Ge, C. Tong, and K. Xu. 2021. Associations among Dietary Omega-3 Polyunsaturated Fatty Acids, the Gut Microbiota, and Intestinal Immunity. Mediators Inflamm. 2021:8879227. doi:10.1155/2021/8879227.

Fujihashi, K., P.N. Boyaka, and J.R. McGhee. 2013. 19 - Host defenses at mucosal surfaces. R.R. Rich, T.A. Fleisher, W.T. Shearer, H.W. Schroeder, A.J. Frew, and C.M.B.T.-C.I. (Fourth E. Weyand, ed. Content Repository Only!, London.

De Gaetano, M., K. Alghamdi, S. Marcone, and O. Belton. 2015. Conjugated linoleic acid induces an atheroprotective macrophage MΦ2 phenotype and limits foam cell formation. J. Inflamm. (United Kingdom) 12:1–14. doi:10.1186/s12950-015-0060-9.

Galamb, E., V. Faigl, M. Keresztes, Z. Csillik, A. Tröscher, P. Elek, M. Kulcsár, G. Huszenicza, H. Fébel, and F. Husvéth. 2017. Effect of pre- and post-partum supplementation with lipid-encapsulated conjugated linoleic acid on milk yield and metabolic status in multiparous high-producing dairy cows. J. Anim. Physiol. Anim. Nutr. (Berl). 101:1026–1035. doi:https://doi.org/10.1111/jpn.12544.

Gilbert, M., G. Nicolas, G. Cinardi, T.P. Van Boeckel, S.O. Vanwambeke, G.R.W. Wint, and T.P. Robinson. 2018. Global distribution data for cattle, buffaloes, horses, sheep, goats, pigs, chickens and ducks in 2010. Sci. Data 5:180227. doi:10.1038/sdata.2018.227.

Giovannetti, E., A. Erozenci, J. Smit, R. Danesi, and G.J. Peters. 2012. Molecular mechanisms underlying the role of microRNAs (miRNAs) in anticancer drug

resistance and implications for clinical practice. Crit. Rev. Oncol. Hematol. 81:103–122. doi:https://doi.org/10.1016/j.critrevonc.2011.03.010.

Glinsky, V. V., and A. Raz. 2009. Modified citrus pectin anti-metastatic properties: one bullet, multiple targets. Carbohydr. Res. 344:1788–1791. doi:10.1016/j.carres.2008.08.038.

Gomez-Lopez, N., F. Vadillo-Ortega, and G. Estrada-Gutierrez. 2011. Combined Boyden-flow cytometry assay improves quantification and provides phenotypification of leukocyte chemotaxis. PLoS One 6:6–13. doi:10.1371/journal.pone.0028771.

Gong, T., L. Liu, W. Jiang, and R. Zhou. 2020. DAMP-sensing receptors in sterile inflammation and inflammatory diseases. Nat. Rev. Immunol. 20:95–112. doi:10.1038/s41577-019-0215-7.

Greco, L.F., J.T.N. Neto, A. Pedrico, R.A. Ferrazza, F.S. Lima, R.S. Bisinotto, N. Martinez, M. Garcia, E.S. Ribeiro, G.C. Gomes, J.H. Shin, M.A. Ballou, W.W. Thatcher, C.R. Staples, and J.E.P. Santos. 2015. Effects of altering the ratio of dietary n-6 to n-3 fatty acids on performance and inflammatory responses to a lipopolysaccharide challenge in lactating Holstein cows. J. Dairy Sci. 98:602–617. doi:https://doi.org/10.3168/jds.2014-8805.

Griinari, J.M., B.A. Corl, S.H. Lacy, P.Y. Chouinard, K.V. V Nurmela, and D.E. Bauman. 2000. Conjugated Linoleic Acid Is Synthesized Endogenously in Lactating Dairy Cows by Δ9-Desaturase. J. Nutr. 130:2285–2291. doi:10.1093/jn/130.9.2285.

Grob, D., I. Conejeros, Z.D. Velásquez, C. Preußer, U. Gärtner, P. Alarcón, R.A. Burgos, C. Hermosilla, and A. Taubert. 2020. Trypanosoma brucei brucei Induces Polymorphonuclear Neutrophil Activation and Neutrophil Extracellular Traps Release . Front. Immunol. 11:2267.

Guan, J.-L. 2005. Cell Migration: Developmental Methods and Protocols. Springer Science & Business Media.

Guo, H.-Y., A.-C. Cheng, M.-S. Wang, Z.-Q. Yin, and R.-Y. Jia. 2020. Exosomes: Potential Therapies for Disease via Regulating TLRs. Mediators Inflamm. 2020:2319616. doi:10.1155/2020/2319616.

Gutiérrez, S., S.L. Svahn, and M.E. Johansson. 2019. Effects of omega-3 fatty acids on immune cells. Int. J. Mol. Sci. 20:5028.

Gutzeit, C., G. Magri, and A. Cerutti. 2014. Intestinal IgA production and its role in host-microbe interaction. Immunol. Rev. 260:76–85. doi:10.1111/imr.12189.

Hafez, H.M., and Y.A. Attia. 2020. Challenges to the Poultry Industry: Current Perspectives and Strategic Future After the COVID-19 Outbreak . Front. Vet. Sci. 7.

Haubold, S., C. Kröger-Koch, A. Starke, A. Tuchscherer, A. Tröscher, H. Kienberger, M. Rychlik, U. Bernabucci, E. Trevisi, and H.M. Hammon. 2020. Effects of abomasal infusion of essential fatty acids and conjugated linoleic acid on performance and fatty acid, antioxidative, and inflammatory status in dairy cows. J. Dairy Sci. 103:972–991. doi:https://doi.org/10.3168/jds.2019-17135.

He, H., K.J. Genovese, D.J. Nisbet, and M.H. Kogut. 2007. Synergy of CpG oligodeoxynucleotide and double-stranded RNA (poly I: C) on nitric oxide induction in chicken peripheral blood monocytes. Mol. Immunol. 44:3234–3242.

Heidari, M., S.D. Fitzgerald, and H. Zhang. 2015. Immune Responses in Cecal Tonsils of Marek's Disease Virus–Infected Chickens. Avian Dis. 59:213–226.

Heo, J.M., J.C. Kim, J. Yoo, and J.R. Pluske. 2015. A between-experiment analysis of relationships linking dietary protein intake and post-weaning diarrhea in weanling pigs under conditions of experimental infection with an enterotoxigenic strain of Escherichia coli. Anim. Sci. J. 86:286–293. doi:https://doi.org/10.1111/asj.12275.

Herrera-Mendez, C.H., S. Becila, X. Blanchet, P. Pelissier, D. Delourme, G. Coulis, M.A. Sentandreu, A. Boudjellal, L. Bremaud, and A. Ouali. 2009. Inhibition of human initiator caspase 8 and effector caspase 3 by cross-class inhibitory bovSERPINA3-1 and A3-3. FEBS Lett. 583:2743–2748.

Hervera, A., F. De Virgiliis, I. Palmisano, L. Zhou, E. Tantardini, G. Kong, T. Hutson, M.C. Danzi, R.B.-T. Perry, C.X.C. Santos, A.N. Kapustin, R.A. Fleck, J.A. Del Río, T. Carroll, V. Lemmon, J.L. Bixby, A.M. Shah, M. Fainzilber, and S. Di Giovanni. 2018. Reactive oxygen species regulate axonal regeneration through the release of exosomal NADPH oxidase 2 complexes into injured axons. Nat. Cell Biol. 20:307–319. doi:10.1038/s41556-018-0039-x.

Hoischen-Taubner, S., J. Habel, V. Uhlig, E.-M. Schwabenbauer, T. Rumphorst, L. Ebert, D. Möller, and A. Sundrum. 2021. The Whole and the Parts—A New Perspective on Production Diseases and Economic Sustainability in Dairy Farming. Sustain. 13. doi:10.3390/su13169044.

Huang-Doran, I., C.-Y. Zhang, and A. Vidal-Puig. 2017. Extracellular Vesicles: Novel Mediators of Cell Communication In Metabolic Disease. Trends Endocrinol. Metab. 28:3–18. doi:https://doi.org/10.1016/j.tem.2016.10.003.

