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In vitro and omics methodologies applied to animal nutrition to address feed-food competition and greenhouse gas emissions

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Abstract

Global population growth and the growing demand for animal-source food are two challenges for global food security and sustainability of agriculture and livestock production. Meanwhile, the use of food-competing feedstuffs and the greenhouse gas emissions from livestock are two major issues that challenge the global feed and food supply chain. Here, we applied several *in vitro* and omics methodologies to estimate the potential inclusion of alternative feeding strategies in the frame of the environmental sustainability of livestock production. We focused on two areas: i) the dietary inclusion of food by-products (e.g. former food products) in the diet of piglets and pigs to evaluate their impact on metabolism, and ii) the dietary inclusion of tannins to reduce methane production from enteric fermentation and ammonia in ruminants. The first research project involved the study of the metabolic impact of the dietary inclusion of salty and sugary former food products (FFPs) in pigs from the weaning phase to the finishing phase. To do this, we applied omics technologies to obtain a broad and comprehensive picture of the metabolism of the pigs fed with FFPs. After assessing that both salty and sugary FFPs diets did not alter the growth performance and the feeding behaviour of the pigs, we observed a limited impact of FFPs on the modulation of the metabolome and the proteome-peptidome of piglets and pigs in multiple districts in liver and plasma. Indeed, we observed only a low number of modulated metabolites and proteins and a weak impact on the activation of specific metabolic pathways. We concluded that FFPs can be safely used as alternative and sustainable feed ingredients to reduce the feed-food competition. The second research project involved the study of the effect of two tannin compounds, called gallic acid (GA) and ellagic acid (EA), on the composition of the rumen microbial community and on the end products of enteric fermentation, with methane and ammonia production under the spotlight. To do this, we simulated rumen fermentation using two *in vitro* approaches, named Hohenheim gas test (short-term batch culture) and Rusitec (long-term continuous culture). The GA and EA molecules, and the combination of both molecules, decreased methane and ammonia production, but they influenced the overall rumen fermentation depending of the dosage used in terms of a reduced production of short-chain fatty acids and degradation of nutrients in rumen. Furthermore, we observed a strong modulation of the rumen bacterial community following tannins' treatment, with limited impact on rumen archaea. The results of the two projects contributed to increase the validity of *in vitro* and omics evaluations as preliminary steps in searching innovative feeding strategies to increase the environmental sustainability of livestock production. Further work is needed to increase the robustness of these innovative and sustainable feeding strategies.

Introduction

Currently, the world is facing several challenges regarding the relationship between humans, animals, and environment. First, the global population is expected to reach 9.6 billion people in 2050 (UN, 2019). This increasing trend is guided by the socio-demographic progresses in the developing countries, related above all to economic conditions and the incidence of poverty in those specific areas of the world (Thornton, 2010). As previously demonstrated, incidence of poverty and population growth are positively correlated (Islam and Karim, 2019), which is why sub-Saharan Africa and other regions with low or rising incomes like India lead the trend of population growth (Molotoks et al., 2021). Along with this, global food demand is expected to increase by around 45% between 2010 and 2050, driven by rising incomes in developing countries (van Dijk et al., 2021). Rising incomes in developing countries are causing a shift in eating habits, from starchy-staple food to animal-source food, more similar to the Western diet (Islam and Karim, 2019). By 2050, the global demand for animal-source food is expected to increase by 60% for meat and 75% for dairy products. The meat demand will be driven by the consumption of poultry meat, then pork and beef (Makkar, 2018). The contribution of meat to the global nutrient availability was reported by Smith et al. (2022a) using the data from the DELTA model, based on the Food Balance Sheets from FAO. Excluding seafood and non-meat animal food products, the authors showed the data only related to meat. In 2018, total meat accounted for the 7% of the total food mass at global level. Poultry meat and pig meat contributed similarly (36% and 37%, respectively) to the total meat supply, whereas ruminants contributed 25% and the other minor species 2%. Regarding the supply of nutrients, animal-source food is a nutrient-dense food and plays a key role in global food security. Meat provided 17% of the Kcal, 21-33% of the protein, and 30% of the dietary fat global consumption. Apart from macronutrients, animal source-food is also rich in essential micronutrients. In particular, the meat contributed almost to 60% of vitamin B12, 19% for zinc, 18% for selenium, and 13% for iron global demand (Smith et al., 2022a; Beal et al., 2023). In addition to meat, milk provides almost half of the global calcium demand, together with a contribution of 15% for dietary fat, 12% for dietary protein and a total 7% for energy global demand, according to the data from the same DELTA model used for meat (Smith et al., 2022b). Furthermore, 86% of feed is human-inedible, such as crop and human food by-products and leftovers, but farm animals and ruminants in particular are able to upgrade human-inedible biomass into proteins and energy available as animal-source food for human consumption (Mottet et al., 2017). Above all, livestock production and what is generated through the production of livestock products (animal-source food, wool, hide, etc.) are considered high-value products, thus providing an essential economic income for farmers and stakeholders. The livestock-related income is much more important in developing and low/middle-income countries, such as some areas of Sub-Saharan Africa and Asia,

which will drive the demand for animal source-food and where the economic return of livestock production will have a greater impact (Herrero et al., 2013). In these countries, farmers and stakeholders rely on livestock not only as a source of saleable products for a direct income, but also as "generators" of socio-economic opportunities, such as for trading agricultural inputs or for investing in parallel economic activities (Baltenweck et al., 2020). Overall, the nutritional and economic value of livestock farming and related products is evident and demonstrates the importance of livestock in the global food production system. However, the growing demand for animal-source food is causing concerns about food security and environmental sustainability. The shift in eating habits in developing countries towards a more animal-based and "Western-style" diet requires the intensification of livestock production. From this perspective, the livestock sector exerts greater pressure on the use of natural resources, without reducing its footprint. This is more valid for developing countries, while the advanced technologies and the economic stability in developed countries make the intensification more geared towards greater efficiency and to the environmental awareness of the impact of livestock production (Thornton, 2010; Islam and Karim, 2019). The livestock sector is called upon satisfying the food demand for animal-source food while reducing its impact on the environment. Below is the current situation of livestock production. Next, a relationship between the major environmental issues and livestock production is reported to provide an overview on the current situation, before discussing two potential mitigation strategies for reducing the environmental footprint of livestock production.

Current trends of livestock production

Current dynamics of the production of livestock and animal-source food show an overall increase in the demand for animal-source food at global level, with developing countries pushing the demand much more than developed countries. This is explained by rising incomes and urbanization trends observed in developing countries, both causing a shift towards Western-style habits, including eating habits. Instead, the higher awareness of consumers in developed countries about the environmental impact of livestock production is slowing the demand for animal-source food. In developing countries, annual per capita consumption of meat is projected to increase +200% and +100% for milk from 1970 to 2050. In developed countries, the same increasing trends are +30% and +10%. These values clearly describe the difference between different areas of the world and their impact on global meat production and, consequently, consumption (FAO, 2006; Thornton, 2010). Projections from 2000 to 2050 show that poultry will be the farm animal species with the highest increase in global meat consumption (+166%), followed by beef cattle (+79%) and pig (+66%) (FAO, 2018). The growing livestock population worldwide is expected to cope with the growing demand for animal-source food but also increase the impact of livestock production on the environment. This is why the sustainable

intensification of livestock production in developed countries could help in counteracting the detrimental consequences on the environment, as sustainable intensification tends to achieve higher productive yields without impacting on the use natural resources (Tedeschi et al., 2015).

Overview of the environmental impact of livestock production

Use of natural resources

Currently, almost the 40% of the terrestrial land surface is used for agricultural purposes (Van Kernebeek et al., 2016), and more than two-thirds of this share are used for livestock, corresponding to 20% of the terrestrial land surface. Furthermore, the arable land used for feed production (mostly cereals) represents 40% of global arable land. The increasing food and animal-source food demand plays a key role in pushing the conversion of grassland into arable land available for crop production. Since 1961, the global cropland has increased by 70% and especially in the developing countries (Van Zanten et al., 2018). In the conversion, perennial vegetation is replaced by annual crops, known to be less able in storing carbon in the plant biomass. The carbon lost from the soil is then transferred to the atmosphere (Kim et al., 2022). This is why this phenomenon has been related to reduced biodiversity and increased greenhouse gas (GHG) emissions (Mottet et al., 2017; Van Zanten et al., 2018). The expansion of arable land must meet the increasing trend of feed and food demand and population growth, since it was observed that the higher the population size, the higher the amount of arable land per capita because, after exploiting the high-yielding soils (e.g. clay soils), the usage rate of low-yielding soils (e.g. sandy soils) increases. Hence, expansion should be carried out within the framework of sustainable intensification, for example: i) increasing the productive yields of cropland already used for agricultural purposes, but here attention should be paid to the use of fertilizers and the resulting condition of the soil (e.g. pH, unbalanced mineral content), or ii) improving the yields of marginal lands, usually poorer in terms of soil nutrients and thus less attractive for starting crop production (Van Kernebeek et al., 2016).

In addition to land use, water is the other major natural resource, the role of which is discussed for agriculture and livestock production and in the framework of sustainable intensification. Currently, 70% of the water use is for agriculture, including livestock and crop production. Indeed, around 30% of the agricultural water is used to produce animal-source food (Mekonnen and Hoekstra, 2012; Ibidhi and Ben Salem, 2020). Among the different estimates of water use (and resources in general), the water footprint represents the total amount of water directly and indirectly used in the entire production chain of a product. The water footprint depends on a plenty of factors such as the product considered (animal or vegetable), the feed conversion ratio and the farming system (when considering animals), the type of soil, and the geographical area considered (Ran et al., 2016; Ibidhi and Ben Salem,

2020). Regarding animal-source food, beef has the highest water footprint among farm animals, followed by pork, poultry meat, eggs, and milk. The water used ranges from 15400 liters of water per kg of beef meat to 1000 liters of water per kg of milk. Water use has been correlated to the feed conversion ratio, which usually shows a decreasing trend from ruminants to pigs and eventually to poultry. Regarding soil type and geographical areas, the humid regions have a lower water footprint than arid areas, because feed production rates and transpiration of soil are positively correlated with soil moisture. Similar to land use above, sustainable intensification of livestock and crop production can lead to a reduced water use. Reducing the water footprint of livestock and crop production can be achieved by acting on feed production. Improving the efficiency of irrigation systems would reduce water losses. Then, even the adoption of low-water consuming crops or of water-rich feedstuffs could reduce the water footprint (Ibidhi and Ben Salem, 2020).

Feed-food competition

Livestock production depends on feed production and consequently on the exploitation of arable land and water. Of all the arable land used for crop production, 40% is currently used for feed production (Boumans et al., 2022). About 15% of all feedstuffs produced consist of feedstuffs that could be directed to human consumption. For each category of farm animals, the quantity of food-competing feedstuffs involved is different. For aquaculture, the share is around 50%, for poultry is close to 70%, for pig is 40%, whereas dairy cow and beef cattle have the lowest shares being 3-4% of their total dry matter intake. The increased demand for animal-source food requires an intensification of livestock production, which in turn should rely on a greater quantity of energy- and protein-rich feedstuffs (Sandström et al., 2022). Among farm animals, different feed ingredients affect the use of natural resources differently. Concentrates from cereal crops (e.g. maize and wheat) are valid protein and energy source for animal nutrition, and are the major ingredient for the diet of monogastric, with a rate of dietary inclusion of concentrates around 50% (Mengesha, 2012; Mottet et al., 2017). Cereals are feed ingredients whose nutritional target, apart from monogastrics, is human. The share of cereals used as feed is therefore subtracted from the human consumption. The issue here is that a nutrient-rich product such as cereals are not available for human consumption (Boumans et al., 2022). In addition to monogastrics, the dietary habits of ruminants are usually based on forage feeds. However, in some cases, they are also fed with human-edible foods such as concentrates, although to a lesser extent than monogastrics. The inclusion of concentrates in ruminant diet provides higher energy density and a proper level of essential amino acids and metabolizable proteins, the latter difficult to achieve by relying only on the microbial protein synthesis in the rumen. The use of concentrates increases the productive yields in terms of higher dry matter intake, generating increased milk and

meat yields from dairy cows and beefs, respectively (Wilkinson and Lee, 2018). The inclusion of concentrates in farm animal feeding and the increased share of concentrates needed to support the demand for animal-source food are the main examples of direct feed-food competition. This competition occurs when humans and animals compete for the same dietary product, in this case cereals. The use of this cereal share for human consumption would contribute to global food security and a lower environmental impact of feed production, but should involve the search for alternative feed ingredients, since animal nutrition has to raise the productive yields to be responsive for the food demand (Boumans et al., 2022; Sandström et al., 2022). Stakeholders seek optimal solutions for future cropping and livestock production in terms of minimal impacts on land and water use, increased investment opportunities and job creation, thus improved social welfare. To address feed-food competition, one option is to implement the use of human-inedible foodstuffs instead of using human-edible products for the formulation of animal feeding. Examples of human-inedible foodstuffs are crop residues and by-products. The crop by-products are also called low-opportunity cost feedstuffs, because they do not require additional energy and resources to be produced but only processing, to make them suitable feed. Farm animals are known to be "bio-converters" (Pinotti et al., 2023), because they are capable of recycling food materials no more suitable for human nutrition into nutrients and energy available for human consumption. If human-inedible foodstuffs were massively used, the environmental impact of farm animals would be decreased because crop by-products do not require more arable land or water for their production, since they are a biomass that would be otherwise discarded (Van Zanten et al., 2018). The replacement of 15% of food-competing feedstuffs currently applied in animal nutrition with crop residues and by/co-products could decrease the feed-food competition while increasing the global supply of kcal by 13% and proteins by 15% (Sandström et al., 2022). In an ideal and circular model, the feed production would not impair the environment and the use of natural resources. Also, animal and human nutrition would not overlap on the same foodstuffs. The end products of arable land would be used for food production, whereas the crop residues, the by/co-products, and the food losses and waste would find a role in animal production as novel and alternative feedstuffs. The alternative feedstuffs do not compete with human nutrition and are beneficial for the environment because they do not subtract any further share of arable land and water for their production (Bikker and Jansman, 2023). The further step of circular production should rewrite the demand for animal-source food, no longer driven by the maximization of the productive yields but only by the availability of crop residues and by/co-products as the only feedstuff available for livestock (Boumans et al., 2022). However, a more complete definition of what the competition between feed and food is should consider also two other elements: fibre and fuel. Currently, cereal crops are used not only for nutritional purposes, but also for industrial applications. Fibre is produced for the clothing industry (e.g. cotton and wool), whereas biofuel is produced by fermenting crops such as corn and

wheat. As seen above, all these cereal crops are already essential for feed and food production, therefore the competition is enlarged to fibre and fuel too. The use of crop residues for the production of non-nutritional crop-related products such as fibre and fuel is an opportunity to recycle this biomass while leaving cereal crops for nutrition, even though the use of crop residues into animal feeding is the most efficient in converting low-quality materials into available energy for human purposes (van Hal et al., 2019).

Greenhouse gas emissions

The main GHG in the atmosphere are carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). In normal conditions, the solar energy passes through the atmosphere and the Earth surface adsorbs the infrared radiations, re-radiating them as heat. The gases normally present in the atmosphere are not able to adsorb the solar radiations, hence part of the radiations escapes back in the space. Instead, the presence of GHG in the atmosphere generates the so-called "greenhouse" effect, because GHG are able to adsorb the solar energy in the form of long-wave infrared radiations. Therefore, the radiations that normally would escape the atmosphere get "trapped" in the atmosphere and released in the form of additional heat (Manabe, 2019). The major sectors responsible for global GHG emission are energy, industry, buildings, transport and AFOLU (agriculture, forestry and other land uses). In 2018, the energy sector was the one with the largest share of direct GHG emissions (34% of total GHG emissions), followed by industry (24%), AFOLU (21%), transport (14%), and buildings (6%). An increasing trend of +0.8%/year has been calculated for the GHG emissions from the AFOLU sector since 2000. The AFOLU sector comprises feed and food production, and in turn livestock production (Lamb et al., 2021). In 2010, the contribution of livestock production to the GHG emissions from the agricultural sector was estimated to be 57% (Caro et al., 2014). The massive amount of crop yields destined to produce feed (around one third of all crop production) relies on the use of natural resources and the application of fertilizers in soil to maintain crop productivity. Then, livestock is involved in the production of food, energy but also manure. Feed production and processing (including use of fertilizers and feed transportation) account for 45% of total livestock GHG emissions, whereas the enteric fermentation occurring in rumen accounts for 39% of total livestock GHG emissions. The rest of the livestock GHG emissions comes from manure management (10%) and food processing and transportation. The CH_4 from enteric fermentation is the major livestock GHG (44%), followed by similar shares of CO_2 and N_2O (around 28% each) (Gerber et al., 2013). Dairy cows and beef cattle contribute 62% of the total livestock GHG emissions, followed by pigs (14%), poultry (9%), buffaloes (8%), and small ruminants (7%). Considering the animal products, meat is responsible for around 60% of total emissions, whereas milk and dairy for the 30%, and eggs for the rest. Instead, by considering the animal species, ruminants

are the major responsible for CH4 emissions, whereas GHG emissions from monogastrics derive mostly from feed production and manure management (Gerber et al., 2013; FAO, 2023). The period 1961-2010 showed a 51% increase of livestock GHG emissions, with livestock CH_4 and N_2O emissions representing 28% and 30% of global CH_4 and N_2O emissions in 2010, respectively (Caro et al., 2014). It was estimated that the CH_4 emissions from enteric fermentation and manure management could increase by 60% from 2000 to 2030 if mitigating strategies would fail (O'Mara, 2011). Global contribution to livestock GHG emissions differs considering specific areas of the world. The developed countries reduced their contribution to total livestock GHG emissions (-23%) starting from 1970 thanks to the application of sustainable intensification strategies, whereas the developing countries showed a 117% increase in the period 1961-2010 (Caro et al., 2014). Considering all these previous data, the role of ruminants and CH_4 emissions at global level is evident. There are several reasons why most of the attention is given to CH_4, rather than CO_2, as main targets to reduce GHG emissions. First, the strategies designed to reduce CH_4 are fast and economically more feasible than for CO_2. Then, 40% of CO_2 emitted in the atmosphere has a lifespan of around 200 years in the atmosphere, whereas the other share (around 60%) is quickly degraded (Blaustein-Rejto and Gambino, 2023). Instead, the lifespan of CH_4 in the atmosphere is around 12 years but its warming potential on the atmosphere is 28-fold higher than CO_2. Therefore, tackling CH_4 emissions would impact faster on mitigating global warming. Last, CH_4 emissions from enteric fermentation and manure represent almost half of the total livestock GHG emissions (Knapp et al., 2014; Grossi et al., 2019). Overall, GHG emissions coming from livestock production account for the 14.5% of total anthropogenic GHG emissions (Gerber et al., 2013). The value of 14.5% was calculated using the data from the reference year 2005, therefore the validity of this data is still debated considering the non-recent time interval and the methodology (Twine, 2021). Regarding the methodology, the calculation of the GWP-100 (global warming potential) values to express the warming impact of each specific GHG species in the atmosphere over 100 years is the standard method. However, the standard GWP-100 does not consider the dynamics of the lifespan of the gases in the atmosphere, which is a key element to consider in the case of CO_2 and CH_4. The further step involved the use of the GWP* calculation, more tailored on the CH_4 emissions, since CH_4 is the GHG with a higher warming potential but with a shorter life span than CO_2 (Blaustein-Rejto and Gambino, 2023). The update of the methodology over time led to different estimation of the total livestock GHG emissions, because with GWP-100 the effect of CH_4 was estimated on 100 years instead of 12 years. The GWP-100 method was used for the estimation of the total livestock GHG emissions of 17.8% considering the interval of time 2001-2004 (Steinfeld et al., 2006) and 14.5% considering the interval 2005 (Gerber et al., 2013), but a re-examination of the data used to generate the 14.5% estimation led Twine (2021) to estimate this value to be 16.5% instead of 14.5%. Overall, it can be stated that the contribution of livestock to the global anthropogenic GHG emissions is around 15%,

with CH_4 from enteric fermentation representing around a quarter of the total anthropogenic CH_4 emissions at global level. In addition to enteric fermentation and feed processing, manure management is the third cause of livestock GHG emissions. Manure is composed of water and organic material as a by-product of the digestion. The mix of manure and urine is called slurry. The major gases emitted from manure are CH_4, N-ammonia (NH_3), and N_2O. The anaerobiosis causes the formation of CH_4 in manure due to the fermentation of the organic material made by the anaerobic bacteria, whereas the aerobiosis transforms NH_3 to N_2O. The CH_4 and N_2O are therefore inversely correlated, depending on the presence or absence of oxygen (Gerber et al., 2013; Wattiaux et al., 2019; Kupper et al., 2020). The practices of manure management are largely responsible for manure-related GHG emissions. Manure storage is one of the most important because it has a reduction potential of 62%, and this is why acting on storage and other practices is the best way to reduce manure GHG emissions. In addition, the use of manure to replace synthetic N-fertilizers could reduce NH_3 emissions and promote both the physical properties of soil, such as its structure and water holding capacity, and the chemical properties of soil, such as microbial immobilization of bioavailable N and subsequent increased N uptake and crop yields (Ti et al., 2019).

Ammonia

Ammonia (NH_3) is the reduced form of N and agriculture contributes to 81% of the total NH_3 emissions at global level (Wyer et al., 2022). A similar share is found in the European Union (EU), where the agricultural sector is estimated to produce 80–90% of European NH_3 emissions, and around 75% of EU NH_3 emissions comes from the livestock production (Webb et al., 2005). The three main sources of agricultural NH_3 are livestock farming, manure management and application of fertilizers in soil. The use of synthetic NH_3 to produce fertilizers ensures around 40% of the current global food production (Ti et al., 2019; Wyer et al., 2022). As seen above, manure and urine are sources of urea, and the aerobic condition favours the conversion of urea and uric acid to CO_2 and NH_3. In addition, the amount of undigested protein is another source of NH_3 release (Wyer et al., 2022). A large part of N-fertilizers applied in soil is released in the atmosphere as NH_3, representing a reason for inefficient feed-food chain processing and causing an excessive N deposit in natural ecosystems such as water basins and soil. After deposition in land and water, NH_3 increases the acidification and nutrient-N enrichment of sensitive habitats (Ti et al., 2019). The higher availability of nutrients causes eutrophication, i.e. the excessive and unbalanced growth of plant and algae whose presence can represent a source of toxins and a reason for O_2 depletion, which is essential for the sustenance of aquatic species (Akinnawo, 2023). In addition to eutrophication, the atmospheric NH_3 can react with atmospheric acids (e.g. sulphuric and nitric acids), contributing to the formation of the particulate matter with a size <2.5 µm,

known to have undesirable health effects on humans and animals at respiratory level (Webb et al., 2005; Wattiaux et al., 2019; Wyer et al., 2022). The strategies to reduce nitrate leaching in the environment involve a more proper application of N-fertilizers tailored to crop requirements (Webb et al., 2005). The adoption of nitrate- or ammonium-based fertilizers is an option to reduce NH_3 and N_2O emissions (Zhang et al., 2013; Ti et al., 2019). Feeding strategies are also seen as a valid opportunity to reduce NH_3 and urea emissions from livestock. The use of dietary additives was seen to reduce NH_3 emissions by 46%, whereas the reduced content of dietary proteins by around 43% (Webb et al., 2005; Ti et al., 2019).

Aim of the research project

The consequences of global warming on the availability of natural resources and of the growing world population on the demand for food and animal-source food make the sustainability of livestock production an important matter. The research topics addressed and investigated here focus on two major areas: i) the metabolic investigation of the impact of the dietary inclusion of food by-products (i.e. former food products) in piglets and pigs' diet ii) the use of tannins as CH_4-mitigating compounds to reduce CH_4 production from enteric fermentation in ruminants. The sustainability issues addressed here involved the application of *in vitro* and omics strategies and the comparison of the different methods used to characterize the same research topic, in the view of giving more reliability to the *in vitro* and omics approaches in the livestock research field. The first research project carried out and presented here involved the application of -omics technologies to evaluate the impact of the dietary inclusion of FFPs in piglets and pigs' diet on the fatty acid profile of the abdominal adipose tissue and on multiple districts such liver proteome and plasma metabolome and peptidome. Metabolomics and proteomics were chosen for their potential in detecting dietary- and metabolism-related metabolites, whose level in the biological samples might have been affected by the presence of FFPs in the diet. Peptidomics was then applied to search for potential bioactive peptides modulated by the experimental diets. Our aim was to observe if this experimental dietary treatment could affect not only the growth performance and feeding behaviour of the animals, but also the lipid deposition in the abdominal adipose tissue and the activation of certain metabolic pathways in response to the experimental diet. The outputs of this research project could be useful to further characterize the use of FFPs as alternative feed ingredient, to better tailor this sustainable feeding strategy in the view of a massive adoption of feedstuffs that do not compete with food products. Instead, the second research project regarded the use of tannins to counteract CH_4 production from ruminants. Two *in vitro* approaches to simulate rumen fermentation were adopted, called Hohenheim gas test (batch culture) and Rusitec (continuous culture). Here, the aim was to investigate the CH_4-mitigating potential of two tannin components, named gallic acid (GA) and ellagic acid (EA), on the composition of the rumen microbial community and on the end products of enteric fermentation, with CH_4 production under the spotlight. Our hypothesis was that the use of single tannin components instead of tannin plant extracts might be useful to better characterize their mode of action on an expected CH_4 mitigation together with a potential and partial depression of rumen fermentation. The outputs of this research project could be useful to better characterize how tannins act in rumen and how the rumen microbiota is modulated in their presence, to assess if these *in vitro* approaches could be moved to an *in vivo* application. To summarize, the final aim of the thesis was to investigate the potential of several *in vitro* and omics methodologies to address issues related to the sustainability of livestock production, in

particular the decrease of the feed-food competition for human-edible feedstuffs and the mitigation of CH_4 emissions from the enteric fermentation in rumen.

Section A – Former food products to address feed-food competition

Overview

The global demand for food of animal origin is continuously increasing in association with the global growing population. Among all the food, animal-source food is one of the leading categories together with vegetables and cereals, therefore it is crucial to design and apply strategies to fill this demand. Regarding meat production, the higher food demand is based on the massive use of natural resources such as water and land for crop production and emissions of GHG (mainly CO_2) from all the activities related to soil and manure management, energy use, transport, processing of crops and use of fertilizers (Chataut et al., 2023). As seen above, about 40% of global arable land is used for feeding livestock. The area used for cereal production intended for the diet of monogastrics is around 20% of the global land used for cereals. The cereals used as feed ingredients for monogastrics are human-edible, therefore this 20% of cereals and the amount of land used to produce them is subtracted from potential human utilization, exacerbating the feed-food competition and reducing the food biomass that could be used to tackle food insecurity (Mottet et al., 2017). In addition, FAO stated that about one third of all the food produced globally is lost or wasted, thus wasting the energy and the resources needed to produce this amount of food and all the nutrient and energy content retained in this food biomass (FAO, 2011).

The environmental impact of feed production and the necessity to find alternative and sustainable feed ingredients led to focus on the reduction of food waste. Overall, food waste drops out at different stages on the food chain, but the term waste is an “umbrella term” which is typically used as synonym of losses, leftovers or also former food. It is important to make a distinction of these subclasses considering their origin and dietary role, since they fall within different regulation. So far, there is not a unique definition of food waste and loss. Usually, food waste refers to food material produced and intended for human consumption that drops out from the later steps of the food supply chain involving human activities, such as the case of retail waste and consumer household food scraps. Instead, food loss refers to a decreased quality or quantity of food material right at the first steps of the food supply chain and involving the lack of infrastructures, such as the post-harvesting activities (Gustaffson et al., 2013; Pinotti et al., 2021). More recently, some authors have questioned these definitions, since they do not consider the final destination of the wasted or lost food material and also without addressing the specific responsibility of this action, so if it is more related to stakeholders or consumers (Van Raamsdonk et al., 2023). Therefore, a broader definition of food waste could help, although it still needs to be detailed (Ostergren et al., 2014; Chaboud and Daviron, 2017).

As reported in the EU Catalogue of Feed Materials (EU, 2013), FFPs are food materials produced in accordance with food regulation and intended for human consumption, thus being still nutritionally valid and safe, but no longer destined for the human diet due to logistical and technical reasons occurring in the food supply chain, such as problems during transport, industrial processing, delivery, or packaging defects (EU, 2013). The main distinction between FFPs and food waste is that FFPs are leftovers legally still suitable for animal consumption, whereas for food waste it is more difficult to generalize, since the nutritional value and the safety of food waste must be addressed before reusing them in the feed chain. Food waste can also include quality-degraded or even contaminated foodstuff, which of course is no longer suitable for both animals and humans, as indicated by feed regulation (EU, 2018; Pinotti et al., 2021).

Our research focused on FFPs because they legally allowed to be used as feed ingredient (EU, 2018), representing a real and valid way to: i) keep nutrients and energy in the feed-food chain, ii) reduce the amount of food waste, and iii) recycle a biomass that would instead be discarded. The European Former Foodstuff Processors Association (EFFPA) reported that currently about 5 out of 60 million tons of former foodstuffs are processed every year in Europe and animal feed represents the best candidate (EFFPA, 2023). The research on the application of FFPs as feed ingredients is therefore crucial to raise the knowledge and the reliability of FFPs in the field of animal nutrition. This would help FFPs processors, stakeholders and farmers to implement the use of FFPs and potentially reduce the cost of their industrial processing. In the current project, we used specific FFPs coming from the industry of ultra-processed and ready-to-eat food, such as sugary and salty snacks, chocolate, candies, biscuits, chips, but also pasta and bread no more suitable for human consumption (Figure 1).

Figure 1. Packed and unpacked industrial food leftovers before being processed by FFPs processors. Images from Pinotti et al. (2021) and EFFPA (www.effpa.eu).

Despite their suitability for human consumption, FFPs contain a higher amount of fats and sugars if compared to the conventional cereals usually included in the animal diet (Giromini et al., 2017). They are a fat and energy-fortified version of conventional cereals and grains included in the diet for monogastrics, and this is the reason why FFPs are a valid feed ingredient especially for swine nutrition. Indeed, swine are the most suitable animal model for the nutritional application of FFPs because they are omnivorous and can easily convert human inedible food into animal proteins within a valid interval of time for the market. In addition, the high energy content and the higher digestibility of sugars and starches make FFPs appropriate for the diet of piglets and growing pigs (Ottoboni et al., 2019). Considering the origin and the nutritional content (detailed below), the FFPs were included into traditional swine diets both as a blend of ingredients with no distinction (Tretola et al., 2019a) and in two different diets considering the characteristics of the food materials the FFPs come from, so those in which the salt content is higher (e.g. salty snacks, crackers, chips, pasta, bread) and those in which the sugar content is higher (e.g. chocolate, biscuits, cakes, croissants, sweet snacks) (Luciano et al., 2021; Mazzoleni et al., 2023a). Table 1 shows the average nutritional content of the FFPs involved in the *in vivo* trials cited above (Tretola et al., 2019a; Luciano et al., 2021), compared to bakery meal and to some of the main cereals usually included as energy sources in the pig diet, in this case maize, wheat and barley.

Table 1. Average nutritional content of FFPs, bakery meal and main cereal crops used as pig feedstuffs. Data for FFPs from Tretola et al. (2019a), Luciano et al. (2020, 2021), Pinotti et al. (2021), for bakery meal from Luciano et al. (2022a) and Liu et al. (2018), and for cereal crops from Varel and Yen (1997) and Stein et al. (2016). For FFPs and bakery meal, the values are presented as mean ± standard deviation (SD).

Item (g/100 g DM)	FFPs	Bakery meal	Maize	Wheat	Barley
DM	90.0 ± 2.0	87.7 ± 5.8	88.2	89.9	89.1
EE	9.1 ± 1.7	6.8 ± 3.7	2.9	1.1	3.0
CF	2.8 ± 1.0	-	4.1	3.2	5.3
NDF	14.6 ± 4.6	13.8	10.2	14.9	18.5
ADF	4.6 ± 2.8	6.2	2.9	3.6	5.8
CP	10.8 ± 0.7	12.4 ± 0.2	8.1	14.0	10.8
Ash	3.0 ± 1.1	4.1 ± 0.7	1.4	2.0	4.1
Starch	46.0 ± 4.4	43.1 ± 2.1	62.1	57.6	49.7
ME (MJ/kg DM)	15.9 ± 1.2	-	14.2	13.8	12.2

Abbreviations: DM = dry matter; EE = ether extracts; CF = crude fibre; NDF = neutral detergent fibre; ADF = acid detergent fibre; CP = crude protein; ME = metabolizable energy.

Despite the slightly higher fat content of FFPs than bakery meal (9.1 vs 6.8 g/100 g DM, respectively), the average nutritional content of the FFPs is similar to the observations reported by Liu et al. (2018). In this study, the authors collected 46 different sources of bakery meal from commercial feed mills all

over US. Bakery meal is another term similar to FFPs, and its ingredient composition is similar to FFPs: bread, pasta, cookies, and breakfast cereals. Furthermore, Liu and authors observed a low coefficient of variation in the nutritional content among the 46 different samples collected, meaning that the supply of multiple bakery meal sources from different geographical areas of US has a limited impact on the chemical composition of the final products. This works in favour of a possible broader application of FFPS in animal nutrition, because farmers and stakeholders would not need to rely only on single FFPs processors located in specific areas, reducing the cost of transportation and the GHG-related emissions (Liu et al., 2018).

As reported in Table 1, a lower CF is observed for FFPs, although the NDF and ADF content is similar to the cereal grains. Another important component is starch, which is lower than in maize, wheat and barley, but it is characterized by a higher digestibility. Indeed, the FFPs are processed and ultra-processed industrial food, therefore they undergo several mechanical and heating processing. All these processes modify the physicochemical structure of starch, leading to the disruption of the food matrix and the gelatinization of starch, thus making it more hydrolysable by the enzymatic action in the gastrointestinal tract, and consequently more digestible (Tretola et al., 2019b; Pinotti et al., 2023).

Fat content

The fat content is the most different nutrient value among FFPs and cereals. The FFPs have a fat content around 4 times higher than cereals (Table 1). What is interesting is the fatty acids profile of FFPs, because it is known that industrial processing of bakery products and FFPs mostly involves margarine, butter and hydrogenated vegetable oils (Albuquerque et al., 2017). The predominant content of palmitic acid (C16:0) among saturated fatty acids (SFA) and oleic acid (C18:1) among mono-unsaturated fatty acids (MUFA) was reported in two different studies dealing with the fat content of sugary and salty bakery and chocolate snacks from different areas of the world, Brazil (Dias et al., 2015) and Turkey (Omeroglu and Ozdal, 2020). The fatty acid profile of the fat fraction of FFPs was reported to be different from a standard pig diet based mainly on cereals. In Mazzoleni et al. (2023a), the FFPs were subdivided depending on their origin in two dietary groups, named salty FFPs (bread, pasta, chips, crackers) and sugary FFPs (chocolate, snacks, cookies, cakes), and included at 30% into a standard pig diet, replacing 30% of cereals. These diets were formulated to be isoenergetic and isonitrogenous. In both these two experimental diets, a higher content of MUFA (+12%) was observed compared to the control diet, whereas the content of poly-unsaturated fatty acids (PUFA) was similar between the two experimental diets and the control diet. Regarding SFA, the sugary FFPs-diet had a higher content (+7%) compared to the salty FFPs-diet. These differences were probably given by the food ingredients used to make FFPs.

Digestibility

After considering the nutritional content of FFPs, the digestibility of these food leftovers needs to be detailed. As reported above, the nature of the starch content in FFPs make them more digestible than conventional cereals, even though this is not the only reason why FFPs are easily-digestible. Giromini et al. (2017) reported that the value of the *in vitro* digestibility of a standard feed sample (wheat) was similar to the average digestibility calculated from six FFPs samples. Further, Ottoboni et al. (2019) measured the digestibility rate and the kinetics of carbohydrate digestion in six FFPs samples. In addition, the authors decided to measure the digestibility of FFPs by including them into a feed ration, in order to evaluate the effect of FFPs on the diet digestibility. Therefore, one standard pig feed sample with or without the 30% inclusion of FFPs and two positive control samples (unprocessed maize and heat-treated wheat) were evaluated. The digestibility was measured by calculating the predicted glycemic index, a value that considers both the potential and the time of digestion. Specifically, the glycemic index measures the time of the clearance of glucose in blood. If high, the consequence is a faster return to hunger, thus raising the feed intake of the animal. The six FFPs samples were characterized by a higher glycemic index than the control samples. Also, the standard pig feed showed a similar value of glycemic index whether it was supplemented or not. However, the supplemented feed sample showed a higher carbohydrate digestion rate compared to the pig feed without FFPs supplementation, thus suggesting a quicker digestibility of carbohydrates (Ottoboni et al., 2019). All these results reported above showed that the FFPs have a higher digestibility than the conventional cereals, and this is mostly because of their nutrient composition and the industrial processing they undergo. An example is the high digestible rate of starch. However, all these results were obtained in *in vitro* assessments, therefore other evaluations were needed to verify the digestibility of FFPs during *in vivo* trials. In two previous studies (Tretola et al., 2019b; Luciano et al., 2021), the apparent total tract digestibility (ATTD) was measured in two trials involving post-weaning piglets fed with a blend of salty and sugary FFPs (Tretola et al., 2019b) and with two different FFPs diets depending on the origin of FFPs (Luciano et al., 2021). In both trials cited here, the ATTD was not altered by the presence of FFPs in the diet of piglets, even though the duration of the *in vivo* trials were 16 and 42 days, respectively. Furthermore, the ATTD was measured in another study involving growing and finishing pigs (Mazzoleni et al., 2023a). Here, the salty FFPs-fed group showed a higher energy ATTD than the salty FFPs-fed and the control groups, whereas the crude protein energy showed a slight decrease in both the feeding groups containing FFPs than the control group. However, these differences did not lead to any significant alteration of the growth performance (Mazzoleni et al., 2023a).