Huang, F.R., Z.P. Zhan, J. Luo, S.W. Jiang, and J. Peng. 2008. Duration of feeding linseed diet influences peroxisome proliferator-activated receptor γ and tumor necrosis factor gene expression, and muscle mass of growing–finishing barrows. Livest. Sci. 119:194–201.

Huntzinger, E., and E. Izaurralde. 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Genet. 12:99–110. doi:10.1038/nrg2936.

Hussen, J., and H.-J. Schuberth. 2017. Heterogeneity of Bovine Peripheral Blood Monocytes.. Front. Immunol. 8:1875. doi:10.3389/fimmu.2017.01875.

Hwang, S.-R., R. Bundey, T. Toneff, and V. Hook. 2009. Endopin Serpin Protease Inhibitors Localize with Neuropeptides in Secretory Vesicles and Neuroendocrine Tissues. Neuroendocrinology 89:210–216. doi:10.1159/000162916.

Hwang, S.-R., B. Steineckert, T. Toneff, R. Bundey, A. V Logvinova, P. Goldsmith, and V.Y.H. Hook. 2002. The Novel Serpin Endopin 2 Demonstrates Cross-Class Inhibition of Papain and Elastase: Localization of Endopin 2 to Regulated Secretory Vesicles of Neuroendocrine Chromaffin Cells. Biochemistry 41:10397–10405. doi:10.1021/bi020088o.

Ijaz, A., E.J.A. Veldhuizen, F. Broere, V.P.M.G. Rutten, and C.A. Jansen. 2021. The Interplay between Salmonella and Intestinal Innate Immune Cells in Chickens. Pathog. 10. doi:10.3390/pathogens10111512.

Ishisono, K., T. Yabe, and K. Kitaguchi. 2017. Citrus pectin attenuates endotoxin shock via suppression of Toll-like receptor signaling in Peyer's patch myeloid cells. J. Nutr. Biochem. 50:38–45.

Izumi, H., M. Tsuda, Y. Sato, N. Kosaka, T. Ochiya, H. Iwamoto, K. Namba, and Y. Takeda. 2015. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. J. Dairy Sci. 98:2920–2933. doi:10.3168/jds.2014-9076.

Janeway, C.A. 2001. How the immune system works to protect the host from infection: a personal view. Proc. Natl. Acad. Sci. 98:7461–7468.

Jha, R., and P. Mishra. 2021. Dietary fiber in poultry nutrition and their effects on nutrient utilization, performance, gut health, and on the environment: a review. J. Anim. Sci. Biotechnol. 12:51. doi:10.1186/s40104-021-00576-0.

Jung, C., J.-P. Hugot, and F. Barreau. 2010. Peyer's Patches: The Immune Sensors of the Intestine. Int. J. Inflam. 2010:1–12. doi:10.4061/2010/823710.

K., F.D., S. Claire, M. Chaouki, J. Steffen, A. Pierre, L.D. R., C. Ana, and G. Frederic.

2006. A Clonogenic Bone Marrow Progenitor Specific for Macrophages and Dendritic Cells. Science (80-.). 311:83–87. doi:10.1126/science.1117729.

Kalluri, R. 2016. The biology and function of exosomes in cancer. J. Clin. Invest. 126:1208–1215. doi:10.1172/JCI81135.

Kalluri, R., and V.S. LeBleu. 2020. The biology, function, and biomedical applications of exosomes. Science (80-.). 367. doi:10.1126/science.aau6977.

Kamiloglu, S., G. Sari, T. Ozdal, and E. Capanoglu. 2020. Guidelines for cell viability assays. Food Front. 1:332–349. doi:https://doi.org/10.1002/fft2.44.

Kang, Ji-Houn and Yang, M.-P. 2008. In vitro evaluation of the effect of trans-10, cis-12 conjugated linoleic acid on phagocytosis by canine peripheral blood polymorphonuclear neutrophilic leukocytes exposed to methylprednisolone sodium succinate. Am. J. Vet. Res. 69:494–500.

Kang, J.H., G.S. Lee, E.B. Jeung, and M.P. Yang. 2007. Trans-10, cis-12-conjugated linoleic acid increases phagocytosis of porcine peripheral blood polymorphonuclear cells in vitro. Br. J. Nutr. 97:117–125. doi:10.1017/S0007114507280584.

Kaspric, N., B. Picard, M. Reichstadt, J. Tournayre, and M. Bonnet. 2015. ProteINSIDE to Easily Investigate Proteomics Data from Ruminants: Application to Mine Proteome of Adipose and Muscle Tissues in Bovine Foetuses. PLoS One 10:e0128086–e0128086. doi:10.1371/journal.pone.0128086.

Kato, H., S. Takahashi, and K. Saito. 2011. Omics and integrated omics for the promotion of food and nutrition science. J. Tradit. Complement. Med. 1:25–30. doi:10.1016/s2225-4110(16)30053-0.

Kaur, N., V. Chugh, and A.K. Gupta. 2014. Essential fatty acids as functional components of foods- a review. J. Food Sci. Technol. 51:2289–2303. doi:10.1007/s13197-012-0677-0.

Klasing, K.C. 2007. Nutrition and the immune system. Br. Poult. Sci. 48:525–537. doi:10.1080/00071660701671336.

Kogut, M.H., M. Iqbal, H. He, V. Philbin, P. Kaiser, and A. Smith. 2005. Expression and function of Toll-like receptors in chicken heterophils. Dev. Comp. Immunol. 29:791–807. doi:https://doi.org/10.1016/j.dci.2005.02.002.

Koussounadis, A., S.P. Langdon, I.H. Um, D.J. Harrison, and V.A. Smith. 2015. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. Sci. Rep. 5:10775. doi:10.1038/srep10775.

Kra, G., N. Nemes-Navon, J.R. Daddam, L. Livshits, S. Jacoby, Y. Levin, M. Zachut, and

U. Moallem. 2021. Proteomic analysis of peripheral blood mononuclear cells and inflammatory status in postpartum dairy cows supplemented with different sources of omega-3 fatty acids. J. Proteomics 246:104313. doi:https://doi.org/10.1016/j.jprot.2021.104313.

Krol, J., I. Loedige, and W. Filipowicz. 2010. The widespread regulation of microRNA biogenesis, function and decay. Nat. Rev. Genet. 11:597–610. doi:10.1038/nrg2843.

Kuper, C.F., M.V.W. Wijnands, and S.A.L. Zander. 2017. Mucosa-Associated Lymphoid Tissues BT - Immunopathology in Toxicology and Drug Development: Volume 2, Organ Systems. G.A. Parker, ed. Springer International Publishing, Cham.

Kussmann, M., S. Rezzi, and H. Daniel. 2008. Profiling techniques in nutrition and health research. Curr. Opin. Biotechnol. 19:83–99.

Lai, K.-L., A.P. Torres-Duarte, and J.Y. Vanderhoek. 2005. 9-trans,11-trans-CLA: Antiproliferative and proapoptotic effects on bovine endothelial cells. Lipids 40:1107–1116. doi:https://doi.org/10.1007/s11745-005-1474-1.

Lai, Y., and R.L. Gallo. 2008. Toll-like receptors in skin infections and inflammatory diseases. Infect. Disord. Drug Targets 8:144–155. doi:10.2174/1871526510808030144.

Langhout, D.J., and J.B. Schutte. 1996. Nutritional Implications of Pectins in Chicks in Relation to Esterification and Origin of Pectins. Poult. Sci. 75:1236–1242. doi:10.3382/ps.0751236.

Lässer, C., V. Seyed Alikhani, K. Ekström, M. Eldh, P. Torregrosa Paredes, A. Bossios, M. Sjöstrand, S. Gabrielsson, J. Lötvall, and H. Valadi. 2011. Human saliva, plasma and breast milk exosomes contain RNA: Uptake by macrophages. J. Transl. Med. 9:9. doi:10.1186/1479-5876-9-9.

Lauvau, G., L. Chorro, E. Spaulding, and S.M. Soudja. 2014. Inflammatory monocyte effector mechanisms. Cell. Immunol. 291:32–40. doi:https://doi.org/10.1016/j.cellimm.2014.07.007.