Safety issues

The FFPs are food leftovers that can be at risk of contamination from packaging remnants and potential microbial contamination. For what concerns packaging, the defects in packaging are one of the reasons why industrial food is removed from the food production chain, and for which there is the possibility of a "second life" as FFPs. The FFPs processors do not manually unpack each food ingredient coming from the industry but everything is processed together, and then a mechanical sorting is performed to remove most of the packaging remnants. The nature of the packaging remnants depends on the food source, but the major components are cellulose, plastic, and aluminum (Luciano et al., 2022b; Mazzoleni et al., 2023b). Several methods were applied to detect the presence of packaging materials. With stereomicroscopy coupled with a computer vision system, Tretola et al. (2017) detected a presence of 0.08% w/w of packaging materials, a value below the legal threshold (0.125% w/w). In addition, the Fourier Transform Infrared Spectroscopy method was applied in detecting packaging remnants in 17 FFPs samples (Mazzoleni et al., 2023b). In this study, the authors observed that about two third of the 17 samples were containing packaging materials, named cellulose, plastic and aluminum in order of abundance. However, the quantification of the detected materials was below the required threshold and, considering the limited percentage of inclusion of the FFPs in feed, the authors concluded that FFPs could be considered a safe and alternative feed ingredient (Mazzoleni et al., 2023b). Regarding the potential biological contamination by microbials, it is important to remind the extensive round of industrial (e.g. thermal and mechanical) processing the FFPs undergo, because the food sources where they come from are at first produced for human consumption and must comply with human food regulation. The industrial processing is intended to give the final product some physical and chemical properties to make it more suitable for human consumption, but it is also useful to reduce the hazard of the microbial content in food (Pinotti et al., 2021). In this regard, Tretola et al. (2017) evaluated the microbial load of 6 FFPs samples. The authors found a limited total viable count falling within the threshold (5 log colony-forming unit per gram of substrate) and the absence of *Salmonella*, which is considered the main hazard for biological contaminations of food (Tretola et al., 2017). To summarize, both the risk of microbial and packaging material into FFPs is limited and within the legal threshold. However, attention must be paid for the evaluation of these aspects, because the food sources of FFPs arrive to the FFPs processors from different areas of the world and industrial food plants.

After discussing the nutritional content and the safety of FFPs, it is important to remind that the sources of the FFPs considered here are, apart from pasta and bread, industrial salty and sugary snacks, chocolate, chips and bakery-processed food. This broad class of food has a similar nutritional content, already detailed for the FFPs, but also a series of food additives used mostly for the food market, such as colourants and flavouring and texture agents (Fardet and Rock, 2020). The detection of metabolic markers of ultra-processed food consumption has been performed only on human subject, because

this class of food is primarily intended for humans. In human serum, several metabolites such as catechol sulphate, stachydrine, caffeine, theobromine, glucose, and mannose were associated with the consumption of ultra-processed food. In this respect, a similar evaluation on farm animals fed with ultra-processed food would depict a clear indication of the downstream effect of the additive compounds in animals (Su et al., 2023).

Environmental sustainability

The issues regarding the sustainability of animal farming are receiving more and more attention due to the pressure on the production system exerted by the demand for animal-source products and the growing world population. The objective is to link the development of animal production to the environmental sustainability, always considering the economic costs of "sustainable strategies" and their impact on production yields (Rauw et al., 2020). The inclusion of food leftovers into animal diets represents a valid way to recycle a biomass full of nutrients and energy that would otherwise be discarded, thus keeping the nutrients in the feed-food chain and reduce the amount of wasted material (Van Raamsdonk et al., 2023). Few studies have assessed the production of animal feed from FFPs, instead dealing more with the broader category of edible and safe food waste as feed ingredient (Kim and Kim, 2010; Alsaleh and Aleisa, 2023). In these two studies, the authors applied life cycle assessment (LCA), a tool used to evaluate the environmental impact of a product throughout its entire life cycle, for example considering the GHG-related emissions and the impact on the use of natural resources (Finnveden and Potting, 2014). In Kim and Kim (2010), the authors proved that food waste could have a lower global warming potential if applied as animal feed instead of sending it to landfill. The animal feed was found to be a valid option for food waste recycling also by Facchini et al. (2023) after conducting a systematic analysis of 101 selected articles focused on the sustainable options to recycle food waste. The main weakness detected was that a quantitative economic evaluation of a real business based on food waste processing and application in animal feeding should be better detailed yet (Facchini et al., 2023). However, an analysis involving the incorporation of environmental, economic, and social impact of processing food waste (collected at the post-consumption step of the food supply chain) into animal feed revealed that the minimum recovery rate of food waste useful to make the process environmentally feasible would be 48%, which is higher than the current rate (13.8%). The only way to achieve this result should be to recover food waste already at the first steps of the food supply chain (Alsaleh and Aleisa, 2023). This is another reason why the use of food waste is important, since it mostly relies on the recovery of food leftovers from the pre-consumption step, such as during the industrial processing (Pinotti et al., 2023).

Feed-food competition

Another crucial objective linked to the use of FFPs and food leftovers is the reduction of feed-food competition. As discussed in the Introduction, this competition comes from the massive reliance of feed production on human-edible food, with cereal crops being the most important example. The 30% of cereal crop production is currently destined to livestock (Schader et al., 2015; Muscat et al., 2020). Hence, the need to increase feed production to face the growing demand for animal-source food would therefore cause an increase of this percentage. This would turn to a lower food security, with even less supply of cereal crops available for human consumption, and a higher environmental impact, since cereal feed production and processing contributes to 45% of total livestock emissions so far (Grossi et al., 2019) and it would require higher rates of land and water (Pinotti et al., 2023). A reduced use of crops for feed and a reuse of food waste products is seen as a considerable opportunity to provide energy and nutrient-rich feedstuffs to livestock (Schader et al., 2015; Makkar, 2018). The reuse of food waste and human-inedible food products also represents the best way to convert a food waste product into high-quality food, such as meat and milk. Ruminants are able to convert low-quality plant fibres into energy and proteins, whereas the omnivore nature of monogastrics makes them suitable "converters" of food waste into animal proteins (Van Zanten et al., 2016). The FFPs respond to each of the environmental questions, because they are human-edible food leftovers and reduced food-competing feedstuffs that are reintroduced in the food chain in replacement of human-edible cereal crops. The advancement in the research on FFPs will provide a stronger evidence on their potential and a higher economic opportunity on their application as feed ingredients.

Former food products in pig nutrition

Table 2 summarizes some of the most recent *in vivo* trials performed involving the use of FFPs and/or bakery meal as feed ingredients for swine nutrition. The criteria of selection of the studies reported in Table 2 was based on the use of the keywords "former food products" and "bakery meal" for the literature research.

Table 2. List of recent studies carrying out *in vivo* trials with inclusion FFPs and/or bakery meal in swine diet.

Animals	Dietary supplement	Inclusion rate	Duration (days)	Parameters	Reference
12 post-weaning piglets	FFPs (blend)	30%	16	Influence on fecal microbiota	Tretola et al., 2019a
				Growth performance, digestibility,	Tretola et al., 2019b

				selected plasma metabolites	
36 post-weaning piglets	Salty FFPs and sugary FFPs	30%	42	Growth performance, digestibility, selected plasma metabolites	Luciano et al., 2021
				Influence on gut microbiota	Tretola et al., 2022
160 growing and finishing pigs	Bakery meal	25, 50, 75, 100%	35	Growth performance	Luciano et al., 2022a
36 growing and finishing pigs	Salty FFPs and sugary FFPs	30%	150	Growth performance, feeding behavior, digestibility, carcass composition	Mazzoleni et al., 2023a
60 pigs (aged 80 days)	Bakery meal	15, 20%	56	Growth performance and behavior	Termatsidou et al., 2023
80 finishing pigs	Bakery meal	20%	100	Growth performance and meat quality	Malamakis et al., 2023
48 growing pigs	Bakery meal (11 sources)	100%	7 (for 21 times)	Amino acids digestibility	Stein et al., (2023)

In this context, the discussed food leftovers, named FFPs and bakery meal, act as energy sources for post-weaning piglets and growing pigs instead of the conventional energy sources listed in Table 1 (corn, wheat, barley). The energy is mostly given by the high fat content, the highly-digestible starch and the content of simple sugars available. The studies listed in Table 2 concern the inclusion of different formulations of FFPs (salty FFPs, sugary FFPs, blend) and bakery meal in the diet of pigs during different life phases (post-weaning phase and growing-finishing phase) and with different doses of inclusion. Despite the different experimental conditions, all these studies agreed in defining FFPs (and bakery meal) as nutritionally valid, safe and sustainable feed ingredients. In particular, the inclusion rate of 15 to 30% of bakery meal did not affect the growth performance in terms of body weight (BW), feed intake (FI), average daily gain (ADG) and feed conversion ratio (FCR) (Luciano et al., 2022a; Malamakis et al., 2023; Termatsidou et al., 2023). However, an inclusion rate of bakery meal higher than 30% and up to the complete substitution of cereal grains (corn up to 100% i.e > than 30% of dietary DM) in the in the diet of post-weaning piglets led to reduced gain to feed ratio, which is a sensible parameter to consider especially for the post-weaning phase (Luciano et al., 2022a). Regarding FFPs, it was observed that a replacement of conventional cereals in the diet of post-weaning piglets

with a 30% of a blend of salty and sugary FFPs did not affect the growth performance and the feeding behavior except for reduced FCR (Tretola et al., 2019a). The inclusion rate of 30% was safe also in the case of subdividing FFPs into salty and sugary, depending on their origin. The use of salty and sugary FFPs as substitutes of cereal grains for the diet of post-weaning piglets (Luciano et al., 2021) and growing and finishing pigs (Mazzoleni et al., 2023a) did not cause any adverse effect of growth performance and feeding behavior in the overall period, despite some fluctuations of the parameters evaluated between the growing and the finishing phases (Mazzoleni et al., 2023a). Furthermore, Mazzoleni et al. (2023a) also observed that salty and sugary FFPs for feeding pigs did not negatively affect the proportion of nutrients (e.g. protein and fat) in the carcass, leading to similar carcass composition between the groups fed with FFPs and the control group (Mazzoleni et al., 2023a). For what concerns the digestibility of the FFPs-supplemented diets, Tretola et al. (2019b) observed a higher *in vitro* and *in vivo* ATTD for the FFPs-diets compared to the control diet. Instead, the use of salty and sugary FFPs in separate diets for post-weaning piglets showed reduced ATTD for the piglets fed with the sugary FFPs-diet, compared to salty FFPs and control diets (Luciano et al., 2021). However, this was a small-scale study performed just for the initial phase of the weaning in a very controlled situation. Indeed, the same diets were then applied in the case of growing and finishing pigs (Mazzoleni et al., 2023a). In this work, the salty and sugary FFPs dietary groups showed different nutrient ATTD. In particular, the salty FFPs diet increased the gross energy ATTD compared to control and sugary FFPs diets and this was probably because of the different level of simple sugars and processed starch content in the two FFPs diets. Also, both FFPs diets decreased the ATTD of CF, possibly because of the lower CF content of the two diets compared to the control diets. Despite all, these differences did not lead to alterations in the growth performance of pigs (Mazzoleni et al., 2023a).

To summarize, the dietary inclusion of 30% FFPs as an alternative and sustainable source of energy and nutrients in pig feeding was proved to be safe for the production performance of piglets and pigs, without causing any relevant alteration of the most important *in vivo* growth parameters, feeding behavior and digestible rates. The differentiation between salty and sugary was useful to better characterize the effect of these two FFPs formulation, but the experimental outputs were most of all similar between the pigs fed either with the salty or the sugary diet, eventually. Therefore, the FFPs could be compared to conventional cereal grains considering their nutritional and functional role: i) nutritional because FFPs could provide high energy in forms of fats, sugars and starch to the animals without impacting on carcass composition and diet digestibility, and ii) functional because the different nutrient composition and the overall higher digestibility rate of FFPs could lead to similar growing trends compared to the animals fed with conventional cereal grains. The substitution of up to 30% of FFPs could thus save 30% of cereal grains, giving the possibility to redirect them to the human

consumption and to reduce the use of natural resources (e.g. water and land) employed for their production and ensure global food security.

Effect of the diet on fatty acid profile of adipose tissue in pigs

As seen above, FFPs have a peculiar fatty acid composition if compared to conventional cereals used as feedstuffs for pigs, with an overall higher level of MUFA. The fatty acid composition of FFPs and of dietary sources in general is crucial to determine the outcome of pork meat. The process of fat deposition in the body depots depends on several factors (diet, breed, sex, age, etc.), but it was observed that the fat deposition of each specific fatty acid is affected by its level in the dietary fat source (Duran-Montgé et al., 2008; Schumacher et al., 2022). The similarity between the fatty acid composition of the diet and of the adipose tissue is more evident in monogastrics than in ruminants, because in monogastrics the lipid deposition rate is higher and in ruminants the unsaturated fatty acids undergo biohydrogenation in rumen. Therefore, the biohydrogenation modifies the original profile of the dietary fatty acids (Fanalli et al., 2022; Schumacher et al., 2022). The dietary fat sources directly target the adipose tissue of pigs by affecting the gene expression of the tissue, as observed by transcriptomics studies involving different levels of dietary fat intake (Yang et al., 2018) and different sources of dietary fat, such as coconut oil, rapeseed oil, and beef tallow (Oczkowicz et al., 2019). The effect on gene expression is then reflected on the fatty acid profile of the adipose tissue, and an example is the use of linseed oil as a dietary source of n-3 fatty acids for pig nutrition to increase the level of n-3 fatty acids in the animal-source products (Kouba and Mourot, 2011).

Application of omics tools

At this point, it was crucial to further characterize the application of FFPs as feed ingredients in a more comprehensive and in-depth direction, trying to consider not only the impact of FFPs on the "macroscopic" variables such as growth and feeding performance, but also on the "microscopic" variables, such as the evaluation of the metabolic activity of animals fed with FFPs. We discussed above the nutrient composition and the FFPs-diet formulation, and even though the diet containing FFPs did not alter the overall status of the animal, it is even possible that the different content of nutrients and compounds from the ultra-processed food chain in the FFPs could exert a perturbation on the metabolism of the animals. Therefore, we decided to focus on a more and comprehensive evaluation of the potential impact of the dietary inclusion of FFPs on the metabolic status of FFPs-fed piglets and pigs. To do this, we explored the use of high throughput omics techniques as gold standard for the in-depth evaluation of the metabolic status of the animals subjected to particular treatments, including experimental dietary regimens. An evaluation of the metabolic perturbation exerted by the use of FFPs

as feed ingredients was already performed on a selected panel of 12 hematological metabolites (Tretola et al., 2019b; Luciano et al., 2021). These metabolites were chosen to be representative of the nutritional health status of the piglets in the post-weaning phase fed with FFPs, which could be homeostatic or stressed depending on the action of FFPs. The results showed that after 16 days of dietary treatments, only glucose was significantly increased and urea was significantly decreased in the FFPs-fed group compared to the control group. Those results were probably given by the higher digestibility of starch and higher glycemic index of FFPs (Tretola et al., 2019b). Instead, any significant difference was observed on a similar panel of hematological metabolites in post-weaning piglets fed with salty and sugary FFPs compared to the control group for a longer period of time, 42 days in this case (Luciano et al., 2021). The results reported above suggested that, apart from minor alterations, the FFPs did not affect the nutritional health status of the post-weaning piglets involved in the study, at least for what could be observed from the panel of hematological metabolites considered. In any case, a broader and more comprehensive assessment of the metabolic status of the FFPs-fed pigs could be useful to encompass if and how FFPs alter the metabolic activity of the animal. This is the reason why the omics technologies can be applied in this context. Overall, the omics technologies are applied more and more in the field of livestock production because of their high throughput potential to screen and evaluate the role of certain factors such as the environmental conditions, the impact of nutrients as "metabolic signals" for the organism in charge of responding to these external stimuli, and the gender on animal physiology and welfare. In the field of animal nutrition, the omics technologies enable to understand the role of nutrients on the metabolic activity of the organism, and in particular how nutrients or some of their constituents affect the metabolic pathways such as gene expression, fat and protein metabolism, in a systematic and holistic approach. Hence, it is now evident the potential role of omics technologies when applied and integrated for achieving a full screening of the animal physiology and welfare (Ribeiro et al., 2020). Below, we discussed the application of three specific omics approaches, named metabolomics, proteomics, and peptidomics, for an in-depth evaluation of the dietary role of nutrients on animal metabolism. In particular, these three experimental approaches were applied in the context of two *in vivo* trials were the FFPs were used to feed pigs.

Metabolomics

Metabolomics enables the qualitative and quantitative study of all the metabolites, also called small molecules, in a specific district and in a specific time. The selected district can be a cell, a tissue, or an organ. The metabolomics investigation can be performed to get an untargeted and comprehensive profiling of all the metabolites detected and identified into a specific sample, but also to direct the

analysis to a small and specific panel of metabolites, for example being part of the same metabolic pathway (Chakraborty et al., 2022). The most common metabolomics tools are nuclear magnetic resonance and mass spectrometry (MS). Usually, the higher sensitivity and the possibility to couple MS with high-resolution separation techniques such as gas or liquid chromatography, make MS the main metabolomics tool for the systematic investigation of the metabolome (Gowda et al., 2008). After obtaining the MS spectrum, several databases such as the Human Metabolome Database (HMDB) and MassBank can be used to identify the metabolites detected in the spectrum starting from their m/z ratio, where m is the mass and z is the charge of the metabolytes detected by MS, and the different metabolites can be discriminated for their different m/z value. Then, several bioinformatics software such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the open-source MetaboAnalyst can be applied for the functional evaluation to observe if and how the identified metabolites are either or not associated with specific metabolic pathways. From the functional analysis, it is also possible to detect omics-derived biomarkers, useful to monitor their level in certain pathological or experimental conditions (Ribeiro et al., 2020). Since we deal with animal trials, the metabolomics results can be eventually correlated with the growth and feeding parameters, to assess if the observed alterations of the metabolome have a correspondence with the phenotypic alterations of the animal (Chakraborty et al., 2022).

The metabolomics tools have been applied to the field of nutrition (animal and human) to study the effect of nutrients on the host metabolism. Specifically, the metabolomics investigation is useful to characterize the metabolic response to the dietary components and predict their effect on the physiology of the animal. Indeed, nutrients affect gene expression and consequently protein expression and the downstream level of specific metabolites in different districts of the organism (Zduńczyk and Pareek, 2009; Kortesniemi et al., 2023). In addition, the metabolomics investigation can also detect novel biomarkers related to specific dietary treatments, for example exposure to ultra-processed food in human subjects (Su et al., 2023). The untargeted metabolomics approach was widely applied to observe the effect of treatments and experimental dietary exposures to high or low levels of nutrients in different districts of pigs, such as plasma and liver (Hanhineva et al., 2013; Jégou et al., 2015; Nørskov et al., 2013; Sun et al., 2015). For example, the muscle tissue was sampled from pigs fed with a low-protein diet either supplemented or not with glycine and evaluated through untargeted metabolomics, to assess the effect of these two diets to potential improvements in meat quality (Jiang et al., 2023). Similarly, Liu et al. (2021a) found that a low-protein diet affected the muscular amino acid and fatty acid profile in an age-related trend. Then, also the dietary starch levels were explored in the diet of pigs fed with different starch sources (e.g. tapioca or pea starch diets) to observe a potential perturbation of the metabolomic profile and of the cecal microbial composition on cecal digesta samples (Yu et al., 2022). As observed, all the studies cite above involve the use of “unbalanced” diets,

where one or more nutrients are increased or decreased. Also, what is still missing is the application of the metabolomics tools in the field of sustainable animal nutrition, to observe the impact of by-products or food leftovers, such as the FFPs, on the metabolome of pigs. The only example found was the study of Belloumi et al. (2023), where the authors supplemented the diet of pigs with dehydrated citrus pulp, a by-product of citrus production with a considerable energetic and nutritional content (e.g. soluble fibre, pectin and sugars). The supplemented diet led to variations in the metabolism of amino acids and fatty acids, known to be the components that are majorly affected by nutrients (Belloumi et al., 2023). The metabolomics evaluation can be applied to a wide variety of samples, but plasma (and also serum) is the most intensively used after urine. The metabolome profile of plasma can be regarded as a summary of the variations occurring in the organism in response to internal and external stimuli, including nutrients (Kiseleva et al., 2022). In this direction, research evidences on the use of plasma samples for untargeted metabolomics have been well documented, mostly to study the effect of specific pathologies on the plasma metabolome and the whole metabolic status of the animal (Kertys et al., 2020; Jiang et al., 2021; Sun et al., 2021).