Lawal, R.A., and O. Hanotte. 2021. Domestic chicken diversity: Origin, distribution, and adaptation. Anim. Genet. 52:385–394. doi:https://doi.org/10.1111/age.13091.

Lecchi, C., C. Catozzi, V. Zamarian, G. Poggi, G. Borriello, A. Martucciello, D. Vecchio, E. DeCarlo, G. Galiero, and F. Ceciliani. 2019. Characterization of circulating miRNA signature in water buffaloes (Bubalus bubalis) during Brucella abortus infection and evaluation as potential biomarkers for non-invasive diagnosis in vaginal fluid. Sci. Rep. 9:1945. doi:10.1038/s41598-018-38365-x.

Lecchi, C., F. Ceciliani, S. Bernasconi, F. Franciosi, V. Bronzo, and P. Sartorelli. 2008. Bovine alpha-1 acid glycoprotein can reduce the chemotaxis of bovine monocytes and modulate CD18 expression. Vet. Res. 39. doi:10.1051/vetres:2008027.

Lecchi, C., G. Invernizzi, A. Agazzi, M. Ferroni, L.F. Pisani, G. Savoini, and F. Ceciliani. 2011. In vitro modulation of caprine monocyte immune functions by ω-3 polyunsaturated fatty acids. Vet. J. 189:353–355. doi:10.1016/j.tvjl.2010.09.001.

Lecchi, C., G. Invernizzi, A. Agazzi, S. Modina, P. Sartorelli, G. Savoini, and F. Ceciliani. 2013a. Effects of EPA and DHA on lipid droplet accumulation and mRNA abundance of PAT proteins in caprine monocytes. Res. Vet. Sci. 94:246–251. doi:https://doi.org/10.1016/j.rvsc.2012.09.019.

Lecchi, C., A.T. Marques, M. Redegalli, S. Meani, L.J. Vinco, V. Bronzo, and F. Ceciliani. 2016a. Circulating extracellular miR-22, miR-155, and miR-365 as candidate biomarkers to assess transport-related stress in turkeys. animal 10:1213–1217. doi:DOI: 10.1017/S1751731115003043.

Lecchi, C., N. Rota, A. Vitali, F. Ceciliani, and N. Lacetera. 2016b. In vitro assessment of the effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in bovine polymorphonuclear cells. Vet. Immunol. Immunopathol. 182:89–94. doi:10.1016/j.vetimm.2016.10.007.

Lecchi, C., A. Scarafoni, V. Bronzo, P.A. Martino, A. Cavallini, P. Sartorelli, and F. Ceciliani. 2013b. α1-Acid glycoprotein modulates phagocytosis and killing of Escherichia coli by bovine polymorphonuclear leucocytes and monocytes. Vet. J. 196:47–51.

Lecchi, C., V. Zamarian, C. Gini, C. Avanzini, A. Polloni, S. Rota Nodari, and F. Ceciliani. 2020. Salivary microRNAs are potential biomarkers for the accurate and precise identification of inflammatory response after tail docking and castration in piglets. J. Anim. Sci. 98:skaa153.

Leclere, L., P. Van Cutsem, and C. Michiels. 2013a. Anti-cancer activities of pH- or heat-modified pectin. Front. Pharmacol. 4 OCT:1–8. doi:10.3389/fphar.2013.00128.

Lee, S.A., N. Whenham, and M.R. Bedford. 2019. Review on docosahexaenoic acid in poultry and swine nutrition: Consequence of enriched animal products on performance and health characteristics. Anim. Nutr. 5:11–21. doi:https://doi.org/10.1016/j.aninu.2018.09.001.

Li, D., and M. Wu. 2021. Pattern recognition receptors in health and diseases. Signal Transduct. Target. Ther. 6:291. doi:10.1038/s41392-021-00687-0.

Li, H., Y. Mao, Y. Xiong, H.H. Zhao, F. Shen, X. Gao, P. Yang, X. Liu, and D. Fu. 2019. A Comprehensive Proteome Analysis of Peripheral Blood Mononuclear Cells (PBMCs) to Identify Candidate Biomarkers of Pancreatic Cancer. Cancer Genomics Proteomics 16:81–89. doi:10.21873/cgp.20114.

Li, P., S. Burke, J. Wang, X. Chen, M. Ortiz, S.C. Lee, D. Lu, L. Campos, D. Goulding, B.L. Ng, G. Dougan, B. Huntly, B. Gottgens, N.A. Jenkins, N.G. Copeland, F. Colucci, and P. Liu. 2010. Reprogramming of T cells to natural killer-like cells upon Bcl11b deletion. Science (80-.). 329:85–89. doi:10.1126/science.1188063.

Lippolis, J.D., E.J. Powell, T.A. Reinhardt, T.C. Thacker, and E. Casas. 2019. Symposium review: Omics in dairy and animal science—Promise, potential, and pitfalls*. J. Dairy Sci. 102:4741–4754. doi:https://doi.org/10.3168/jds.2018-15267.

Liu, Y., M. Dong, Z. Yang, and S. Pan. 2016. Anti-diabetic effect of citrus pectin in diabetic rats and potential mechanism via PI3K/Akt signaling pathway. Int. J. Biol. Macromol. 89:484–488. doi:https://doi.org/10.1016/j.ijbiomac.2016.05.015.

Lonnerdal, B., X. Du, Y. Liao, and J. Li. 2015. Human milk exosomes resist digestion in vitro and are internalized by human intestinal cells. FASEB J. 29:121.3. doi:https://doi.org/10.1096/fasebj.29.1_supplement.121.3.

Lu, Y., M. Zhang, P. Zhao, M. Jia, B. Liu, Q. Jia, J. Guo, L. Dou, and J. Li. 2017. Modified citrus pectin inhibits galectin-3 function to reduce atherosclerotic lesions in apoE-deficient mice. Mol Med Rep 16:647–653. doi:10.3892/mmr.2017.6646.

Lycke, N. 2012. Recent progress in mucosal vaccine development: potential and limitations. Nat. Rev. Immunol. 12:592–605. doi:10.1038/nri3251.

Ma, N., P. Guo, J. Zhang, T. He, S.W. Kim, G. Zhang, and X. Ma. 2018. Nutrients mediate intestinal bacteria-mucosal immune crosstalk. Front. Immunol. 9:1–16. doi:10.3389/fimmu.2018.00005.

Maes, D.G.D., J. Dewulf, C. Piñeiro, S. Edwards, and I. Kyriazakis. 2020. A critical reflection on intensive pork production with an emphasis on animal health and welfare. J. Anim. Sci. 98:S15–S26. doi:10.1093/jas/skz362.

Manca, S., B. Upadhyaya, E. Mutai, A.T. Desaulniers, R.A. Cederberg, B.R. White, and J. Zempleni. 2018. Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. Sci. Rep. 8:1–11. doi:10.1038/s41598-018-29780-1.

Mann, M., and O.N. Jensen. 2003. Proteomic analysis of post-translational modifications. Nat. Biotechnol. 21:255–261.

Manzoni, C., D.A. Kia, J. Vandrovcova, J. Hardy, N.W. Wood, P.A. Lewis, and R. Ferrari. 2018. Genome, transcriptome and proteome: the rise of omics data and their integration in biomedical sciences. Brief. Bioinform. 19:286–302. doi:10.1093/bib/bbw114.

Martyniuk, C.J. 2020. Perspectives on transcriptomics in animal physiology studies. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 250:110490. doi:https://doi.org/10.1016/j.cbpb.2020.110490.

Masi, M., Y. Vecchio, G. Pauselli, J. Di Pasquale, and F. Adinolfi. 2021. A Typological Classification for Assessing Farm Sustainability in the Italian Bovine Dairy Sector. Sustain. 13. doi:10.3390/su13137097.

May, R.C., and L.M. Machesky. 2001. Phagocytosis and the actin cytoskeleton. J. Cell Sci. 114:1061–1077. doi:10.1242/jcs.114.6.1061.

Mayne, S., J. McCaughey, and C. Ferris. 2011. Dairy Farm Management Systems | Non-Seasonal, Pasture-Based Milk Production Systems in Western Europe. J.W.B.T.-E. of D.S. (Second E. Fuquay, ed. Academic Press, San Diego.