Proteomics and peptidomics

The proteomics tools are applied to analyse all the proteins present in a specific cell, tissue, or organ and in a definite time point. Proteins are the endpoint of genome expression, representing the final step of the influence of exogenous triggers on the organism. Proteome is the protein endpoint of the genome expression and is a highly dynamic element responding to different exogenous and endogenous stimuli, in contrast to the "static" nature of the genome (Zduńczyk and Pareek, 2009). The variation of the protein abundance in a specific experimental condition compared to a control condition can provide insights on how the experimental stimuli (e.g. environment, drugs, pathological conditions, dietary treatments) can impact on the proteome, and if the altered proteins are involved in specific metabolic pathways. The main techniques involved in proteomics are chromatography (gas or liquid) and MS. The two techniques are often coupled, in order to use chromatography to better separate the single protein components before being further analysed with MS-based approaches. Usually, between chromatography and MS, the protein pool isolated from a cell, tissue, or organ, is digested by a protease enzyme of which the cleavage site is knows (e.g. trypsin). The enzymatic digestion is a crucial step for two reasons. First, because it provides peptides with a suitable size of the amino acid sequence for the subsequent MS analysis. Then, because knowing the cleavage site of the peptides makes the downstream matching of the peptide fragments to the MS spectrum easier, to better identify the peptides starting from the known first and last amino acid of their sequence (Laskay et al., 2013; Chakraborty et al., 2022). Several features of proteomics are similar to metabolomics,

peptidomics and to the other omics techniques, such as the possibility to detect specific biomarkers of experimental conditions (Alharbi, 2020). Peptidomics is a tool for the detection and identification of peptides within a biological sample in a specific interval of time. The experimental approaches of peptidomics and proteomics are similar, for example the application of MS. Indeed, the two techniques are often coupled to extend the power of the investigation on a biological sample (Di Meo et al., 2016). Usually, the peptides synthesized by the organism that have a bioactive function are the target of peptidomics but are less abundant (1-5% of total peptides) than the majority of peptides coming from the physiological turnover rate of proteins. In order to target the endogenous and bioactive peptides, peptidomics tool does not involve the enzymatic digestion of the sample because peptidomics is oriented to the pre-existing peptides in the sample, whereas proteomics involves the controlled enzymatic digestion of the sample to make the analysis easier. Therefore, enzymatic digestion is not performed in peptidomics to maintain the endogenous content of peptides while avoiding the modification of the content by "artificially" producing peptides from the enzymatic protein degradation (Dudley et al., 2010; Wardman and Fricker, 2011).

The proteomics tools are usually applied on plasma, urine and liver samples of pigs challenged with pathologies or experimental dietary regimens. Proteomics was applied in trials involving pigs affected by specific pathological conditions, such as intrauterine growth restriction (Wang et al., 2010) and infective agents (Jiang et al., 2020). In addition, other researchers applied proteomics on the comparison of different pig breeds to identify candidate genes and gene products to improve the meat quality (Yang et al., 2016). Also, Bovo et al. (2018) applied proteomics on the liver tissue of different pig breeds to unravel the link between the liver metabolism and the related production traits, such as feed efficiency, carcass composition and meat quality. The authors identified 25 differentially modulated proteins between two Italian heavy pig breeds (Duroc and Large White) that were useful to discriminate between liver proteome profile and the related production potential of the two pig breeds. The differentially expressed proteins were mostly involved in the metabolism of exogenous compounds, such as nutrients and drugs (Bovo et al., 2018). Meat quality and characteristics of pork meat were also addressed with the application of peptidomics tools, to assess how the cooking process could alter the *in vitro* digestibility of pork meat (Wen et al., 2015) and to identify potential biomarkers for the freshness of pork meat (Wei et al., 2022). Furthermore, proteomics was applied also in the field of animal nutrition. For example, Li et al. (2018) observed that long-term protein restriction in pigs led to altered metabolism of amino acids, detectable by a different abundance of blood amino acids. Then, Lepczyński et al. (2019) provided two experimental diets containing inulin in two different forms (from chicory extract or from chicory root) to growing pigs, to observe the influence of inulin on the ileal proteome and related functions. Chicory is known for its content of nutraceutical compounds (e.g. inulin-like fructans). The authors observed that both the experimental diets altered the ileal proteome

associated with the energy metabolism and cell reorganization and iron metabolism, with a potential direct action of the chicory on the maturation of ileal mucosa and iron absorption in the gut (Lepczyński et al., 2019). The liver tissue is a suitable target for proteomics studies because it is the major metabolic centre in the organism. An example of the application of proteomics on the liver tissue of pigs fed with alternative feed compounds comes from Ribeiro et al. (2022), who evaluated the use of a microalga species named *Chlorella vulgaris* as an alternative protein source with a lower environmental impact than the conventional protein sources, such as soybean meal. The authors evaluated also the coupled effect of the enzymatic action of CAZyme, used to disrupt the cell wall of plant cells and thus raise the bioavailability of proteins and n-3 PUFA. Any relevant impact on liver function was observed, concluding that *Chlorella vulgaris* could be considered an interesting alternative feed ingredient to be further characterized (Ribeiro et al., 2022). The importance of liver proteome in pigs was depicted by Golovan et al. (2008), who provided the largest liver proteome profile by that date, with 880 proteins with a considerably low level of false discovery rate (5%). The identified proteins were mostly involved in energy and nutrient metabolism. Of these 880 pig liver proteins, 80% was shared with human and mouse liver proteomes, being considered part of the "proteome core" crucial for liver physiology (Golovan et al., 2008).

The effect of nutrients and dietary treatments is reflected on the hepatic proteome mostly involving the proteins related to amino acids (Eugenio et al., 2023) and fatty acids metabolism (Szostak et al., 2016; Hodson et al., 2020). Specifically, liver is directly involved in the metabolic processes of lipid oxidation, amino acids metabolism and consequent protein secretion in blood, and elimination of waste metabolites (e.g. drug-derived metabolites, urea, etc.) (Bovo et al., 2018). In livestock production, the key role of liver in the metabolism is strictly related to the production traits such as growth performance and feed efficiency (Zduńczyk and Pareek, 2009). Considering our interest in the impact of FFPs on the physiology of pigs and in particular on liver proteome, we explored the literature dealing with the major nutritional features of FFPs. First, the digestion rate is known to affect the liver function in terms of regulation of blood glucose, insulin level and lipid metabolism (Yin et al., 2011). The use of low- or high-digestible starch feed ingredients in pig nutrition was observed to raise the triglyceride and cholesterol level in liver, with an increased expression of lipogenesis-related genes and decreased lipolytic-related genes in the case of the dietary treatment with the high-digestible starch feed source, which was eventually associated with fat accumulation in liver (Jun et al., 2010). The low digestibility of starch was associated with low cholesterol level in liver and increased lipid oxidation also by Sun et al. (2016). In addition, the ultra-processed "nature" of FFPs involves the presence of particular compounds from the food industry, such as caffeine. The liver is involved in the elimination of caffeine and theobromine, but it is known that these two compounds are related to higher lipid oxidation (Sinha et al., 2014; Wei et al., 2020).

Aim of the project (Section A)

As reported in the literature discussed above, in most cases the experimental trials involved dietary regimens with an altered level of a specific nutrient than the control diet. These cases were just reported to provide examples of the potential findings obtained after omics application. In our case, the experimental diets with salty or sugary FFPs were formulated to be isonitrogenous and isoenergetic with the control diet, so the observed impact on metabolome and proteome could be ascribed to the nutritional composition of the diet rather than to an unbalanced supply of a specific nutrient. Moreover, we decided to apply advanced high-throughput omics techniques in the "niche" of the use alternative feed ingredients as sustainable dietary treatments, to characterize their impact on animal welfare and physiology and increase the liability on their application. Therefore, the aim of the application of the omics tools was to obtain a more in-depth insight on how the FFPs-based experimental diets could influence the composition of the plasma metabolome and peptidome and the liver proteome of piglets and pigs fed with salty or sugary FFPs diets. Specifically, the untargeted metabolomics investigation was performed on plasma samples collected from piglets fed with salty and sugary FFPs. Then, a label-free proteomics and peptidomics investigation was applied on liver tissue and plasma samples (respectively) to evaluate the impact of FFPs on the liver proteome and plasma peptidome of pigs fed with salty and sugary FFPs-based diets.

Section B – Tannins to address greenhouse gas emissions from enteric fermentation

Overview

Evidences of global warming and consumers' awareness towards the environmental issues of animal husbandry are pushing livestock production to reduce the environmental impact and the use of natural resources, for a more sustainable food production and animal welfare (Fouts et al., 2022). Animal-source food provides high-quality nutrients (e.g. proteins, minerals, vitamins, etc.) and energy, a key element for ensuring food security, especially in the developing countries. Also, it is a source of income especially for small farmers and stakeholders in developing countries (Reynolds et al., 2015; Hoque et al., 2022). Farm animals can convert feed and food leftovers of various nature into high-quality food. For example, monogastrics and especially swine can be fed with food by-products falling into the broad category of food waste, giving the same productive yields as if fed with conventional cereals (Luciano et al., 2021; Mazzoleni et al., 2023). Obviously, the regulation about food waste is strict to ensure feed safety and animal welfare, but this does not mean that these alternative feed sources are less valuable than cereal crops. Among farm animals, ruminants are the only capable of converting plant fibres and forages from grasslands into energy and high-quality proteins. These forages have a low nutritional quality considering their nutritional content, mostly fibres, and humans are not able to digest it and so to derive value. The "bioconversion" is possible because of the presence of a microbial community in the rumen, able to break down plant fibres and produce volatile short-chain fatty acids (SCFA) from fermentation as a source of energy, together with other end products. The action of the rumen microbial community is what make ruminants such valid "bioconverters", because they can add value to what humans are not capable just by their mechanism of digestion and metabolization of nutrients (Smith et al., 2022c). However, conversion of these natural resources into energy available for the animal also involves a 2 to 12% loss of energy because the mechanism of metabolization is not as efficient as possible (Johnson and Johnson, 1995). This energy loss is primarily represented by CH_4 production from enteric fermentation, which accounts for half of the GHG emissions from livestock production (Gerber et al., 2013; Rojas-Downing et al., 2017). The CH_4 production has no nutritional value and it represent a loss of dietary energy for ruminants (Boadi et al., 2004). In addition to CH_4, ruminants are also responsible for the emissions of CO_2 mostly from enteric fermentation but also feed production, processing, and transportation, and N_2O from the use of fertilizers and the manure management (Wu et al., 2019). The impact of GHG from ruminant production is the reason why ruminants are considered the most impacting farm animal species on the environment (Du et al.,

2022). Below, an overview of the ruminant gastrointestinal system is reported to show which are the main factors and processes needed by ruminants to digest fibrous feed, but also to elaborate how this system could be altered to reduce the production of CH_4 and ammonia.

Rumen structure

The gastrointestinal tract of ruminants is characterized by the presence of functional and anatomic adaptations aimed at drawing energy from plant fibres. The key element is a multi-chamber forestomach that works as a large fermentation chamber able to degrade fibres from the ingested feed, before reaching the stomach. This fermentation chamber is subdivided in three sections, named rumen, reticulum, and omasum, plus the abomasum, this latter considered the "actual" stomach of ruminants because of the presence of gastric glands (Figure 2).

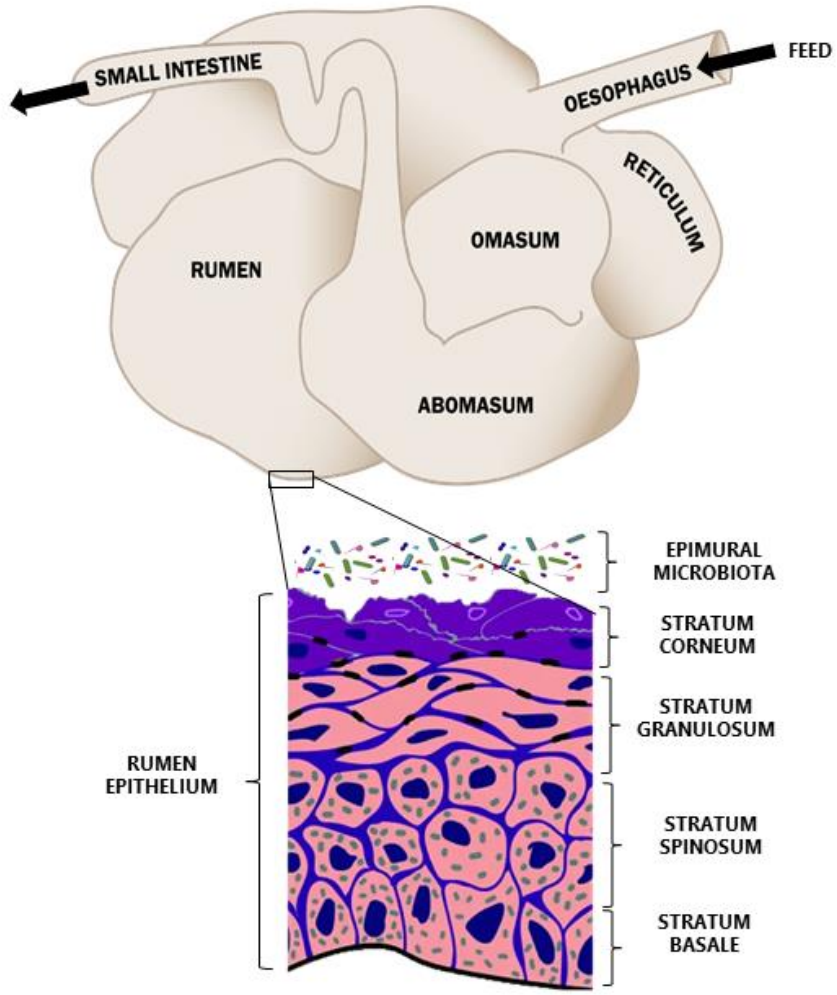

Figure 2. Structure of rumen and rumen epithelium. Adapted from Steele et al. (2016).

Rumen is the largest anaerobic and fermenting chamber among the forestomach of ruminants, with a common mean temperature of 39 °C and always buffered by saliva and by the organic acids from fermentation. Rumen can be considered a continuous fermentation chamber in which output (or outflow) of nutrients and fluids like water and saliva is continuous and proportional to the input. This input-output link is called rumen turnover, expressed as the solid and liquid content leaving the rumen per hour. The rumen turnover is influenced by the amount of the total microbial mass flowing in the rumen, called microbial yield. The efficiency of the microbial yield, expressed and the amount of microbial proteins produced per kg of organic matter ingested by the animal, is affected by the turnover rate of rumen (Hoover and Miller, 1991). Here, we introduced the concept of microbial yield because the rumen is a suitable environment for the growth and colonization of a huge and various symbiotic microbial community mostly made by anaerobic bacteria, protozoa, and fungi. Ruminants are dependent upon the microbial community of rumen for the degradation of the ingested feed, since they are not able to disrupt the complex molecular structure of plant fibres (Hoover and Miller, 1991). After degrading the polysaccharides, the resulting end products are a pool of small and soluble oligosaccharides or single sugars in the fermentation medium of rumen, used by the microbial community as a source of energy for their growth and metabolism (Hoover and Miller, 1991). Indeed, the main function of rumen is to degrade the solid feed into semisolid feed and let the ingested bolus proceed for further digestion. The fermenting action of the rumen microbial community is needed to produce the molecular building blocks (e.g. single sugars from complex carbohydrates) used to produce SCFA and microbial proteins, sources of energy and amino acids for the host, respectively. In addition to SCFA, the enteric fermentation is also source of gases, with CO_2 and CH_4 being the largest proportion of gases produced, then H_2. The CO_2 has a higher production rate than CH_4, but then H_2 is used to reduce CO_2 to CH_4, therefore their concentrations get more similar closer with time (Czerkawski, 1986; Hoover and Miller, 1991). The symbiotic microbial community interacts through cross-feeding of substrates, when specific cellulose-degrading bacterial species do not further metabolize the end products of their fermentation of cellulose, but make these end products available to other microbial species which are only capable of hydrolysing hemicellulose but not cellulose (Dijkstra et al., 2002). Another important component of rumen is its physical barrier, e.g. the rumen epithelial cell wall. This is a multi-functional barrier and is organized as a squamous stratified barrier with four layers of cells, protecting rumen from potential diffusion of harmful microbes or compounds, and controlling the absorption, transport and metabolism of SCFA and other nutrients. The first epithelial layer of the luminal side of rumen epithelial cell wall is called stratum corneum and is a keratinized cell layer useful to prevent the diffusion of harmful microbes and exogenous compounds through the rumen cell wall and is also a barrier for the potential colonization of rumen epithelial cells by the rumen host microbiota (Baldwin and Connor, 2017) (Figure 2). The rumen epithelium is

colonized with a diverse microbial population, called epimural microbiota, with different composition and function compared to the rumen host microbiota. The epimural microbiota is physically attached to the rumen epithelial cell wall, with the phyla Firmicutes, Proteobacteria and Bacteroidetes representing around 80% of the microbial core conserved among species. The epimural microbiota is needed to digest the keratinized cells to improve cell turnover and tissue recycling. Then, it is involved in urea degradation, because urea can derive from diffusion across the epithelium or by the diet. Urea degradation produces ammonia and CO_2. The last recognized function of epimural microbiota is the O_2 scavenging action made by facultative epimural bacterial species to maintain the strict anaerobiosis in the inner part of rumen and let enteric fermentation proceed properly by the action of obligated anaerobic bacteria (Schmitz-Esser, 2021; Na and Guan, 2022). In addition, the luminal side of rumen cell wall is characterized by the presence of rumen papillae that serve to increase the surface area available for SCFA absorption. This complex structure is not completely developed at birth, but needs to be physically stimulated by solid feed intake and the start of enteric fermentation in rumen, which is the cause of the metabolic and physical maturation of rumen and rumen cell wall and the absorption of SCFA through the rumen cell wall (Baldwin and Connor, 2017; Ji et al., 2021).

Rumen microbiota

Rumen microbiota is a various and symbiotic community of microbes that colonizes the rumen just after birth and first colostrum intake. During weaning, the microbiota colonizes and adapts to the ruminal environment. The final maturation takes place after weaning, when the gastrointestinal tract is initiated to fibrous feed for the first time and enteric fermentation starts to occur. Indeed, diet is the main factor of variation of the abundance of microbial species in rumen (Arshad et al., 2021). The rumen microbiota colonizes rumen because this environment has some physiological characteristics that make it a suitable environment for optimal microbial growth of certain bacterial species, such as the strict anaerobiosis and the favourable conditions of temperature and pH. The rumen microbiota consists of anaerobic bacteria, methanogen archaea, protozoa, fungi, and viruses in order of abundance, e.g. the number of genetic sequences detected by advanced sequencing techniques. Indeed, bacteria are the most abundant microbes in rumen, accounting for 10^{10}-10^{11} cells/ml. Then, methanogenic archaea are in the 10^{6}-10^{8} cells/ml range, followed by protozoa, with 10^{4}-10^{6} cells/ml. In terms of microbial mass, bacteria and protozoa contribute similarly, with a share of around 45% for each. This is possible even if protozoa are less than bacteria because protozoa have a larger size than bacteria (Newbold et al., 2015; Matthews et al., 2019). A further classification is based on the metabolic role of the rumen microbial species, depending on which class of feed components they are able to degrade and ferment. For example, bacteria can be classified as fibre- or protein-degrading

bacteria, and then the fibre-degrading bacteria can be further sub-classified as cellulolytic, amylolytic, or hemicellulolytic bacteria (Henderson et al., 2015). In 2015, Henderson et al. developed a database reporting the composition of rumen microbiota observed in samples of rumen content collected from a cohort of 742 ruminants from 32 different species and from different areas of the world. The aim was to identify the common features and the presence of dominant microbial species that constitute the "core microbiome". The authors observed that 30 bacterial groups were shared by 90% of all the samples considered. Seven of them were constituting the "core microbiome", covering 70% of all the bacterial genetic sequences detected: Prevotella, Butyrivibrio, Ruminococcus, Lachnospiraceae, Ruminococcaceae, Bateroidales, and Clostridiales. Since bacteria are the main microbial species involved in feed degradation in rumen, it is supposed that the core bacterial groups are the ones involved the most (Henderson et al., 2015). The main fibrolytic bacterial species are *Fibrobacter succinogenes, Ruminococcus flavefaciens, Ruminococcus albus, Butyrivibrio fibrisolvens, Prevotella ruminicola, Eubacterium cellulosolvens* and *Eubacterium ruminantium*. Among these 7 predominant species, *F. succinogenes, R. flavefaciens and R. albus* are the most important cellulolytic bacteria (Koike and Kobayashi, 2009). The fibrolytic activity of rumen bacteria is exerted through different mechanisms. An example is the cellulosome, defined as a multi-enzymatic complex associated with the cell surface, able to mediate the adhesion of bacteria to feed particles to be degraded. The cellulosome consists of several catalytic enzymatic domains interacting with each other to degrade feed, in most cases cellulose. Among all the catalytic subunits, the scaffolding system that permits cellulosome to be cohesive and attached to the cell surface is a dockerin domain present in each of the catalytic subunits of the cellulosome. Another example is the polysaccharide utilization loci (PUL), specific genomic regions encoding for a set of enzymes involved in the degradation of a particular glycan molecule. The enzymatic action involves binding, depolimerization and transport of the released oligosaccharides. The fibre-degrading genes of Bacteroidetes, Firmicutes and Prevotella are organized as PUL co-expressed together and that act synergistically on feed (Terry et al., 2019). It was reported that the cellulolytic activity of *R. flavefaciens* is retained in 65 genes organised as a cellulosome. This is not the case of *F. succinogenes*, for which none of the genes encoding for cellulose degradation enzymes is similar to cellulosome-related enzymes expressed by other cellulolytic bacteria (Koike and Kobayashi, 2009).

The majority of the archaeal population in rumen consists of methanogens. A sub-classification of methanogens can be made depending on the substrate used to form CH_4. In Henderson et al. (2015), around 80% of detected methanogens were hydrogenotrophic, reducing CO_2 to CH_4 through H_2, whereas around the remaining 20% were methylotrophic methanogens, able to use methyl groups from methylated compounds to produce CH_4. A remaining negligible percentage of methanogens detected in the study was capable of producing CH_4 from acetate through the aceticlastic pathway

(Henderson et al., 2015). These results were further confirmed in another study, in which the authors analysed the genomic sequences of 501 bacterial and archaeal cultures, part of which are reported in the Hungate1000 Genome Catalogue. This is a catalogue of reference genomes from all the rumen bacterial and archaeal species that were cultivated, sequenced and taxonomically characterized (Seshadri et al., 2018). The achaeal groups Methanobrevibacter, Metahnosphaera and Methanomassiliiococcaceae were identified as the predominant methanogens, comprising around 90% of total rumen archaea. Compared to bacteria, archaea are more conserved across species (Henderson et al., 2015; Tapio et al., 2017).

The main populations of protozoa in rumen are the ciliated Holotricha and Entodiniomorphida (Patel and Ambalam, 2018). Rumen protozoa are a metabolically active population, able to affect fermentation pathways and act as H_2 supply and hosts for methanogens (Morgavi et al., 2010). The H_2 from protozoa is produced in the hydrogenosome, named mitochondria-like organelles present in protozoa. The methanogens take advantage of the H_2 flow coming from protozoa, and can establish a mutualistic relationship with protozoa. This is a clear example of how the interspecies H_2 transfer can work. Methanogens can be associated with protozoa as intracytoplasmic symbionts or bound to the cell surface (Henderson et al., 2015; Tapio et al., 2017). However, defaunation (complete artificial removal of the protozoa population from rumen) was correlated with reduced CH_4 production but not with a lower relative abundance of methanogens. This suggests that despite the observed association between protozoa and methanogens, there must be other metabolic pathways to dispose H_2 apart from CH_4 formation (Knapp et al., 2014). The importance of the contribution of protozoa to methanogenesis is still debated and needs further investigation. Protozoa in rumen are also known to possess a fibrolytic activity exerted by enzymatic domains, which are encoded by gene sequences that probably reached protozoa through horizontal gene transfer from fibrolytic bacteria (Newbold et al., 2015). Even though the presence of protozoa in rumen is not essential for animal survival, is was observed that defaunated animals showed lower NDF and ADF digestibility, thus limiting feed efficiency (Newbold et al., 2015).

The minor components of rumen microbiota are fungi and viruses. Fungi account for 10^3-10^6 zoospores/ml, around 8-12% of the total microbial biomass. The Neocallimasticaceae is the major family of fungi in rumen. Anaerobic fungal species are capable of initiating the degradation of lignocellulosic and recalcitrant plant fibres through the production of enzymes organized as cellulosomes (Akin and Borneman, 1990; Matthews et al., 2019). The presence of lignin is seen as a physical barrier that limits the accessibility and the subsequent degradability of cellulose or xylans by the bacterial enzymes. Fungi colonize lignocellulosic fibre particles and establish a rhizoid to “dig” into the lignin cell wall. Therefore, the degrading activity of fungi is essential to support bacterial fermentation of plant polysaccharides. An alternative degradation of lignin can occur thanks to the

action of aerobic fungi adherent to the feed particles in micro-aerobic conditions, before reaching the strict anaerobic environment of the inner part of rumen (Bhagat et al., 2023). It has been hypothesized that the cellulolytic activity of rumen fungi would derive from horizontal gene transfer of genetic sequences encoding for cellulases from fibrolytic bacteria, such as *F. succinogenes* (Garcia-Vallvé et al., 2000).

The predominant viruses in rumen are bacteriophages, occurring in 10^7-10^9 particles/g of digested feed in rumen, with the order Caudovirales being the main one. Bacteriophages were associated with the main fibrolytic bacterial groups. Even though the function has not yet been fully assumed, bacteriophages seem to play a major role in horizontal gene transfer and cell lysis. The virus-mediated lysis of microbial cells is vital for the release of new viral particles, but in parallel also because it allows the release of intact enzymes, amino acids, and nutrients that are used by other microbes though the mechanism of intra-ruminal recycling (Matthews et al., 2019; Gilbert et al., 2020).

Enteric fermentation

The anaerobic environment of rumen allows feed fermentation through the action of rumen host microbiota. The symbiotic action of primary fibrolytic and cellulolytic bacteria enables degradation of feed particles into polymers, then secondary fermenting bacteria degrade polymers into monomers, mostly hexose sugars. Usually, the main metabolic pathway for monomers in rumen is glycolysis, providing pyruvate, ATP and NADH. The main end products of fibre fermentation are SCFA, microbial proteins and gases (CO_2 and H_2). The main SCFA produced are acetate, butyrate and propionate. The produced gases are the major electron acceptor and donor species, and the reducing environment of rumen "pushes" the H_2-mediated reduction of CO_2 to CH_4 by the methanogenic archaea in rumen (hydrogenotrophic methanogenesis), which are at the bottom of the trophic chain of enteric fermentation (Morgavi et al., 2010, Knapp et al., 2014). Methanogens are able to reach the H_2 reserve and use it to mediate CO_2 reduction because of the process of interspecies H_2 transfer (Figure 3).

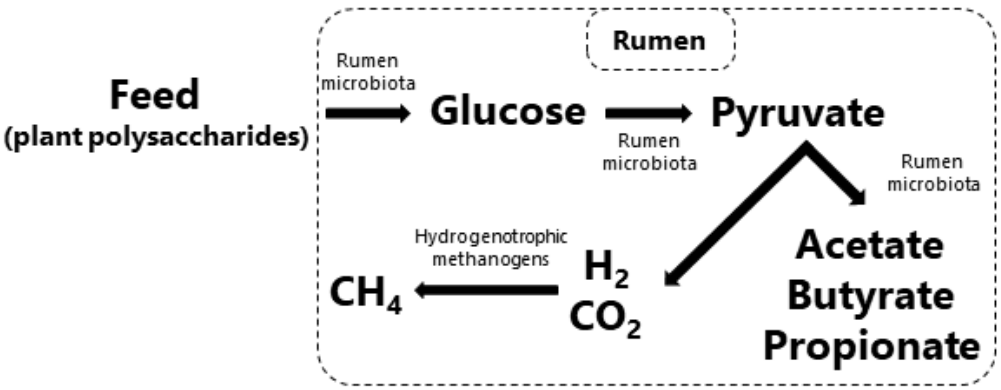

Figure 3. Schematic representation of enteric methane production.

This is a syntrophic process allowing H_2 transfer from the fermenting rumen microbial components, such as fibrolytic bacteria, protozoa and fungi, to the final users, the methanogenic archaea (Lan and Yang, 2019). The production of CH_4 is a physiological process to dispose metabolic H_2 production and therefore reduce the pressure of H_2 production in rumen. This is an efficient metabolic process to maintain rumen homeostasis and let fermentation occur. Indeed, during sugar oxidation in rumen, NAD+ is reduced to NADH but the fermentation requires the re-oxidation of NADH to NAD+. The oxidation involves the dehydrogenation of NADH to NAD+ and the subsequent release of H_2. The absence of O_2 in the anaerobic rumen environment requires different H_2 acceptors than O_2, and the reduction of CO_2 to CH_4 is the most suitable reaction available. The correct cycling of NAD+/NADH oxidation and reduction reactions is needed to avoid both the accumulation of H_2 and the lack of reduced cofactors (NADH) due to the inhibition of dehydrogenase activity NADH and subsequently the reduced rate of enteric fermentation (McAllister and Newbold, 2008; Martin et al., 2010). Hydrogenotrophic methanogenesis is not the only way to counteract H_2 pressure through CO_2 reduction, because other non-methanogenic microbial species in rumen are able to use different electron acceptors than CO_2 to oxidize H_2, thus reducing the H_2 reserve available for methanogenesis. The alternative electron acceptors are mainly sulphate, nitrate, and fumarate. The sulphate-, nitrate-, and fumarate-reducing bacterial species compete with hydrogenotrophic methanogens for the use of H_2 to produce acetate, sulphate, and succinate, respectively. In any case, the CO_2-mediated CH_4 production is the predominant in rumen (Ungerfeld, 2020; Choudhury et al., 2022). The production of fermentation end products such as SCFA and gases is affected by feed intake and feed quality (e.g. digestibility rate), and by the health condition of the animal. In this sense, the production of SCFA and CH_4 is related, since the production of acetate and butyrate provides metabolic H_2 available for methanogens, whereas propionate is an alternative H_2 sink that consumes reducing equivalents and leads to reduced CH_4 production (Knapp et al., 2014; Ungerfeld, 2020).

Digestion

The characteristics of the major components of feed particles that reach rumen as ingested feed are reported below. In relation to feed, each microbial community in rumen has a specific pattern of feed degradation derived from how the colonization of the feed particles by the microbial enzymatic pool works. Here, the term “colonization” is used to give the idea of how is the real mechanism of feed degradation in rumen, where the microbial enzymatic activity has to face the diversity of the feed particles in terms of size, composition, solubility, and accessibility.

Fibre

The fibres ingested by ruminants are mainly composed of plant cell wall polysaccharides such as cellulose, hemicellulose and pectin. These polysaccharides are cross-linked with other components like lignin, proteins, hydroxycinnamic acids, ions, and water by molecular bonds of different nature (e.g. ionic, hydrogen-bound, covalent). The cross-linked molecules are useful for building up a three-dimensional matrix capable of keeping polysaccharides within the cell wall (Terry et al., 2019) (Figure 4).

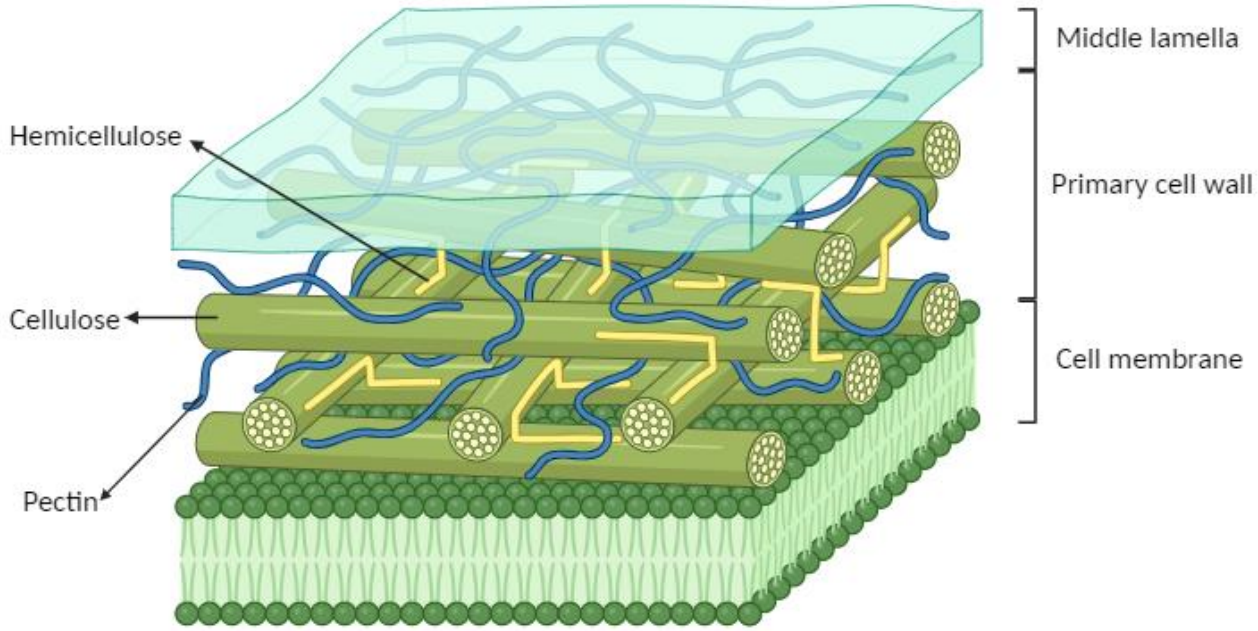

Figure 4. Schematic representation of the molecular structure of the plant cell wall. Created with BioRender.com.

Ruminants alone are not able to produce the required enzymes to degrade polysaccharides. Rumen microbiota allows ruminants to have and use a large consortium of fibre-degrading enzymes, which are the only way to degrade, ferment and produce the molecular compounds of interest (e.g. SCFA, amino acids) from these low-quality feed sources. The degradation of the cell wall by microbial hydrolases in rumen must be able to degrade the cross-links of the cell wall matrix before reaching the polysaccharides (Wang et al., 2002). As reported above, the cellulolytic bacteria *F. succinogenes, R. flavefaciens* and *R. albus* are primarily involved in the plant cell wall degradation, for which the adhesion to the feed particles is required to start the enzymatic degradation process (Terry et al., 2019).

Proteins

Dietary proteins are the major source of nitrogen (N) for the animals. The proteolytic activity of rumen microbial proteases requires the attachment of bacterial to feed particles to provide a substrate for their protease activity. The protein degradation rate depends on their retention time in rumen, accessibility to enzymes, solubility, and presence of molecular bonds within and between the tertiary and quaternary protein chains. As seen for the plant cell wall, lipids or other water-insoluble components can encapsulate proteins in rumen, thus reducing the accessible surface for microbial proteases. The microbial proteases mediate protein degradation to single amino acids and oligopeptides. The bacterial peptidases and deaminases metabolize these protein components and use this N pool for microbial protein synthesis. The excess amino acids not used for microbial growth are deaminated to SCFA and ammonia, released in rumen. The synthesis of microbial proteins relies on dietary N sources and on ammonia content in rumen. The animal takes advantage by adsorbing and digesting the 50-80% of microbial proteins in the small intestine (Leng and Nolan, 1984; Bach et al., 2004). Protozoa also play a crucial role in protein degradation, because they can bind to polysaccharides, subtracting them from bacteria. Polysaccharides are the main energy source for bacteria and they use this energy for the synthesis of bacterial proteins. Therefore, less polysaccharides available reduce bacterial N uptake and subsequent protein synthesis. Protozoa can also directly engulf bacteria and impair their metabolic activity (Leng and Nolan, 1984; Bach et al., 2005).

Lipids

Dietary lipids in the ruminant diet are usually glycolipids and phospholipids in herbage and triglycerides in seeds (Buccioni et al., 2012). Lipids can depress enteric fermentation of carbohydrates in rumen and reduce the amount of lipids reaching the absorption sites in the gastrointestinal tract (Jenkins, 1993). This is because lipids can naturally stick to the solid feed particles in rumen, thus reducing the possibility of adherence of the bacteria responsible for the degradation of plant fibres (Hoover and Miller, 1991, Jenkins, 1993). In rumen, dietary lipids undergo two chemical transformations, called lipolysis and biohydrogenation. Lipolysis occurs when bacterial lipases hydrolyse the ester bonds and release free fatty acids, glycerol, and mono- and di-glycerides to a lesser extent. The cellulolytic *B. fibrisolvens* is able to hydrolyse phospholipids, whereas the bacterial strain *Anaerovibrio fibrisolvens* is known to hydrolyse only mono- and di-glycerides (Bauman and Lock, 2006; Buccioni et al., 2012). After lipolysis, the unsaturated free fatty acids are rapidly hydrogenated because they can be toxic to bacteria. The bacterial hydrogenation of double bonds of the unsaturated fatty acids leads to the production of saturated fatty acids, of which palmitic (16:0) and stearic (18:0) are the major ones.

Then, mono-unsaturated fatty acids are also produced but to a lesser extent (15-20% of total bacterial fatty acids). Therefore, the lipids leaving the rumen and reaching the duodenum for the absorption originate from dietary sources (mostly long chain unsaturated fatty acids that escape biohydrogenation) and bacterial de novo synthesis (Jenkins, 1993; Bauman and Lock, 2006).

Physical interaction with feed

The physical interaction between rumen microbes and feed particles is the starting point for feed degradation. The adherence on feed particles is also the only way for microbes to be retained more time in rumen, avoiding rumen turnover and consequent washout. The microbial components in rumen are functionally distributed according to their characteristics. The case of bacteria helps to clear this concept. Non-adherent bacteria localize in rumen fluid to metabolize the soluble polysaccharides and proteins or because they are newly detached from feed particles, whereas adherent bacteria can loosely or strongly adhere to feed particles to hydrolyse the insoluble polysaccharides and the other components of plant cell wall (Wang et al., 2002). The idea is that bacteria compartmentalize both in the liquid fraction and in the solid fraction of rumen according to nutrient availability and their enzymatic specialization. Indeed, bacteria seem to be chemotactically attracted where they can find feed particles to be degraded (Hoover and Miller, 1991). Furthermore, the rate of degradation and solubility of feed particles affects their retention time in rumen. This allows slow-growing rumen microbes, such as protozoa and fungi, to adhere to these feed particles and extend their retention time in rumen and therefore their survival. Protozoa and fungi can also adhere to the rumen wall to avoid the washout. In general, the compartmentalization of protozoa and fungi is similar to what already reported for bacteria (Hoover and Miller, 1991; Wang et al., 2002).