McClelland, S., C. Cox, R. O'Connor, M. de Gaetano, C. McCarthy, L. Cryan, D. Fitzgerald, and O. Belton. 2010. Conjugated linoleic acid suppresses the migratory and inflammatory phenotype of the monocyte/macrophage cell. Atherosclerosis 211:96–102. doi:10.1016/j.atherosclerosis.2010.02.003.

McGuffey, R.K., and J.E. Shirley. 2011. Introduction | History of Dairy Farming. J.W.B.T.-E. of D.S. (Second E. Fuquay, ed. Academic Press, San Diego.

Melnik, B.C., W. Stremmel, R. Weiskirchen, S.M. John, and G. Schmitz. 2021. Exosome-Derived MicroRNAs of Human Milk and Their Effects on Infant Health and Development. Biomolecules 11:851. doi:10.3390/biom11060851.

Miretti, S., C. Lecchi, F. Ceciliani, and M. Baratta. 2020. MicroRNAs as Biomarkers for Animal Health and Welfare in Livestock . Front. Vet. Sci. 7.

Misra, B.B., C. Langefeld, M. Olivier, and L.A. Cox. 2019. Integrated omics: tools, advances and future approaches. J. Mol. Endocrinol. 62:R21–R45. doi:10.1530/JME-18-0055.

Mittelbrunn, M., C. Gutiérrez-Vázquez, C. Villarroya-Beltri, S. González, F. Sánchez-Cabo, M.Á. González, A. Bernad, and F. Sánchez-Madrid. 2011. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat. Commun. 2:282. doi:10.1038/ncomms1285.

Moffitt, K.L., S.L. Martin, and B. Walker. 2007. The emerging role of serine proteases

in apoptosis. Biochem. Soc. Trans. 35:559–560. doi:10.1042/BST0350559.

Mogensen, T.H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin. Microbiol. Rev. 22:240–273. doi:10.1128/CMR.00046-08.

Møller, S., and C. Lauridsen. 2006. Dietary fatty acid composition rather than vitamin E supplementation influence ex vivo cytokine and eicosanoid response of porcine alveolar macrophages. Cytokine 35:6–12. doi:https://doi.org/10.1016/j.cyto.2006.07.001.

Montecalvo, A., A.T. Larregina, W.J. Shufesky, D. Beer Stolz, M.L.G. Sullivan, J.M. Karlsson, C.J. Baty, G.A. Gibson, G. Erdos, Z. Wang, J. Milosevic, O.A. Tkacheva, S.J. Divito, R. Jordan, J. Lyons-Weiler, S.C. Watkins, and A.E. Morelli. 2012. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 119:756–766. doi:10.1182/blood-2011-02-338004.

Mowat, A.M., and W.W. Agace. 2014. Regional specialization within the intestinal immune system. Nat. Rev. Immunol. 14:667–685. doi:10.1038/nri3738.

Mudroňová, D., M. Kožár, L. Molnár, and A. Trbolová. 2014. Viability and discrimination of avian peripheral blood mononuclear cells and thrombocytes intended for improvement of wound healing in birds. Acta Vet. Hung. 62:334–339.

Mulongo, M., T. Prysliak, E. Scruten, S. Napper, and J. Perez-Casal. 2014. In vitro infection of bovine monocytes with Mycoplasma bovis delays apoptosis and suppresses production of gamma interferon and tumor necrosis factor alpha but not interleukin-10. Infect. Immun. 82:62–71.

Munir, J., M. Lee, and S. Ryu. 2020. Exosomes in Food: Health Benefits and Clinical Relevance in Diseases. Adv. Nutr. 11:687–696. doi:10.1093/advances/nmz123.

Murch, S. 2021. 5 - Gastrointestinal Mucosal Immunology and Mechanisms of Inflammation. R. Wyllie, J.S. Hyams, and M.B.T.-P.G. and L.D. (Sixth E. Kay, ed. Elsevier, Philadelphia.

Murray, P.J., and T.A. Wynn. 2011. Protective and pathogenic functions of macrophage subsets. Nat. Rev. Immunol. 11:723–737. doi:10.1038/nri3073.

Nangia-Makker, P., V. Hogan, Y. Honjo, S. Baccarini, L. Tait, R. Bresalier, and A. Raz. 2002. Inhibition of Human Cancer Cell Growth and Metastasis in Nude Mice by Oral Intake of Modified Citrus Pectin. JNCI J. Natl. Cancer Inst. 94:1854–1862. doi:10.1093/jnci/94.24.1854.

Newsholme, P., R. Curi, S. Gordon, and E.A. Newsholme. 1986. Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages.

Biochem. J. 239:121–125.

Nguyen, M.-A., D. Karunakaran, M. Geoffrion, H.S. Cheng, K. Tandoc, L. Perisic Matic, U. Hedin, L. Maegdefessel, J.E. Fish, and K.J. Rayner. 2018. Extracellular Vesicles Secreted by Atherogenic Macrophages Transfer MicroRNA to Inhibit Cell Migration. Arterioscler. Thromb. Vasc. Biol. 38:49–63. doi:10.1161/ATVBAHA.117.309795.

Ning, Z., H. Zhou, F. Wang, M. Abu-Farha, and D. Figeys. 2011. Analytical Aspects of Proteomics: 2009–2010. Anal. Chem. 83:4407–4426. doi:10.1021/ac200857t.

O'Connell, A.R., and C. Stenson-Cox. 2007. A more serine way to die: Defining the characteristics of serine protease-mediated cell death cascades. Biochim. Biophys. Acta - Mol. Cell Res. 1773:1491–1499. doi:https://doi.org/10.1016/j.bbamcr.2007.08.002.

Okumura, R., and K. Takeda. 2017. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. Exp. Mol. Med. 49:e338–e338. doi:10.1038/emm.2017.20.

Oláh, I., and B. Glick. 1984. Meckel's diverticulum. I. Extramedullary myelopoiesis in the yolk sac of hatched chickens (Gallus domesticus). Anat. Rec. 208:243–252. doi:https://doi.org/10.1002/ar.1092080211.

Olah, I., B. Glick, and R.L. Taylor Jr. 1984. Meckel's diverticulum. II. A novel lymphoepithelial organ in the chicken. Anat. Rec. 208:253–263.

Ozsolak, F., and P.M. Milos. 2011. RNA sequencing: advances, challenges and opportunities. Nat. Rev. Genet. 12:87–98. doi:10.1038/nrg2934.

Pabst, O., and A.M. Mowat. 2012. Oral tolerance to food protein. Mucosal Immunol. 5:232–239. doi:10.1038/mi.2012.4.

Paek, J., J.H. Kang, S.S. Kim, K.A. Son, M.R. Park, and M.P. Yang. 2010. Trans-10, cis-12 conjugated linoleic acid directly enhances the chemotactic activity of porcine peripheral blood polymorphonuclear neutrophillic leukocytes by activating F-actin polymerization in vitro. Res. Vet. Sci. 89:191–195. doi:10.1016/j.rvsc.2010.02.021.

Pan, D., Y. Li, F. Yang, Z. Lv, S. Zhu, Y. Shao, Y. Huang, G. Ning, and S. Feng. 2021. Increasing toll-like receptor 2 on astrocytes induced by Schwann cell-derived exosomes promotes recovery by inhibiting CSPGs deposition after spinal cord injury. J. Neuroinflammation 18:172. doi:10.1186/s12974-021-02215-x.

Papathanasopoulos, A., and M. Camilleri. 2010. Dietary Fiber Supplements: Effects in Obesity and Metabolic Syndrome and Relationship to Gastrointestinal Functions. Gastroenterology 138:65-72.e2. doi:https://doi.org/10.1053/j.gastro.2009.11.045.

Parada, N., A. Romero-Trujillo, N. Georges, and F. Alcayaga-Miranda. 2021. Camouflage strategies for therapeutic exosomes evasion from phagocytosis. J. Adv. Res. 31:61–74. doi:https://doi.org/10.1016/j.jare.2021.01.001.

Pardanani, A., E.D. Wieben, T.C. Spelsberg, and A. Tefferi. 2002. Primer on medical genomics part IV: expression proteomics. Pages 1185–1196 in Mayo Clinic Proceedings. Elsevier.