Effect of the diet on the composition of rumen microbiota

Different dietary regimens modulate the composition of rumen microbiota by either favouring or suppressing specific microbial populations, thus impacting also on the fermentation activity occurring in rumen (Henderson et al., 2015; Liu et al., 2021b). In a study involving 32 ruminant species and sub-species, it was observed that the rumen composition of forage-fed animals was comparable and differed from the one of concentrate-fed animals, regardless of the animal species considered. For example, the bacterial populations *Bacteroidales* and *Ruminococcaceae* are associated with forage-rich diets, whereas *Prevotella* and *Succinivibrionaceae* are associated with concentrate-rich diets (Henderson et al., 2015). A group of 16 Holstein heifers was used to evaluate the dietary supplementation of three grass and two clover silages on rumen fermentation and microbiota. The clover-fed heifers had a significantly higher DM intake (DMI) and a lower CH_4 production per kg DMI

than grass-fed heifers. Around 70% of all the sequences detected belonged to *Firmicutes* and *Bacteroidetes*. Of the 34 predominant bacterial orders detected, 8 were more abundant in clover-fed heifers (of which 3 within *Bacteroidales* and 4 within *Clostridiales*), whereas 7 were more abundant in grass-fed heifers (2 within *Bacteroidales* and 5 within *Clostridiales*). Looking at these last results, there is a similarity between the two dietary groups, even though the single bacterial species were different between groups (Parnian-Khajehdizaj et al., 2023). Furthermore, high-concentrate or high-lipid diets are known to affect rumen fermentation because they are less fermentable than plant fibres, and this can be observed on rumen microbiota. The fatty acid composition and degree of unsaturation are correlated with rumen microbes and in particular protozoa: the higher the unsaturation, the lower the protozoa in rumen because of the toxic effect of lipids (Kargar et al., 2023). A cohort of 18 Alpine goats was fed with two diets either supplemented with linseed oil or hempseed oils, to observe the effect on rumen fermentation and microbiota. These two oils differ for the unsaturated fatty acid composition, with linseed oil being richer in α-linolenic acid (C18:3n3) and hempseed oil richer in linoleic acid (C18:2n6). Independently from the dietary group, *Bacteroidetes* and *Firmicutes* were the dominant bacteria, whereas Methanobacteriaceae and Methanomassiliicoccaceae the dominant archaea. However, linseed reduced the abundance of *Prevotella* (-12%) and increased *Succinivibrio* (+50%) and *Fibrobacter* (+75%) compared to control, whereas hempseed supplementation did not alter the abundance of any bacterial group (Cremonesi et al., 2018).

Solutions against environmental impact of livestock production

As reported above, CH_4 emissions from enteric fermentation and, overall, GHG emissions from livestock and feed production strongly contribute to global warming. The revolution of livestock production should consider to implement strategies within the production chain to massively tackle GHG emissions without reducing the productive yields in terms of feed and animal-source food from the same amount of resources. Among the strategies discussed in the literature, the application of technologies and practices belonging to the concept of Precision Agriculture (PA) and Precision Livestock Farming (PLF) could help in reaching the productive goals at animal level while managing environmental sustainability issues and animal welfare. The starting point of PA and PLF is to “produce more with less” (Tullo et al., 2019; Monteiro et al., 2021). This is possible by applying a set of processes aimed at measuring and recording any biological response of the animal to adapt and then apply best practices as precisely as possible for improving animal welfare, productivity, and environmental impact (Gerber et al., 2013; Tullo et al., 2019). Mitigation strategies can be subdivided in three main areas: i) sustainable intensification of animal productivity, ii) technical operations to modify animal breeding and farming, and iii) reduced use of natural resources and demand for livestock end products.

Specifically, several practical strategies compliant with PLF are the use of sensors to monitor grazing behaviour and animal health in real time, genetic selection to breed animals with genetic traits of interest, and management of feed strategies to: recover nutrients and energy from feed and food by-products, use low-fermenting feed ingredients (e.g. concentrates, lipids), or use GHG-tackling feed additives (e.g. enzymes, prebiotics, probiotics, and plant phytochemicals) (Herrero et al., 2016; Monteiro et al., 2021). Sensors are the clearer example to explain the application of PLF principles, because they are intended to detect and constantly record data from farm and animals without animal manipulation, so being less invasive than previous methods and allowing farmers and stakeholders to better decision-making (Morrone et al., 2022). An example of how the improvement of productivity can help in reducing emissions is the fact that higher emission rates are more related to low efficient farming systems rather than large intensive farming systems. This is usually because smaller farming systems rely more on low-digestible feed ingredients because of economic feasibility, and this reduces their production efficiency. The use of low-digestible feed ingredients reduces the animal digestion in the gastrointestinal tract, slowing the growth rate of the animal and in turn leading to higher slaughtering age, usually related to more GHG emissions. The optimization of feeding strategies could raise the productive yields through higher feed-to-product conversion ratio, together with lower emission rates. This strategy is usually accompanied by a reduction of herd size, because fewer high-producing animals emit less GHG than more low-producing animals (Gerber et al., 2013; Herrero et al., 2016). In any case, the increased production efficiency is accompanied by higher feed-related GHG emissions, in particular higher CO_2 and N_2O emissions from crop harvesting and feed processing to sustain the intensification of the production. This is why improved efficiency and intensification must be considered within the whole feed-food chain (Beauchemin et al., 2020). Another example involves animal breeding and genetic selection, in particular the selection of high-producing animals or low GHG-emitting animals. For the first ones, the rationale is that the higher the nutrient utilization, the higher the production yields (e.g. meat and milk), the lower the GHG-related emissions. Usually, genetic selection involves both productive and non-productive traits, such as animal welfare and fertility traits (Wall et al., 2010). Instead of focusing on productive traits, the genetic selection can be oriented on low GHG-producing traits. By tackling GHG emissions, the animal redirects the share of feed normally used for maintenance (as reported above, gases are physiological products of enteric fermentation) to production. The consequence is that the amount of CH_4 and GHG in general per unit of conversion from feed to product (e.g. meat and milk) is reduced (Beauchemin et al., 2022; Strandén et al., 2022). Kamalanathan et al. (2023) analysed the genetic heritability of three CH_4 genetic traits in a cohort of 1765 individual records of CH_4 emissions from 330 Holstein cattles: daily CH_4 production, CH_4 yield (g CH_4/g DM), and CH_4 intensity (g CH_4/kg milk). The authors observed a high genetic correlation between the three traits and high heritability rates, suggesting that these genetic traits are

potential targets for selection breeding (Kamalanathan et al., 2023). Also, Stepanchenko et al. (2023) obtained different results from low vs high CH_4-yield Holstein dairy cows. The authors observed that, despite similar body weight, DM intake, and feed efficiency, the CH_4 production of high-yield cows was significantly +24% higher than low-yield cows. These latter showed higher propionate (+22%) and lower acetate (-8%) production than high-yield. Bacterial richness and diversity were lower for low-yield cows than high-yield cows. Among archaea, *Methanobrevibacter* was the predominant in the high-yield group, whereas the lactate-producing *Methanosphaera* was the predominant in the low-yield group. Among bacteria, the low-yield group was characterized by an increased abundance of H_2-utilizing bacteria, such as *Succinivibrionaceae* and *Veillonellaceae*, both known to be associated with propionate production (Stepanchenko et al., 2023).

Mitigation of rumen methane emissions

After discussing how the application of PLF can increase the sustainability of livestock production through higher production efficiency, here the major strategies aimed at mitigating CH_4 emissions from rumen enteric fermentation are discussed (Figure 5).

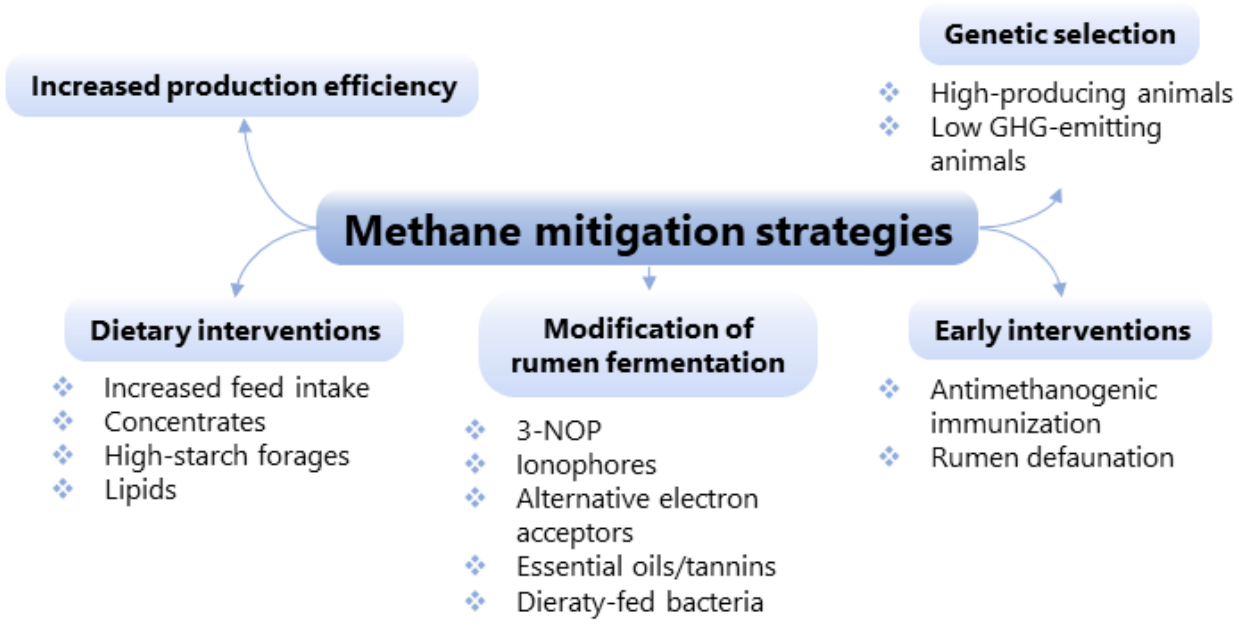

Figure 5. Summary of the major strategies to mitigate enteric methane production.

The first essential disclaimer is that mitigating strategies must consider that the production of CH_4 has a physiological role in rumen aimed at preventing the disruption of the fermentative processes occurring in rumen, therefore CH_4 production must be targeted but other strategies to adsorb H_2 pressure need to be considered (Llonch et al., 2017). Moreover, any strategy applied for CH_4 (or GHG)

reduction should tend to ameliorate the health and/or the productivity of the animal. The co-benefits of CH_4 mitigation and H_2 alternative utilization could redirect the 2-12% energy loss usually caused by CH_4 to animal growth and higher productivity (Kelly et al., 2022). The mitigating strategies can be grouped in three classes: i) dietary interventions, ii) modification of rumen fermentation, and iii) interventions at early stages of life. Increasing feed intake is an option to reduce the retention time in rumen and consequently the lower accessibility of rumen microbes to organic matter (OM). This would turn in lower fermentation and CH_4 yield, ameliorating feed efficiency. Specific feed ingredients can be applied, such as concentrates and high-starch forages (e.g. cereal grains), known to increase the energy density of the diet and also have a different chemical structure than plant fibres, resulting less prone to microbial fermentation. Lipids are another feed ingredient used to reduce the fermentation of the diet, because the microbial populations in rumen can digest lipids (as seen above) but not ferment them as fibres. The biohydrogenation of unsaturated fatty acids in rumen is useful to both reduce their toxicity and be a H_2 sink, alternative to methanogenesis. The modification of rumen fermentation processes involves natural and synthetic compounds to redirect some metabolic pathways or alter the action of enzymes involved in the methanogenesis. This latter is the case of 3-nitrooxypropanol (3-NOP), a synthetic compound that is a structural analogue of methyl-coenzyme M (MCM). MCM transfers a methyl group to methyl-coenzyme M reductase (MCR). MCR is an enzyme with a nickel ion group, and 3-NOP is capable or targeting the active site of MCR, irreversibly oxidising the nickel ion and thus blocking the catalytic activity of MCR (Romero-Perez et al., 2014; Duin et al., 2016). The effect of 3-NOP was also observed in *in vivo* trials, showing an average 30% reduction of CH_4 emissions when used as a feed additive for dairy cows and beef cattle. The MCR targeting of 3-NOP is highly specific also because any effect on the bacterial growth of the major fibrolytic bacterial species in rumen (e.g. *R. albus, R. flavefaciens, F. succinogenes,* and *B. fibriosolvens*) was observed (Duin et al., 2016; Kebreab et al., 2023). Other compounds are ionophores, known to increase the permeability of the cell membrane of gram-positive bacteria and protozoa, both sources of H_2. The higher permeability causes cell death and in turn lower H_2 production. Furthermore, the use of feed additives can also be oriented to H_2 production rather than directly targeting CH_4 production. The use of electron acceptors alternative to CO_2, such as fumarate, malate, nitrate, and sulphate redirects H_2 from CO_2-mediated methanogenesis to other pathways. The reduction of nitrate and sulphide provides building blocks for the synthesis of microbial proteins. However, nitrate can be also converted to N_2O, the latter representing a hazard for soil and water when released in the environment. The last dietary intervention discussed involves dietary-fed bacteria, to promote alternative H_2 utilization, such as acetate- or propionate-reducing bacteria. Acetate-producing bacteria reduce CO_2 to acetate, even though this reaction is less favourable than the H_2-mediated CO_2 reduction to CH_4. Instead, propionate production is a valid alternative H_2 sink and is associated with reduced CH_4 production (Lan and Yang,

2019). Another way to act on H_2 is to decrease its production, for example through rumen defaunation at early stages of life, since protozoa supply methanogens with H_2, as discussed above (McAllister and Newbold, 2008; Beauchemin et al., 2022). Finally, early-stage immunization with an antimethanogenic vaccine able to stimulate an immune response against archaeal methanogens is seen as a promising strategy to modify the rumen fermentative processes. An issue is the choice of the antigen to be targeted, and the research involved the use of a mix of different archaeal strains (e.g. from *Methanobrevibacter* spp.) or the identification of extracellular peptides expressed on the cell membrane of methanogenic archaea. Also, saliva has been recognised as main carrier of salivary antibodies in rumen, therefore the stability of salivary antibodies in rumen is another issue (Baca-González et al., 2022; Beauchemin et al., 2022).

Plant secondary metabolites

Among the several dietary interventions to alter rumen fermentation, the use of plant-secondary metabolites (PSM) has been seen for decades as unfavourable, due to their anti-nutritional and toxic effect on rumen fermentation and rumen microbiota. By tailoring the dietary supplementation with PSM it is possible to "isolate" the beneficial effect of PSM on the modulation of the fermentative processes in rumen while lowering their suppressing effect on enteric fermentation and energy production from ingested feed. Examples of PSM are mostly phenolic compounds such as tannins, saponins, essential oils, and flavonoids. These are non-nutritive compounds that are physiologically produced by the plants as protecting agents against parasites and herbivore animals (Bodas et al., 2012; Ku-Vera et al., 2020). At rumen level, the major effects recognised to PSM are: i) binding to dietary macromolecules, mostly proteins and polysaccharides, and ii) binding to microbial enzymes. The first effect lowers ruminal degradation of PSM-macromolecules complexes, which are less accessible to degrading enzymes. The reduced degradability of polysaccharides in rumen can lead to lower SCFA production and alteration of SCFA profile, deceasing feed efficiency and animal performance. However, PSM-protein complexes prevent rumen degradation of proteins, increasing the by-pass flow of these complexes to the small intestine and N excretion. In the small intestine, the absorption of this higher amount of undegraded proteins depends on the possibility to break the binding between PSM and proteins. PSM can also bind microbial enzymes and cell membrane molecules, with direct consequences on nutrient degradation and CH_4 production. By binding fibrolytic and cellulolytic enzymes, PSM suppress the fibre-degrading activity of bacteria and overall the feed utilization in rumen. For what concerns CH_4 production, PSM can directly bind methanogens' enzymes and impair the methanogenic pathway, or bind other microbial components such as methanogen-associated protozoa and reduce their H_2 production (Bodas et al., 2012; Aboagye and Beauchemin,

2019). The effect of PSM is more evident in *in vitro* than *in vivo* studies. The *in vitro* trials let the operators to control all the parameters involved in the study, and in most cases the use of PSM can be more oriented to their beneficial effects on rumen fermentation (e.g. lower CH_4 production) rather than their depressing effects on rumen fermentation. These parameters are the distribution rate of PSM in the medium, faster and more homogeneous when *in vitro* batch cultures are used, and the exposure time for rumen microbes to a specific dose of supplementation of PSM (Bodas et al., 2012). Despite the easier application of PSM *in vitro*, the *in vivo* application is still debated because it is still difficult to have a clear idea on the effects of PSM on rumen fermentation due to the lack of full knowledge on the mode of action of the different PSM compounds (Aboagye and Beauchemin et al., 2019). This is why the use of PSM cannot be generalized, because any deduction on mode of action and exposure time can be easily inferred. Regarding the mode of action, the research mostly involved PSM extracts (Jayanegara et al., 2009; Bueno et al., 2020), for which is it hard to identify the single and specific bioactive molecule that exerts the observed effect. The bioactive molecule can be identified and discriminated from all the other extract components only when the potential bioactive molecules are supplemented alone (Bodas et al., 2012).

Tannins

Tannins are water-soluble PSM composed of hydroxyl groups attached to aromatic rings, giving them high binding capability with other molecular components. Tannins are usually grouped in two classes: condensed tannins (CT) and hydrolysable tannins (HT). The CT, also called proanthocyanidins, are larger molecules with a molecular weight ranging from 1900 to 28000 Da, consisting of polymers of flavan-3-ol (epi)catechin and (epi)gallocatechin subunits. Instead, HT are smaller molecules (500-3000 Da) consisting of a polyol core, usually glucose, esterified with gallic acid (GA). The further esterification of HT subdivides HT into gallotannins and ellagitannins. The GA is the first molecular group coupled to gallotannins, whereas ellagic acid (EA) is a condensed dimer of GA and is the molecular group that characterizes ellagitannins (Patra and Saxena, 2010; Aboagye and Beauchemin, 2019; Ku-Vera et al., 2020) (Figure 6).

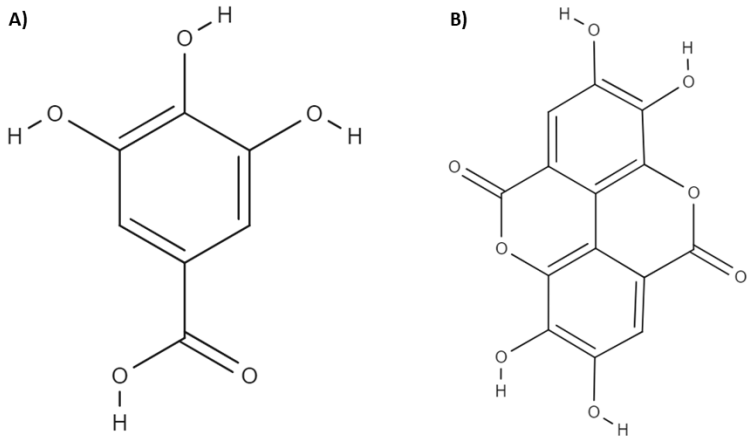

Figure 6. Molecular structure of A) gallic acid and B) ellagic acid.