Pariza, M.W., Y. Park, and M.E. Cook. 2001. The biologically active isomers of conjugated linoleic acid. Prog. Lipid Res. 40:283–298. doi:https://doi.org/10.1016/S0163-7827(01)00008-X.

Park, Y.M., M. Febbraio, and R.L. Silverstein. 2009. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. J. Clin. Invest. 119:136–145. doi:10.1172/JCI35535.

Paschoal, V.A., M.A.R. Vinolo, A.R. Crisma, J. Magdalon, and R. Curi. 2013. Eicosapentaenoic (EPA) and Docosahexaenoic (DHA) Acid Differentially Modulate Rat Neutrophil Function In Vitro. Lipids 48:93–103. doi:10.1007/s11745-012-3726-6.

Pedersen, L.J. 2018. Chapter 1 - Overview of commercial pig production systems and their main welfare challenges. M.B.T.-A. in P.W. Špinka, ed. Woodhead Publishing.

Pisani, L.F., C. Lecchi, G. Invernizzi, P. Sartorelli, G. Savoini, and F. Ceciliani. 2009. In vitro modulatory effect of ω-3 polyunsaturated fatty acid (EPA and DHA) on phagocytosis and ROS production of goat neutrophils. Vet. Immunol. Immunopathol. 131:79–85. doi:10.1016/j.vetimm.2009.03.018.

Plotnikova, O., A. Baranova, and M. Skoblov. 2019. Comprehensive Analysis of Human microRNA–mRNA Interactome . Front. Genet. 10.

do Prado, S.B.R., T.M. Shiga, Y. Harazono, V.A. Hogan, A. Raz, N.C. Carpita, and J.P. Fabi. 2019. Migration and proliferation of cancer cells in culture are differentially affected by molecular size of modified citrus pectin. Carbohydr. Polym. 211:141–151. doi:https://doi.org/10.1016/j.carbpol.2019.02.010.

Raimondo, S., F. Naselli, S. Fontana, F. Monteleone, A. Lo Dico, L. Saieva, G. Zito, A. Flugy, M. Manno, M.A. Di Bella, G. De Leo, and R. Alessandro. 2015. Citrus limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-mediated cell death. Oncotarget 6:19514–19527. doi:10.18632/oncotarget.4004.

Ramakrishna, B.S. 2013. Role of the gut microbiota in human nutrition and metabolism. J. Gastroenterol. Hepatol. 28:9–17. doi:10.1111/jgh.12294.

Ramakrishnan, D.P., R.A. Hajj-Ali, Y. Chen, and R.L. Silverstein. 2016. Extracellular Vesicles Activate a CD36-Dependent Signaling Pathway to Inhibit Microvascular Endothelial Cell Migration and Tube Formation. Arterioscler. Thromb. Vasc. Biol. 36:534–544. doi:10.1161/ATVBAHA.115.307085.

Raposo, G., H.W. Nijman, W. Stoorvogel, R. Liejendekker, C. V Harding, C.J. Melief, and H.J. Geuze. 1996. B lymphocytes secrete antigen-presenting vesicles.. J. Exp. Med. 183:1161–1172. doi:10.1084/jem.183.3.1161.

Rendra, E., V. Riabov, D.M. Mossel, T. Sevastyanova, M.C. Harmsen, and J. Kzhyshkowska. 2019. Reactive oxygen species (ROS) in macrophage activation and function in diabetes. Immunobiology 224:242–253. doi:https://doi.org/10.1016/j.imbio.2018.11.010.

Renner, L., S. Kersten, A. Duevel, H.-J. Schuberth, and S. Dänicke. 2013. Effects of cis-9,trans-11 and trans-10,cis-12 Conjugated Linoleic Acid, Linoleic Acid, Phytanic Acid and the Combination of Various Fatty Acids on Proliferation and Cytokine Expression of Bovine Peripheral Blood Mononuclear Cells. Nutr. 5. doi:10.3390/nu5072667.

Renner, L., J. Pappritz, R. Kramer, S. Kersten, G. Jahreis, and S. Dänicke. 2012. Fatty acid profile and proliferation of bovine blood mononuclear cells after conjugated linoleic acid supplementation. Lipids Health Dis. 11:63. doi:10.1186/1476-511X-11-63.

Ridley, B.L., M.A. O'Neill, and D. Mohnen. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 57:929–967. doi:https://doi.org/10.1016/S0031-9422(01)00113-3.

Rinaldi, M., F. Ceciliani, C. Lecchi, P. Moroni, and D.D. Bannerman. 2008. Differential effects of α1-acid glycoprotein on bovine neutrophil respiratory burst activity and IL-8 production. Vet. Immunol. Immunopathol. 126:199–210. doi:10.1016/j.vetimm.2008.07.001.

Rinaldi, M., P. Moroni, M.J. Paape, and D.D. Bannerman. 2007. Evaluation of assays for the measurement of bovine neutrophil reactive oxygen species.. Vet. Immunol. Immunopathol. 115:107–25. doi:10.1016/j.vetimm.2006.09.009.

Ritchie, M.D., E.R. Holzinger, R. Li, S.A. Pendergrass, and D. Kim. 2015. Methods of integrating data to uncover genotype–phenotype interactions. Nat. Rev. Genet.

16:85–97. doi:10.1038/nrg3868.

Rivero, F., B. Koppel, B. Peracino, S. Bozzaro, F. Siegert, C.J. Weijer, M. Schleicher, R. Albrecht, and A.A. Noegel. 1996. The role of the cortical cytoskeleton: F-actin crosslinking proteins protect against osmotic stress, ensure cell size, cell shape and motility, and contribute to phagocytosis and development. J. Cell Sci. 109:2679–2691. doi:10.1242/jcs.109.11.2679.

Rowe, E., M.S. Dawkins, and S.G. Gebhardt-Henrich. 2019. A Systematic Review of Precision Livestock Farming in the Poultry Sector: Is Technology Focussed on Improving Bird Welfare?. Anim. 9. doi:10.3390/ani9090614.

Roy, N.H., and J.K. Burkhardt. 2018. The Actin Cytoskeleton: A Mechanical Intermediate for Signal Integration at the Immunological Synapse . Front. Cell Dev. Biol. 6.

Sabour, S., S.A. Tabeidian, and G. Sadeghi. 2019. Dietary organic acid and fiber sources affect performance, intestinal morphology, immune responses and gut microflora in broilers. Anim. Nutr. (Zhongguo xu mu shou yi xue hui) 5:156–162. doi:10.1016/j.aninu.2018.07.004.

Sahasrabudhe, N.M., M. Beukema, L. Tian, B. Troost, J. Scholte, E. Bruininx, G. Bruggeman, M. van den Berg, A. Scheurink, H.A. Schols, M.M. Faas, and P. de Vos. 2018. Dietary Fiber Pectin Directly Blocks Toll-Like Receptor 2–1 and Prevents Doxorubicin-Induced Ileitis . Front. Immunol. 9.

Salehi, F., H. Behboudi, G. Kavoosi, and S.K. Ardestani. 2018. Oxidative DNA damage induced by ROS-modulating agents with the ability to target DNA: A comparison of the biological characteristics of citrus pectin and apple pectin. Sci. Rep. 8:13902. doi:10.1038/s41598-018-32308-2.

Salman, H., M. Bergman, M. Djaldetti, J. Orlin, and H. Bessler. 2008. Citrus pectin affects cytokine production by human peripheral blood mononuclear cells. Biomed. Pharmacother. 62:579–582. doi:https://doi.org/10.1016/j.biopha.2008.07.058.

Sanders, L.M., C.E. Henderson, M.Y. Hong, R. Barhoumi, R.C. Burghardt, N. Wang, C.M. Spinka, R.J. Carroll, N.D. Turner, R.S. Chapkin, and J.R. Lupton. 2004. An Increase in Reactive Oxygen Species by Dietary Fish Oil Coupled with the Attenuation of Antioxidant Defenses by Dietary Pectin Enhances Rat Colonocyte Apoptosis. J. Nutr. 134:3233–3238. doi:10.1093/jn/134.12.3233.

Savoini, G., F. Omodei Zorini, G. Farina, A. Agazzi, D. Cattaneo, and G. Invernizzi. 2019. Effects of Fat Supplementation in Dairy Goats on Lipid Metabolism and

Health Status. Anim. 9. doi:10.3390/ani9110917.

Schmidt, E.B., K. Varming, J.O. Pedersen, H.-H. Lervang, N. Grunnet, C. Jersild, and J. Dyerberg. 1992. Long-term supplementation with n-3 fatty acids, II: effect on neutrophil and monocyte chemotaxis. Scand. J. Clin. Lab. Invest. 52:229–236. doi:10.3109/00365519209088790.

Scholten, M.C.T., I.J.M. de Boer, B. Gremmen, and C. Lokhorst. 2013. Livestock Farming with Care: towards sustainable production of animal-source food. NJAS Wageningen J. Life Sci. 66:3–5. doi:10.1016/j.njas.2013.05.009.

Schönfeld, P., and L. Wojtczak. 2008. Fatty acids as modulators of the cellular production of reactive oxygen species. Free Radic. Biol. Med. 45:231–241. doi:https://doi.org/10.1016/j.freeradbiomed.2008.04.029.

Selberg, K.T., A.C. Lowe, C.R. Staples, N.D. Luchini, and L. Badinga. 2004. Production and Metabolic Responses of Periparturient Holstein Cows to Dietary Conjugated Linoleic Acid and trans-Octadecenoic Acids*. J. Dairy Sci. 87:158–168. doi:https://doi.org/10.3168/jds.S0022-0302(04)73153-7.

Serhan, C.N., C.B. Clish, J. Brannon, S.P. Colgan, N. Chiang, and K. Gronert. 2000. Novel Functional Sets of Lipid-Derived Mediators with Antiinflammatory Actions Generated from Omega-3 Fatty Acids via Cyclooxygenase 2–Nonsteroidal Antiinflammatory Drugs and Transcellular Processing. J. Exp. Med. 192:1197–1204. doi:10.1084/jem.192.8.1197.

Shang, Q., H. Liu, D. Wu, S. Mahfuz, and X. Piao. 2021. Source of fiber influences growth, immune responses, gut barrier function and microbiota in weaned piglets fed antibiotic-free diets. Anim. Nutr. 7:315–325. doi:https://doi.org/10.1016/j.aninu.2020.12.008.

Shen, Q., L. Zhao, and K.M. Tuohy. 2012. High-level dietary fibre up-regulates colonic fermentation and relative abundance of saccharolytic bacteria within the human faecal microbiota in vitro. Eur. J. Nutr. 51:693–705. doi:10.1007/s00394-011-0248-6.

Silva-Sanchez, A., and T.D. Randall. 2020. Anatomical Uniqueness of the Mucosal Immune System (GALT, NALT, iBALT) for the Induction and Regulation of Mucosal Immunity and Tolerance. Mucosal Vaccines 21–54. doi:10.1016/B978-0-12-811924-2.00002-X.

Silva, C., C. Santa, S.I. Anjo, and B. Manadas. 2016. A reference library of peripheral blood mononuclear cells for SWATH-MS analysis. Proteomics–Clinical Appl.

10:760–764.

Silva, V.K., V. de Souza Morita, and I.C. Boleli. 2013. Effect of pectin extracted from citrus pulp on digesta characteristics and nutrient digestibility in broilers chickens. Rev. Bras. Zootec. 42:575–583. doi:10.1590/S1516-35982013000800007.

Silverstein, R.L., and M. Febbraio. 2009. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Sci. Signal. 2:re3–re3.

Sinkora, M., K. Stepanova, J.E. Butler, D. Francis, K. Santiago-Mateo, H. Potockova, K. Karova, and J. Sinkorova. 2011. Ileal Peyer's Patches Are Not Necessary for Systemic B Cell Development and Maintenance and Do Not Contribute Significantly to the Overall B Cell Pool in Swine. J. Immunol. 187:5150–5161. doi:10.4049/jimmunol.1101879.

Sipka, S., I. Dey, C. Buda, J. Csongor, G. Szegedi, and T. Farkas. 1996. The Mechanism of Inhibitory Effect of Eicosapentaenoic Acid on Phagocytic Activity and Chemotaxis of Human Neutrophil Granulocytes. Clin. Immunol. Immunopathol. 79:224–228. doi:https://doi.org/10.1006/clin.1996.0072.

Smith, N.C., M.L. Rise, and S.L. Christian. 2019. A Comparison of the Innate and Adaptive Immune Systems in Cartilaginous Fish, Ray-Finned Fish, and Lobe-Finned Fish . Front. Immunol. 10.

Soares, R., C. Franco, E. Pires, M. Ventosa, R. Palhinhas, K. Koci, A. Martinho de Almeida, and A. Varela Coelho. 2012. Mass spectrometry and animal science: Protein identification strategies and particularities of farm animal species. J. Proteomics 75:4190–4206. doi:https://doi.org/10.1016/j.jprot.2012.04.009.

Song, H.-J., I. Grant, D. Rotondo, I. Mohede, N. Sattar, S.D. Heys, and K.W.J. Wahle. 2005. Effect of CLA supplementation on immune function in young healthy volunteers. Eur. J. Clin. Nutr. 59:508–517. doi:10.1038/sj.ejcn.1602102.

Sordillo, L.M. 2016. Nutritional strategies to optimize dairy cattle immunity1. J. Dairy Sci. 99:4967–4982. doi:https://doi.org/10.3168/jds.2015-10354.

Stachowska, E., M. Baśkiewicz-Masiuk, V. Dziedziejko, I. Gutowska, I. Baranowska-Bosiacka, M. Marchlewicz, B. Dołęgowska, B. Wiszniewska, B. Machaliński, and D. Chlubek. 2008. Conjugated linoleic acid increases intracellular ROS synthesis and oxygenation of arachidonic acid in macrophages. Nutrition 24:187–199. doi:https://doi.org/10.1016/j.nut.2007.10.018.

Stone, K.D., C. Prussin, and D.D. Metcalfe. 2011. NIH Public Access. J. Allergy 125:1–16. doi:10.1016/j.jaci.2009.11.017.IgE.

Strober, W., I. Fuss, and P. Mannon. 2007. The fundamental basis of inflammatory bowel disease. J. Clin. Invest. 117:514–521. doi:10.1172/JCI30587.

Suh, H.-J., H.-S. Yang, K.-S. Ra, D.-O. Noh, K.-H. Kwon, J.-H. Hwang, and K.-W. Yu. 2013. Peyer's patch-mediated intestinal immune system modulating activity of pectic-type polysaccharide from peel of Citrus unshiu. Food Chem. 138:1079–1086.

Sundaram, T.S., C. Giromini, R. Rebucci, and A. Baldi. 2020. Omega-3 polyunsaturated fatty acids counteract inflammatory and oxidative damage of non-transformed porcine enterocytes. Animals 10:956.

Svensson, K.J., H.C. Christianson, A. Wittrup, E. Bourseau-Guilmain, E. Lindqvist, L.M. Svensson, M. Mörgelin, and M. Belting. 2013. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. J. Biol. Chem. 288:17713–17724. doi:10.1074/jbc.M112.445403.

Swatton, J.E., S. Prabakaran, N.A. Karp, K.S. Lilley, and S. Bahn. 2004. Protein profiling of human postmortem brain using 2-dimensional fluorescence difference gel electrophoresis (2-D DIGE). Mol. Psychiatry 9:128–143. doi:10.1038/sj.mp.4001475.

Swiatczak, B., and I.R. Cohen. 2015. Gut feelings of safety: tolerance to the microbiota mediated by innate immune receptors. Microbiol. Immunol. 59:573–585.

Takiishi, T., C.I.M. Fenero, and N.O.S. Câmara. 2017. Intestinal barrier and gut microbiota: Shaping our immune responses throughout life. Tissue barriers 5:e1373208–e1373208. doi:10.1080/21688370.2017.1373208.