As seen for PSM, tannins are able to form large molecular complexes with proteins and plant polysaccharides in rumen, preventing them from microbial degradation, thus reducing the fermentation rate of fibre and the degradation of proteins in rumen. The binding can be directed either to the dietary components or to rumen microbes, specifically enzymes or membrane molecules (e.g. adhesins). The direct binding with rumen microbes impairs their metabolic activity, evidenced by slower feed degradation rates, lower SCFA and in turn reduced CH_4 production. Since one is detrimental (lower SCFA) and the other beneficial (lower CH_4), the use of tannins must be well balanced depending on the feed ingredients used and on the modulation of rumen microbiota. Due to their effect on CH_4, tannins are studied and applied as antimethanogenic phytochemicals. The possible actions of tannins on CH_4 are: i) direct binding with methanogen archaea to prevent their enzymatic action or the coupling with protozoa, and in turn the interspecies H_2 transfer, ii) binding with protozoa or other bacterial H_2 producers to decrease the H_2 flow available for methanogenesis, and iii) formation of molecular complexes with feed components, preventing their degradation and the formation of H_2 and CH_4 (Aboagye and Beauchemin et al., 2019; Ku-Vera et al., 2020; Manoni et al., 2023). The debate on which are best tannin candidates to target CH_4 emissions without negatively affecting rumen fermentation is open. The CT have more binding sites than HT, therefore they can form more and larger complexes with macromolecules, but this can easily affect feed degradation, whereas the smaller size of HT can help them to better target methanogens for a higher accessibility to the binding sites of methanogens than CT, but this higher accessibility can in turn be also directed to fibrolytic bacteria (Jayanegara et al., 2015; Aboagye and Beauchemin et al., 2019). Jayanegara et al. (2015) set up an *in vitro* study to simulate rumen fermentation and assess the effect of CT and HT using tannin plant extracts up to 1 mg/ml. The authors observed that CT had a higher protein binding capacity than HT, whereas both CT and HT linearly decreased CH_4 production, but CT tended to decrease SCFA and OM digestibility more than HT (Jayanegara et al., 2015). A meta-analysis conducted by Berça et al.

(2023) on the relationship between CT and rumen fermentation partially confirmed what discussed above. A linear increase of CT decreased the *in vitro* CH_4 production, and this was accompanied by reduced digestibility of feed (e.g. OM, DM), but here the authors observed a higher total SCFA and propionate formation. The higher propionate rate could partially explain the reduced CH_4 formation, along with the suppressing action of CT on methanogens and on rumen fermentation.

In vitro simulation of rumen fermentation

In vivo experimental approaches to monitor rumen fermentation and measure the outputs comprehend the entire physiological complexity of the animal, which is difficult to "transfer" in an *in vitro* approach. However, the *in vivo* experimental conditions are time and cost consuming and it is hard to standardize the methods due to the variability to which animals are exposed, such as diet, nutrient intake, and environmental conditions (Alende et al., 2018). In light of this, several approaches based on the *in vitro* simulation of rumen fermentative processes were designed and applied. All the *in vitro* methods are based on the collection of fresh rumen material from donor animals (e.g. rumen fluid, rumen epithelial cells, isolation of specific microbial species) to characterize the fermentation end products or to observe the interaction with exogenous compounds or environmental conditions. The idea to monitor and set all the parameters involved in the rumen enteric fermentation allows to standardize the experimental conditions to increase the repeatability and the robustness of the results, with a higher level of adherence to the *in vivo* conditions. The *in vitro* techniques also allow the reduction of experimental time and cost and the number of animals involved in the study, thus complying with the three R for animal research (reduce, refine, and replace) (Vinyard and Faciola, 2022). The factors influencing the *in vitro* approaches to rumen fermentation are multiple. The donor animal species used to collect the fresh rumen fluid for inoculum is important to consider because, in addition to the presence of a core microbiota in rumen, different animal species have a different microbial composition and this affects their interaction with the CH_4 mitigation solutions tested. The diet the donor animals are fed is also crucial because of the strong interaction between nutrient intake and composition of rumen microbiota, as discussed above. Indeed, the feed substrate used for the *in vitro* experiment should be the same or at least similar to the diet used to feed the donor animals. Animals fed a high forage diet are characterized by a higher presence of fibrolytic bacteria and methanogenic archaea than high-concentrate-fed animals. Also, the higher the DM intake, the lower the retention time and the accessibility of degrading bacteria to the ingested feed in rumen. These last dietary features should be depicted also in the *in vitro* feed substrate. Above all, the discussion about the adherence between *in vitro* and *in vivo* results is still open because the data are variable (Yáñez-Ruiz et al., 2016). Hatew et al. (2015) conducted two simultaneous experiments to compare the results

using the same lactating dairy cows and the same diet and feed substrate for the *in vitro* and *in vivo* trials. The authors found that the *in vitro* results could predict the *in vivo* ones by observing a moderate correlation between the *in vitro* 24-hours CH_4 production (expressed per unit of incubated OM) with the *in vivo* CH_4 (per unit of estimated rumen fermentable OM) using diets differing for the source and level of starch (R^2 = 0.54, P = 0.04). A similar approach was adopted by Martínez-Fernández et al. (2013), who tested the effect of several plant compounds on rumen fermentation and CH_4 mitigation with two 24-h and 72-h *in vitro* rumen fermentation trials using goat rumen fluid as inoculum. Then, the *in vitro* results guided the design of the *in vivo* trial, in which the authors treated 16 adult goats with diets supplemented with similar doses of the effective plant compounds observed *in vitro*. The *in vivo* results on CH_4 mitigation were lower than the *in vitro* ones but followed the same trend (e.g. the higher the supplementation dose, the lower the CH_4 production). For example, the same dose of propyl propane thiosulfinate reduced by 33% the *in vivo* and by 87% the *in vitro* CH_4 production. The discrepancy could be explained by a heterogeneous distribution and a higher dilution rate of the tested compounds in the rumen in the *in vivo* condition rather than the *in vitro* condition, and a probably different composition of the rumen microbiota in the two experimental conditions (Martínez-Fernández et al., 2013). These factors affecting the *in vitro* and *in vivo* trials were similarly discussed by Yáñez-Ruiz et al. (2016). The first attempt to study rumen fermentation *in vitro* involved the isolation and the pure culture of specific rumen microbial populations, to observe the effect of exogenous molecules and the physiological response of microbial cells. The main limitation is that in pure cultures the microbial cells are not in their rumen physiological environment and this affects their growth rate and kinetics. A further step was the development of the *in vitro* batch cultures, based on the mixing of the fresh collected rumen fluid and the feed substrate of interest in a closed system. The closed system allows the measurement of feed degradation and gas production, but the fermentation products retained in the system all along the trial can impair the end products by altering the pH stability of the fluid. Because of this, batch culture is usually applied for the preliminary analysis of fermentation rate of feed substrates with or without additives. Batch culture is a short-term approach that gives fast results and, even though it does not fully resemble the *in vivo* condition, it can drive the further research on the substrate used (Yáñez-Ruiz et al., 2016; Vinyard and Faciola, 2022). The Hohenheim gas test (HGT) is an example of batch culture and is based on the use of glass syringes (Figure 7A) filled with freshly collected rumen fluid (liquid phase) put in direct contact with the feed substrate of interest (solid phase). Before the beginning of the experiment, the air within the syringe is removed by pushing the plunger until the mixture of rumen fluid and feed substrate. The syringes are placed at controlled temperature (e.g. 39 °C) and in constant rotation (Figure 7B), to let the fermentation run with the feed substrates homogenously distributed in the rumen fluid.

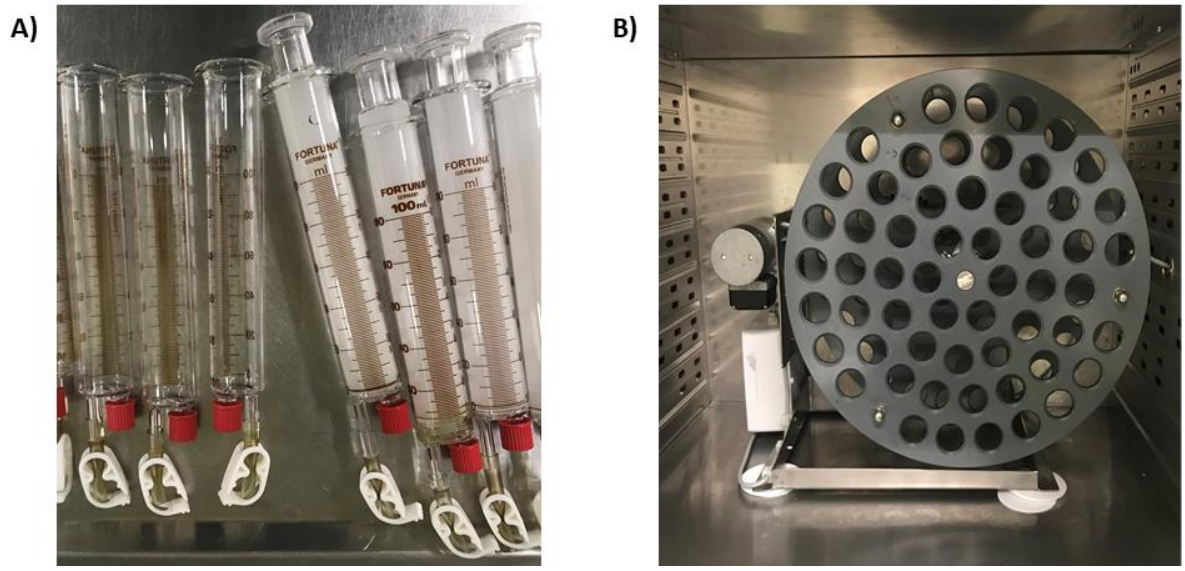

Figure 7. 100 ml glass syringes (A) and rotor placed into the incubator (B). Pictures by the author.

The subsequent displacement of the glass plunger of the syringe is related to the rate of gas production in the syringe by feed fermentation (Figure 8). Usually, HGT is set up to last 24 hours, so it is useful to monitor, compare, and obtain quick indications about the impact of different feed substrates on rumen fermentation (Yáñez-Ruiz et al., 2016; Manoni et al., 2023).

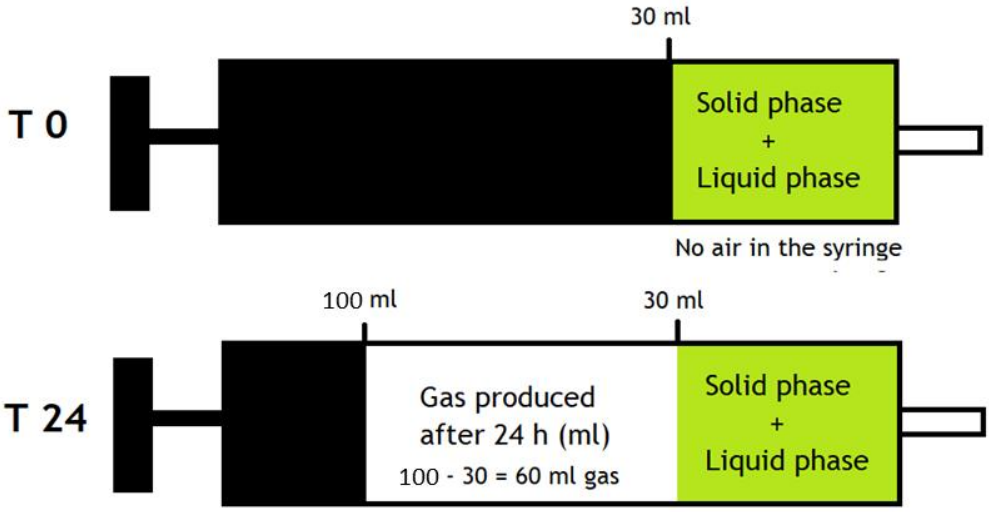

Figure 8. Schematic representation of plunger displacement following gas production after a 24-h HGT trial.

Further batch culture approaches were developed involving: i) periodical release and collection of gas, to reduce the gas pressure within the system, and ii) real-time monitoring and automated analysis of gas production through gas chromatography (e.g. Ankom batch system). The further step to "correct" the batch culture came with the development of continuous cultures, based on maintenance of the culture for longer time than batch cultures and removal of the excess products, such as liquid and

gases, to obtain a more stable culture. These are the major conditions making continuous cultures more similar to the *in vivo* condition, but other important elements are the stratification of the feed particles based on their density and rate of degradation and the constant inflow of salivary buffer (Alende et al., 2018; Vinyard and Faciola, 2022). A limitation of the continuous cultures is the set up and the maintenance of the system, which is time and cost consuming. Another weakness is that the maintenance of protozoa in the culture is difficult, and this can affect the enteric fermentation because an important character in rumen such as protozoa would not be involved. Protozoa usually have a slower replication rate than bacteria and are attached to feed particles, therefore the constant outflow of fluid decreases the presence of protozoa in the system. The protozoa issue is less valid for the batch cultures only because of the faster experimental time and the lack of outflow (Vinyard and Faciola, 2022). A meta-analysis from Hristov et al. (2012) showed that by comparing results from continuous culture and *in vivo* trials, the continuous cultures led to an overall decreased total SCFA, acetate, low abundance of protozoa and reduced OM and NDF digestibility than the *in vivo* trials (Hristov et al., 2012). The Rusitec (rumen simulation technique) is a single-flow continuous culture, firstly developed by Czerkawski and Breckenridge (1977). It is called single because the outflow does not discriminate between solid (e.g. feed) and liquid (e.g. rumen fluid and buffer) components of the solution that is constantly displaced by the system (Vinyard and Faciola, 2022). This is a well-established, continuous and standardized *in vitro* culture useful to let the system and the rumen microbiota adapt to the experimental conditions, to better allow the persistency and the consistency of the effects to be investigated if compared to batch culture approaches such as HGT (García-González et al., 2010). Rusitec is based on the use of fermenting units inserted into a water bath kept at 39-40 °C. The fermenter units are filled with fresh rumen fluid and continuously buffered with artificial saliva. In each fermenter unit, the feed substrates are inserted into nylon bags, to allow the interaction between feed and microbes but to retain the solid phase from the liquid one for the subsequent analysis of nutrient digestibility. Usually, the feed bags are replaced with new feed bags every 24-48 hours, to mimic the intake of fresh feed. Two feed bags are usually inserted in the fermenter units, but the first day of the experiment one of the two bags is filled with solid rumen digesta, to “push” the fermentation and allow the transfer of larger and feed-adherent microbes such as protozoa. The fermenters are closed and anaerobiosis is made by flushing the fermenters with N_2. Continuous or intermittent stirring allows the homogeneous distribution of microbes and feed particles within the whole fermenter. The surplus gas is collected into specific aluminium bags, whereas the liquid surplus is collected into glass bottles, to measure the liquid outflow (Figure 9). To let the rumen microbiota adapt to the artificial environment, the whole duration of the experimental plan is split in two, with the first half called “adaptation phase”, and the second one “experimental phase”. In the experimental phase it is supposed that enough time has passed for microbiota to adapt and establish the enteric fermentation

processes, therefore this is the phase when sampling is recommended and when the system variations are given more by the experimental treatment rather than external factors (Soliva et al., 2011; Li et al., 2021). However, the adaptation phase is useful to set up the system but it also causes a potential alteration of the original *in vivo* rumen inoculum, with protozoa usually being the microbial population most negatively correlated with time because of the high turnover rate in Rusitec and their consequent washout (Lengowski et al., 2016).

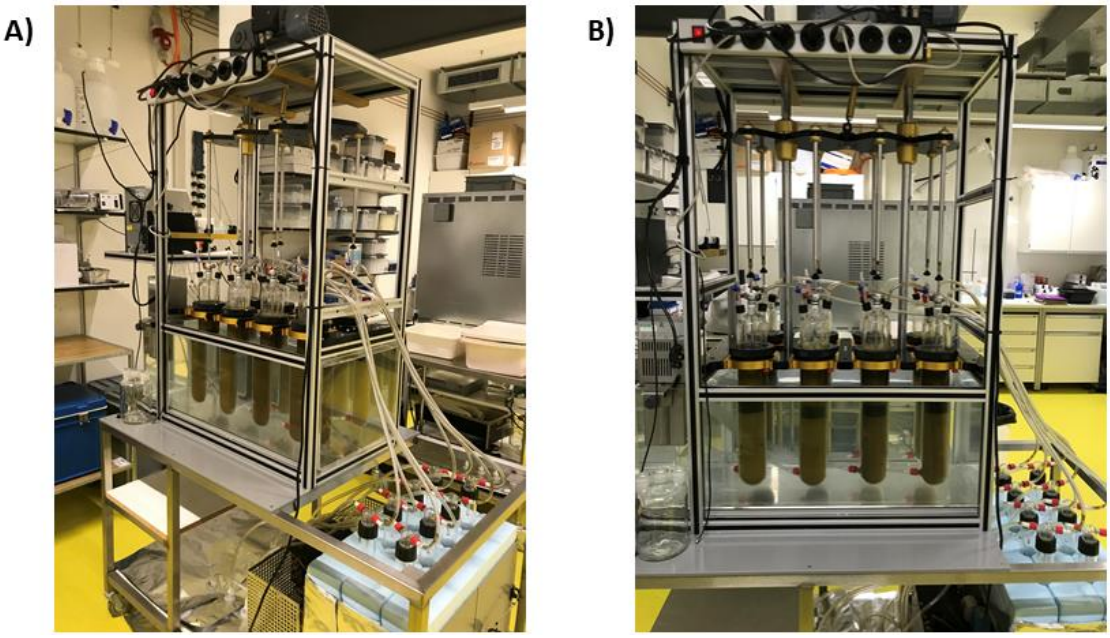

Figure 9. Rusitec system used for the experiments of this research project. Pictures by the author.

Differences among the *in vitro* approaches were discussed by Shaw et al. (2023). The rumen fluid from a single Holstein dairy cow was applied to a batch system (e.g. Ankom) and two Rusitec-like systems for five days of experiment and in parallel to compare the influence of different systems on a single sample and on fermentation outputs. The authors observed that the variation of CO_2 and CH_4 production rates over time (0-120 h) was higher in Ankom batch system than in Rusitec-like semi-continuous systems. Also, the authors observed that up to 48 h all the three systems resembled the "physiological" rumen conditions of the *in vivo* sample, but after 48 h only the Rusitec-like systems were adherent to the physiological *in vivo* condition in terms of gas and SCFA production and composition of rumen microbiota, possibly due to the nature of Rusitec that allows the continuous removal and buffering of the rumen inoculum. Instead, the Ankom batch system was proposed to be used for preliminary short-term trials up to 48 h (Shaw et al., 2023).