Teng, Y., Y. Ren, M. Sayed, X. Hu, C. Lei, A. Kumar, E. Hutchins, J. Mu, Z. Deng, C. Luo, K. Sundaram, M.K. Sriwastva, L. Zhang, M. Hsieh, R. Reiman, B. Haribabu, J. Yan, V.R. Jala, D.M. Miller, K. Van Keuren-Jensen, M.L. Merchant, C.J. McClain, J.W. Park, N.K. Egilmez, and H.-G. Zhang. 2018. Plant-Derived Exosomal MicroRNAs Shape the Gut Microbiota. Cell Host Microbe 24:637-652.e8. doi:https://doi.org/10.1016/j.chom.2018.10.001.

Tian, L., G. Bruggeman, M. van den Berg, K. Borewicz, A.J.W. Scheurink, E. Bruininx, P. de Vos, H. Smidt, H.A. Schols, and H. Gruppen. 2017. Effects of pectin on fermentation characteristics, carbohydrate utilization, and microbial community composition in the gastrointestinal tract of weaning pigs. Mol. Nutr. Food Res. 61:1600186. doi:https://doi.org/10.1002/mnfr.201600186.

Trevisi, E., P. Grossi, F.P. Cappelli, S. Cogrossi, and G. Bertoni. 2011. Attenuation of

inflammatory response phenomena in periparturient dairy cows by the administration of an ω3 rumen protected supplement containing vitamin E. Ital. J. Anim. Sci. 10:e61. doi:10.4081/ijas.2011.e61.

Triantafilou, M., F.G.J. Gamper, R.M. Haston, M.A. Mouratis, S. Morath, T. Hartung, and K. Triantafilou. 2006. Membrane Sorting of Toll-like Receptor (TLR)-2/6 and TLR2/1 Heterodimers at the Cell Surface Determines Heterotypic Associations with CD36 and Intracellular Targeting * . J. Biol. Chem. 281:31002–31011. doi:10.1074/jbc.M602794200.

Uribe-Querol, E., and C. Rosales. 2020. Phagocytosis: Our Current Understanding of a Universal Biological Process . Front. Immunol. 11.

Utsunomiya, Y.T., M. Milanesi, M.R.S. Fortes, L.R. Porto-Neto, A.T.H. Utsunomiya, M. Silva, J.F. Garcia, and P. Ajmone-Marsan. 2019. Genomic clues of the evolutionary history of Bos indicus cattle. Anim. Genet. 50:557–568.

Utz, P.J., and P. Anderson. 2000. Life and death decisions: regulation of apoptosis by proteolysis of signaling molecules. Cell Death Differ. 7:589–602. doi:10.1038/sj.cdd.4400696.

Vallés, P.G., A.G. Lorenzo, V. Bocanegra, and R. Vallés. 2014. Acute kidney injury: what part do toll-like receptors play?. Int. J. Nephrol. Renovasc. Dis. 7:241–251. doi:10.2147/IJNRD.S37891.

Varol, C., L. Landsman, D.K. Fogg, L. Greenshtein, B. Gildor, R. Margalit, V. Kalchenko, F. Geissmann, and S. Jung. 2006. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells . J. Exp. Med. 204:171–180. doi:10.1084/jem.20061011.

de Veth, M.J., D.E. Bauman, W. Koch, G.E. Mann, A.M. Pfeiffer, and W.R. Butler. 2009. Efficacy of conjugated linoleic acid for improving reproduction: A multi-study analysis in early-lactation dairy cows1. J. Dairy Sci. 92:2662–2669. doi:https://doi.org/10.3168/jds.2008-1845.

Viladomiu, M., R. Hontecillas, and J. Bassaganya-Riera. 2016. Modulation of inflammation and immunity by dietary conjugated linoleic acid. Eur. J. Pharmacol. 785:87–95. doi:https://doi.org/10.1016/j.ejphar.2015.03.095.

Vlasova, A.N., and L.J. Saif. 2021. Bovine Immunology: Implications for Dairy Cattle . Front. Immunol. 12.

Walter, M.R. V, and D.W. Morck. 2002. Chemotaxis, phagocytosis, and oxidative metabolism in bovine macrophages exposed to a novel interdigital phlegmon (foot

rot) lesion isolate, Porphyromonas levii. Am. J. Vet. Res. 63:757–762.

Wang, B., V. Kumar, A. Olson, and D. Ware. 2019. Reviving the Transcriptome Studies: An Insight Into the Emergence of Single-Molecule Transcriptome Sequencing . Front. Genet. 10.

Wang, J., Z. Liu, Z. Han, Z. Wei, Y. Zhang, K. Wang, and Z. Yang. 2020. Fumonisin B1 triggers the formation of bovine neutrophil extracellular traps. Toxicol. Lett. 332:140–145. doi:https://doi.org/10.1016/j.toxlet.2020.07.006.

Wen, C., R.C. Seeger, M. Fabbri, L. Wang, A.S. Wayne, and A.Y. Jong. 2017. Biological roles and potential applications of immune cell-derived extracellular vesicles. J. Extracell. Vesicles 6:1–19. doi:10.1080/20013078.2017.1400370.

Wigley, P. 2017. Immunology of Birds. eLS 1–8. doi:https://doi.org/10.1002/9780470015902.a0026259.

Wils-Plotz, E.L., M.C. Jenkins, and R.N. Dilger. 2013. Modulation of the intestinal environment, innate immune response, and barrier function by dietary threonine and purified fiber during a coccidiosis challenge in broiler chicks. Poult. Sci. 92:735–745.

Wolf, T., S.R. Baier, and J. Zempleni. 2015. The Intestinal Transport of Bovine Milk Exosomes Is Mediated by Endocytosis in Human Colon Carcinoma Caco-2 Cells and Rat Small Intestinal IEC-6 Cells. J. Nutr. 145:2201–2206. doi:10.3945/jn.115.218586.

WU, D. 2005. Signaling mechanisms for regulation of chemotaxis. Cell Res. 15:52–56. doi:10.1038/sj.cr.7290265.

Wu, D., E.D. Lewis, M. Pae, and S.N. Meydani. 2019. Nutritional Modulation of Immune Function: Analysis of Evidence, Mechanisms, and Clinical Relevance. Front. Immunol. 9:3160. doi:10.3389/fimmu.2018.03160.

Yang, J., L. Zhang, C. Yu, X.F. Yang, and H. Wang. 2014. Monocyte and macrophage differentiation: Circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark. Res. 2:1–9. doi:10.1186/2050-7771-2-1.

Yates 3rd, J.R. 2019. Recent technical advances in proteomics. F1000Research 8:F1000 Faculty Rev-351. doi:10.12688/f1000research.16987.1.

Zamaraeva, M. V, R.Z. Sabirov, E. Maeno, Y. Ando-Akatsuka, S. V Bessonova, and Y. Okada. 2005. Cells die with increased cytosolic ATP during apoptosis: a bioluminescence study with intracellular luciferase. Cell Death Differ. 12:1390–1397. doi:10.1038/sj.cdd.4401661.

Zaritskaya, L., M.R. Shurin, T.J. Sayers, and A.M. Malyguine. 2010. New flow cytometric assays for monitoring cell-mediated cytotoxicity. Expert Rev. Vaccines 9:601–616.

Zhou, Q., M. Li, X. Wang, Q. Li, T. Wang, Q. Zhu, X. Zhou, X. Wang, X. Gao, and X. Li. 2012. Immune-related microRNAs are abundant in breast milk exosomes. Int. J. Biol. Sci. 8:118–123. doi:10.7150/ijbs.8.118.

Zhu, F., W. Yue, and Y. Wang. 2014. The nuclear factor kappa B (NF-κB) activation is required for phagocytosis of staphylococcus aureus by RAW 264.7 cells. Exp. Cell Res. 327:256–263. doi:https://doi.org/10.1016/j.yexcr.2014.04.018.

Zindel, J., and P. Kubes. 2020. DAMPs, PAMPs, and LAMPs in Immunity and Sterile Inflammation. Annu. Rev. Pathol. Mech. Dis. 15:493–518. doi:10.1146/annurev-pathmechdis-012419-032847.

List of papers published, submitted or in preparation

Published papers

1. Ávila G, Catozzi C, Pravettoni D, Sala G, Martino P, Meroni G, Lecchi C, Ceciliani F. *In vitro* effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes. Journal of Dairy Science. 2020 Sep;103(9):8554-8563. doi: 10.3168/jds.2020-18659. Epub 2020 Jul 16. PMID: 32684447.
2. G.Ávila, D.De Leonardis, G.Grilli, C.Lecchi, F.Ceciliani. Anti-inflammatory activity of citrus pectin on chicken monocytes' immune response (Short communication). Veterinary Immunology and Immunopathology. 2021. 237: 110269 (1-6).

Manuscripts submitted

1. G. Ávila, S. Di Mauro, J. Filipe, Alessandro Agazzi, Marcello Comic, Cristina Lecchi, Fabrizio Ceciliani. Immunomodulatory effects of long-chain n-3 polyunsaturated fatty acids (PUFA) on porcine monocytes (CD14+) immune response *in vitro*. **[Submitted in Veterinary Immunology and Immunopathology].**

Manuscripts to be submitted

1. G. Ávila, D. De Leonardis, J. Filipe, R. Furioso Ferreira, A. Agazzi, H. Sauerwein, M. Comi, V. Mrljak, C. Lecchi, F. Ceciliani. Porcine milk exosomes modulate the immune functions of CD14+ monocytes *in vitro*. **[To be submitted in Scientific Reports].**
2. G. Ávila, F. Ceciliani, D., Viala, S. Dejean, D. Pravettoni, G. Sala, C. Lecchi, M.Bonnet. Conjugated Linoleic Acid (CLA) modulates bovine peripheral blood mononuclear cells (PBMC) proteome *in vitro*. **[To be submitted in Journal of Proteomics]**.

3. G. Ávila, M.Bonnet, D. Viala, S. Dejean, G. Grilli, C. Lecchi, F. Ceciliani. Citrus pectin (CP) modulates chicken peripheral blood mononuclear cells (PBMC) proteome *in vitro.* **[To be submitted in Journal of Proteomics]**.
4. G. Ávila, M.Bonnet, D. Viala, S. Dejean, A. Agazzi, M. Comi, C. Lecchi, F. Ceciliani. Porcine milk exosomes modulate porcine peripheral blood mononuclear cells (PBMC) proteome *in vitro.* **[To be submitted in Journal of Proteomics]**.

Other published papers not related to the present thesis project

1. Catozzi C, Ávila G, Zamarian V, Pravettoni D, Sala G, Ceciliani F, Lacetera N, Lecchi C. *In vitro* effect of heat stress on bovine monocytes lifespan and polarization. Immunobiology. 2020 Mar;225(2):151888. doi: 10.1016/j.imbio.2019.11.023. Epub 2019 Nov 29. PMID: 31843259.
2. Ceciliani F, Ávila Morales G, De Matteis G, Grandoni F, Furioso Ferreira R, Roccabianca P, Lecchi C. Methods in isolation and characterization of bovine monocytes and macrophages. Methods. 2020 Jul 2: S1046-2023(20)30105-5. doi: 10.1016/j.ymeth.2020.06.017. Epub ahead of print. PMID: 32622986.

Activities performed during PhD

Courses

Courses	Year	CFU	Observations
Core Training Course in Molecular Animal Nutrition (MANNA)	2018	-	Certificate of participation
Bioinformatic tools to study omic data	2019	2	Approved
Basic and advanced techniques for optical microscopy in biological and preclinical research	2019	2	Approved

Digital imaging and image integrity in scientific publication	2019	2	Approved
Corso de perfezionamento: Benessere dell'animale da laboratorio ed animal care (lagomorfi-modelli acquatici-roditori)	2019	-	Certificate of participation
Winter school ISCCA - flow cytometry for beginners	2019	-	Certificate of participation
MANNA I Summer School in OMICS	2019	-	MANNA evaluation/Certificate of participation
Writing in the Sciences (University of Stanford online course)	2020	-	Approved (Certificate)
Cellular Models in Neurodegenerative Diseases	2020	2	Approved
MANNA II Summer School in Essential Management Skills and Media Communication (Online).	2020	-	MANNA evaluation/Certificate of participation
Proteomics: From study design to scientific publication. (Proteomics tutorials under the framework of MANNA project	2020	6 h	Certificate of participation
The Animal Microbiome: Basic Concepts and Implications for Veterinary Medicine And Animal Productions	2020	3	30 (Lode)
Flow Cytometry in Biomedical Research	2020	4	Approved
Course in Research Papers Veterinary from Università Autònoma de Barcelona (UAB)	2020/ 652021	-	Approved and Certificate of participation
MANNA Bioinformatics Workshop (Online)	2021	-	Certificate of participation
MANNA Transcriptomics and Proteomics interactive workshop (Online)	2021	-	Certificate of participation

Course: Hands-on Introduction to R, held by the Core Facility CUBA of the University of Bonn	2021	-	Approved and Certificate of participation
MANNA III Summer School School in Essential Management Skills and Media Communication (Online)	2021	-	MANNA evaluation/Certificate of participation
Transferable Skills Courses	2018-2021	-	-

Communications (abstracts) to meetings or congresses

1. Abstract accepted for oral presentation at EAAP 73rd Annual Meeting 2022. Porto, Portugal (September 5th-9th), entitled: "Citrus Pectin (CP) modulates chicken PBMC proteome". **(First author)**
2. Oral presentation at EAAP 72nd Annual Meeting 2021. Davos, Switzerland (August 30th -3rd of September), entitled: "milk-derived exosomes modulate porcine monocyte immune function". **(First author)**
3. Oral presentation at EAAP 72nd Annual Meeting 2021. Davos, Switzerland. (August 30th -3rd of September), entitled: "In vitro immunomodulatory effects of PUFA on porcine monocytes". **(First author)**
4. Poster presentation at British Society Proteomics Research (BSPR) Interact Online Conference 2021 (December 6th -8th of July), entitled: "Citrus Pectin (CP) modulates chicken PBMC proteome". **(First author)**
5. Oral presentation at EAAP Virtual Meeting 2020 (December 1st -4th of December), entitled: "In vitro impact of citrus pectin (CP) on chicken monocytes' immune response". **(First author)**
6. Poster presentation at EAAP Virtual Meeting 2020 (December 1st -4th of December), entitled: "In vitro impact of conjugated linoleic acid (CLA) on bovine monocytes' immune response". **(First author)**
7. Poster presentation at EAAP Virtual Meeting 2020 (December 1st -4th of December), entitled: "Milk-derived exosomes uptake by bovine monocytes". **(First author)**

8. Poster presentation at EAAP Annual Meeting 2019. Ghent, Belgium. (26th -30th of August), entitled: “Effect of 9c,11t and 10t,12c conjugated linoleic acid (CLA) on bovine PBMC apoptosis and viability”. **(First author)**
9. Oral presentation at 23rd National Congress of the Animal Science and Production Association (ASPA) 2019. Sorrento, Italy (11th -14th of June), entitled: “Effect of heat stress on monocytes and lymphocytes in dairy cattle”. **(Co-author)**

Acknowledgements

I would like first to thank my supervisor Fabrizio Ceciliani for believing in me and giving me the opportunity to work with him and with his team, for always guiding and helping me to grow both, professionally and personally. To Cristina Lecchi for her valuable support and teaching along these three years. To my second supervisors Armand and Muriel for providing guidance, feedback and precious teaching throughout this project.

To my colleagues and friends Carlotta and Valentina, for showing me the real meaning of team work and always being there for each other. Thanks for making me feel like home! To Daria and Susanna, for letting me be part of their professional journey and growth, and for always supporting me and cheering my days. To Joel for his precious help along these years and for sharing with me his experience. To Alba and Salma, that even if it was for just a short period, made my days lighter and more joyful.

To Ale and Teo (and Lea), for sharing your home with me and making me feel part of your family.

To my parents and my sisters, for giving me everything I have and for making me the person I am today. Thanks also for supporting me always with my dreams, and most importantly for loving me. A mi abuelita por inspirarme y darme fuerza siempre. Los amo.

Last but not least, to mi Mori, Jan, for being my greatest friend, supporter since day one, and life partner. Without you this would have not been possible. Ich liebe dich.

"Life is not easy for any of us. But what of that? We must have perseverance and, above all, confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained".
-Marie Curie-