Use of tannins in in vitro fermentation

HGT

The CH_4 reduction potential of 17 plant materials was assessed by Jayanegara et al. (2009) through HGT. The authors observed a significant negative correlation between CH_4 production (and in turn a positive with the CH_4 reduction potential) and the total tannin content of the plant materials, but the content of CT was not significantly correlated with lower CH_4 production. The authors also stated that HGT is a valid screening method to quickly obtain information about the potential of plant materials towards CH_4 mitigation (Jayanegara et al., 2009). These findings were further confirmed in a meta-analysis by Jayanegara et al. (2012), in which the authors built up a database of only studies involving HGT and tannin plant materials, although the different effect of CT and HT was not recorded. The authors observed a negative correlation between tannin concentration in tannin plant materials and total gas, CH_4, and SCFA production, and with digestibility of OM, CP, and NDF. Instead, the correlation with a reduced abundance of protozoa was not observed, even though it was discussed above that an indirect effect of tannins on protozoa could reduce H_2 and in turn CH_4 production. The effective tannin supplementation was found to range from 20 to 100 g/kg DM (Jayanegara et al, 2012). A direct inhibiting effect on CH_4 production probably given by a reduced abundance of methanogenic archaea and protozoa was observed by testing a total of six different HT and CT plant sources including chestnut and quebracho added until 250 mg/g to a basal diet (Bhatta et al., 2009). Giller et al. (2021) used six fruit pomaces with a different CT and HT content and observed that no differences were detected by using the fruit pomaces with doses lower than 150 g/kg DM. Then, pomegranate pomace used up to 500 g/kg DM, being the one with the highest HT content, did not affect total gas production and nutrient digestibility but significantly reduced CH_4 production and total SCFA. The authors indicated that HT could more effectively reduce CH_4 production than CT (Giller et al., 2021). The combined effect of tannins and essential oils as CH_4 mitigation agents was investigated with HGT by Foggi et al. (2022). This study included quebracho-CT tannins and chestnut-HT tannins used either alone or in combination with four essential oils and five essential oil compounds, with a maximum supplementation dose of 20 g/kg DM. The single tannin treatments were able to reduce total gas, CH_4 and ammonia-N production even without the combination with essential oils. In contrast with Jayanegara et al. (2015), here the authors reported a lower value of organic matter digestibility with HT-rich chestnut than with CT-rich quebracho (Foggi et al., 2022).

Rusitec

Jayanegara et al. (2018) analysed six Rusitec trials involving different tannin plant extracts, such as chestnut, sainfoin, and acacia. The authors observed a linear decrease of nutrient digestibility (CP, NDF,

ADF) correlated with an increased dietary tannin supplementation, supporting the idea that tannins form large complexes with macromolecules and subtract them from rumen degradation. Along with an increased tannins level, CH_4 production significantly decreased despite any alteration of total gas production was observed. Interestingly, dietary tannins had no effect on total SCFA production and abundance of bacteria and protozoa, these latter usually found to be reduced by the action of tannins, even though the effect of tannins on protozoa is not unique (Jayanegara et al., 2018). The effect of chestnut tannins was also evaluated and compared to the use of medium chain fatty acids (MCFA) as a CH_4 mitigation strategy through Rusitec (Pérez-Ruiz et al., 2023). In the cited study, the authors compared the impact on rumen fermentation of: i) a standard total mixed ration (TMR) supplemented with 10 g/kg DM of tannin extract composed of chestnut and quebracho plus the addition of tannic acid, and ii) a TMR supplemented with 10 g/kg DM of a mixture of MUFA. The rumen inoculum was collected from 3 Holstein × Jersey cows. Total gas and SCFA production were not affected by the treatments, but the tannin treatment significantly decreased ammonia production compared to the control and the MCFA treatment. The nutrient digestibility of DM, CP, and NDF was significantly reduced by tannins rather than control and MCFA treatments. Regarding the composition of rumen microbiota, a decreased overall bacterial richness was observed over time, but both the tannin and MCFA treatments increased the abundance of *Succinivibrio* and only tannins reduced *Methanobrevibacter*, whereas *Fibrobacter* were not altered. The authors concluded that tannins reduced protein degradation in rumen and methanogens abundance (Pérez-Ruiz et al., 2023). The quebracho tannin extract was also found to be effective in modulating rumen fermentation by Makkar et al. (1995), using rumen fluid from oxen and by adding quebracho tannins up to 0.4 mg/ml to a wheat-based feed substrate. In the 8-day Rusitec trial, the CT from quebracho were not degraded. Tannins did not alter DM digestibility and total SCFA production but raised the level of propionate and reduced the level of acetate. This was related to a significantly decreased abundance of protozoa (Makkar et al., 1995). To conclude, Wischer et al. (2013) screened with HGT the effect of ten different tannin plant sources (e.g. quebracho, mimosa, chestnut, grape seed, etc.) and four rapeseed tannin monomers on rumen fermentation supplemented at 5 and 10% DM. Then, the authors selected the five best candidates for a further step with a 14-d Rusitec trial. Grass silage was used in both experiments as the standard feed substrate and the rumen fluid was collected from two Holstein-Friesian cows. Four tannin plant extract including chestnut (HT-rich) and grape seed (CT-rich) were selected for Rusitec and used at 10% DM because in the HGT trial they reduced CH_4 production (-14% and -28% than control, respectively) without impairing total gas production. With Rusitec, chestnut exerted the highest CH_4 reduction (-63%) compared to control. Moreover, chestnut extract reduced total SCFA production and ammonia-N, but it did not reduce nutrient digestibility such as grape seed, which reduced CP digestibility. Chestnut supplementation at 10% DM reduced CH_4 production but also

impaired rumen fermentation (e.g. lower SCFA production) (Wischer et al., 2013). Considering all these results, the negative correlation between the concentration of tannins and CH_4 production can be observed, but for almost all the other parameters discussed (e.g. total SCFA, nutrient digestibility, microbial abundance) the correlation is not fully clear and statistically demonstrated, because the experimental conditions and the tannin sources affect the endpoints of the fermentation.

Aim of the project (Section B)

The research about rumen fermentation has to consider multiple aspects such as ruminant physiology and role of the rumen microbial community in feed fermentation and gas production in rumen. Therefore, the aim of this research project was to use two *in vitro* fermentation approaches, first HGT and then Rusitec, to evaluate the impact of tannins on rumen fermentation parameters and on the composition and metabolism of rumen microbiota. The 24-h HGT trial was adopted because it is a valid and reliable preliminary approach to evaluate experimental feed supplements and it is time- and cost-saving. We used HGT to set up a further trial, this time involving a 10-d Rusitec trial, a more robust and representative approach than HGT. Rusitec was applied to obtain long-term insights on the influence and the potential microbial adaptation to the use of tannins. The GA and EA compounds were applied either alone or as co-treatment. Here, the decision was driven by the idea of precisely targeting the bioactive effect of single HT components, rather than using tannin plant extracts, known to be effective in modulating gas production in rumen but for which the single bioactive component is not known. The doses of supplementation applied in the HGT trial were slightly higher than usual doses of supplementation, to better discriminate between the effects of a higher (15% DM) and a lower (7.5% DM) supplementation. Since the lower supplementation dose of 7.5% DM gave promising results mostly for EA, we decided to set up the Rusitec by keeping this lower supplementation dose.

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List of articles:

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Section A – Article 1

Ex-foods diets affect the fatty acid profile of the abdominal adipose tissue without significantly affecting the plasma metabolome of post-weaning piglets

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Abstract

Former food products (FFP) are sustainable feed ingredients, although their metabolic impact has never been investigated. In this study, the aim was to investigate the effect of partially replacing conventional cereals with FFP on the quality of the abdominal fat and on the modulation of plasma metabolome of post-weaning piglets. Thirty-six Large White × Landrace post-weaning piglets (28 days old) were randomly divided into three dietary groups: control (CTR), 30% replacement of the control diet with salty FFP (SA), 30% replacement of the control diet with sugary FFP (SU). The body weight (BW) and feed intake (FI) were measured to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). The fatty acid profile of the abdominal adipose tissue was determined. Moreover, an untargeted metabolomics evaluation via ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS-MS) was performed on plasma. No significant differences were observed between the groups in terms of growth performance ($P > 0.05$). Despite some differences observed in the fatty acid profiles of the three diets, the piglets rebalanced the fatty acid profile of the abdominal adipose tissue. The CTR group showed lower monounsaturated fatty acids (MUFA) compared to the SA and SU FFP groups ($P < 0.001$), whereas the polyunsaturated fatty acids (PUFA) were higher in the CTR and SA groups compared to the SU group ($P < 0.05$). An increasing n-6/n-3 ratio was observed between the CTR, SA, and SU groups, respectively ($P < 0.001$). The plasma metabolic changes were more closely related to the effect of time than to the experimental diets. Six metabolites were altered in the two FFP groups compared to the CTR: caffeine, theobromine, proline-betaine, dipalmitoyl-phosphatidylcholine (PC 32:0), spermidine, and L-tryptophan ($P < 0.05$). Furthermore, caffeine and glycerophospholipid pathways were significantly different between the CTR and both FFP groups ($P < 0.05$). No differences were observed in the plasma metabolomes between

the SA and SU diets. Overall, the limited impact of FFP on the piglets' abdominal fat, plasma metabolome, and related pathways demonstrates the value of FFP as innovative and sustainable feed ingredients. Future studies should determine the influence of FFP-based diets on the mechanisms of fat deposition and the perturbation of the metabolome in pigs.

Introduction

Livestock production is currently facing an increasing demand for animal-source food (Mottet et al., 2017) while being responsible for 14.5% of all anthropogenic global greenhouse gas emissions, of which 45% stem from feed production (Gerber et al., 2013). In this context, one-third of global food production is lost or wasted annually (FAO, 2011). The reuse of lost or wasted food as animal feed could be beneficial for both the environment and the global food supply due to reducing the wastage of natural resources and reducing the feed–food competition (Sandström et al., 2022).

Food waste, food losses, and ex-foods/former food products all refer to food effluents, which mainly differ for: i) the stage of the food chain from which they drop out; and ii) their legal status regarding animal nutrition (Pinotti et al., 2021). Food waste cannot be used as animal feed due to the risk of disease transmission for both animals and humans. Instead, former food products (FFP) are foodstuffs, other than catering reflux, manufactured for human consumption according to the European Union (EU) food regulations but no longer intended for humans for practical or logistical reasons, or due to manufacturing or packaging defects, and which do not present health risks if used as feed (EU, 2013). The FFP are also referred to as bakery meals, especially in the United States (Liu et al., 2018; Stein et al., 2023), and are not classified as food waste by law, at least not in the EU and United States (Pinotti et al., 2021). The FFP are typically processed by feed business operators starting from food, before being delivered to compound feed manufacturers or, in some cases, directly to livestock farmers. Depending on the needs, the feed business operator processes FFP from various food sources to ensure a constant production flow and stable nutrient composition (EFFPA, 2018; Pinotti et al. 2021). The FFP are generated in large quantities and are still usable in various sectors due to their energy and nutrient content. One option to both address food waste and promote sustainable livestock production is to use FFP as feed ingredients, thereby upgrading food losses into valuable feed products (Sandström et al., 2022; Pinotti et al., 2023).

The FFP do not pose any risks to animal health when used as raw material for feed (EU, 2017). The FFP used in the present study are classified as salty bakery FFP (SA) and sugary confectionery FFP (SU). Generally, FFP exhibit a higher content of energy, lipids, and high-digestible starch than conventional cereals. By using a balanced combination of standard feed ingredients and FFP, it is possible to partially replace "conventional" energy and protein sources, such as cereals, in animal diets. This study extends

the works by Luciano et al. (2021) and Tretola et al. (2022), in which it was shown that diets containing 70% conventional cereals and 30% FFP did not adversely affect the diet digestibility, growth performance, gut microbiota, or blood metabolites of post-weaning piglets. The FFP-containing diets were also found to be safe in terms of microbiological contamination (Tretola et al., 2017) and to not exceed the safety threshold for packaging contaminants established by the German Federal Ministry of Food (van Raamsdonk et al., 2011; Mazzoleni et al., 2023a). These encouraging results can serve as the starting point for more in-depth and comprehensive analyses of aspects such as the potential impact of FFP on the quality of the abdominal adipose tissue, the plasma metabolome, and the related metabolic pathways.

Adipose tissue plays a key role in the regulation of lipid metabolism. It is responsible for energy storage in the form of lipids, and in pigs it is the major source of circulating free fatty acids. Adipose tissue is related to the overall fatty acid synthesis, thus affecting other lipid-target organs, such as muscles and liver. A relationship exists between the fatty acid composition of products of animal origin (e.g. eggs, milk, meat), the biosynthesis of fatty acids in adipose tissue, and the fatty acid composition of ingested lipids (Kouba and Mourot, 2011). The influence of the composition of dietary fat on the fat deposition in adipose tissue is more evident in monogastrics than ruminants. Indeed, the dietary unsaturated fatty acids are hydrogenated in the rumen, thus altering the original fatty acid profile of dietary lipids. This process does not occur in monogastrics, in which the rate of fat deposition in adipose tissue is higher than in ruminants and the fatty acid profile of adipose tissue more clearly reflects the original dietary lipids (Madsen et al., 2009; Kouba and Mourot, 2011; Schumacher et al., 2022).

Metabolomics enables the investigation of nutrient–metabolism interactions, allowing assessment of the possible activation of specific metabolic pathways or the variation in the levels of certain metabolites (Zduńczyk and Pareek, 2009; Sun et al., 2015). Over the past decade, several studies have used untargeted metabolomics on pig plasma and/or urine to identify metabolites as biomarkers of specific dietary treatments in an effort to better understand the diet–health relationship (Dannenberger et al., 2016). To date, the metabolic status of piglets fed with FFP-supplemented diets (or other food by-products) has not been investigated by applying metabolomics, possibly due to concerns regarding the nutritional and safety features of FFP, which require further evaluation. The hypothesis tested in this study is that the metabolic impact of diets containing up to 30% FFP may be minimal, considering the previous positive results reported above (Luciano et al., 2021; Tretola et al., 2022). Therefore, the aim of this study was to determine the impact of the dietary inclusion of 30% SA or SU FFP on the fatty acid profile of the abdominal fat and the plasma metabolome of post-weaning piglets by using an untargeted metabolomics approach.

Material and methods

Animals and experimental design

Thirty-six post-weaning female piglets (Large White × Landrace, 28 days old) with a body weight (BW) of 6.5 ± 1.1 kg were housed in individual pens in the same room under controlled temperature and air flow conditions. Small plastic balls were provided as environmental enrichment, which accorded with the EU guidelines (2008). After 7 days of adaptation, the piglets were randomly divided into three dietary groups: control (CTR), 30% replacement of the control diet with salty FFPs (SA), or 30% replacement of the control diet with sugary FFPs (SU). They were then given ad-libitum access to feed and water for 42 days. At day 42, six piglets per group were slaughtered so that samples could be collected for further investigation.

Experimental diets

The diets were analysed in terms of their compliance with the EU regulation concerning methods of sampling and analysis for the official control of feed (EU, 2009). The piglets assigned to the CTR group received a standard diet, whereas those in the SA and SU groups were fed diets in which 30% of the CTR diet was replaced with SA and SU FFP, respectively. The analytical composition of the SA and SU ingredients and the ingredient composition and analytical composition of all three diets complied with the requirements of the National Research Council (NRC, 2012) and are reported in Tretola et al. (2022). All the three diets were formulated to be iso-energetic (14.0 MJ/kg dry matter (DM)) and iso-nitrogenous (19.0% DM).

Analysis of the fatty acid profiles of the experimental diets

Fatty acid extraction from feed

The extraction of fatty acids from the three experimental diets was performed as described by Bligh and Dyer (1959). More specifically, an aliquot of feed (3.00 ± 0.05 g) was weighed into a 50 mL polypropylene centrifuge tube. The target analytes were then extracted from the matrix using 11.25 mL of a 1:2 (v/v) solution of chloroform:methanol. Next, the samples were stirred for 30 min in a wrist shaker and centrifuged for 10 minutes at 2000 rpm. Afterwards, 3.75 mL of chloroform and 3.75 mL Milli-Q water were added, with the sample undergoing further stirring after each solvent addition. The samples were then centrifuged at room temperature for 10 minutes at 2000 rpm. The supernatant was collected and, following filtration, dried in previously weighed flasks.

Methyl ester preparation

The previously obtained extracts were resuspended in 1 mL of hexane. An aliquot corresponding to 30 mg of abdominal adipose tissue was dried under a nitrogen flow in a screw-capped tube for the derivatization step, as described by Moltò-Puigmarti et al. (2007). Briefly, the samples were incubated at 80 °C for 10 minutes following the addition of 0.5 mL of sodium methoxide solution. Once cooled to 37 °C, 0.5 mL of bromine trifluoride solution was added. The samples were incubated again for three minutes at 80 °C to allow the formation of fatty acid methyl esters. Then, the methyl esters were extracted by adding 0.5 mL of hexane and vortexing the samples for one minute. After the addition of both 1 mL of a saturated solution of sodium chloride and 100 mg of sodium sulphate anhydrous, the sample was gently shaken, and following centrifugation, the supernatant was removed and dried under a flow of nitrogen. Finally, the samples were resuspended with 1 mL of hexane and subjected to instrumental gas chromatography-flame ionization detection (GC-FID) analysis.

Gas chromatography (GC) detection

The analysis of the methyl esters was performed by means of a TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific, Rodano, Italy) equipped with a flame ionization detector. An RT-2560 fused silica capillary column (100 m x 0.25 mm x 0.25 µm film thickness; Restek, Milan, Italy) was used with a temperature range programmed from 70 °C to 250 °C at 2 °C/min. The carrier gas was nitrogen at 1.0 mL/min, with an inlet pressure of 16.9 psi. A quantitative procedure was used in which 1 mL of internal standard (1 mg/mL 23:0 methyl ester; N-23-M; Nu-Chek Prep Inc., Elysian, MN, USA) was added prior to methylation. The fatty acid methyl ester (FAME) content was quantified by weight and expressed as the g/100g of the total FAMEs. The detailed fatty acid profile of the three diets is reported in Table 1.

Table 1. Fatty acid profiles of the three experimental diets. Data are expressed as the percentage (%) of the total fatty acids.

Fatty acid	**CTR[1]**	**SA[2]**	**SU[3]**
C6:0	0.03	0.04	0.06
C8:0	0.20	0.38	0.30
C10:0	022	0.44	0.41
C11:0	-	-	-
C12:0	1.29	2.29	1.67
C13:0	-	-	-
C14:0	0.50	0.66	1.28
C14:1	0.01	0.02	0.06
C15:0	0.07	0.08	0.13
C15:1	-	-	-
C16:0	18.82	17.82	21.89
C16:1	0.47	0.37	0.46
C17:0	0.14	0.11	0.15

C17:1	-	-	-
C18:0	5.98	5.04	8.63
C18:1n9t	0.05	0.07	0.10
C18:1n9c	22.94	36.48	36.98
C18:2n6t	-	-	-
C18:2n6c	42.83	31.57	24.18
C20:0	0.29	0.28	0.34
C18:3n6	0.01	-	-
C20:1	0.47	0.38	0.30
C18:3n3	4.13	2.69	1.97
C21:0	0.02	0.01	0.01
C20:2	0.10	0.04	0.04
C22:0	0.29	0.37	0.30
C20:3n6	0.01	-	0.01
C22:1n9	0.04	0.03	0.03
C20:3n3	0.04	-	-
C20:4n6	0.12	0.06	0.08
C22:2	0.04	0.02	0.01
C24:0	0.15	0.17	0.15
C20:5n3	0.39	0.28	0.18
C24:1	0.06	0.05	0.04
C22:6n3	0.29	0.25	0.23
SFA[4]	28.02	27.68	35.32
MUFA[5]	24.03	37.40	37.97
PUFA[6]	47.95	34.92	26.70
n-6/n-3	8.87	9.81	10.17

[1] CTR = control diet.
[2] SA = 30% replacement of the control diet with salty FFPs.
[3] SU = 30% replacement of the control diet with sugary FFPs.
[4] SFA = saturated fatty acids.
[5] MUFA = monounsaturated fatty acids.
[6] PUFA = polyunsaturated fatty acids.

Growth performance

For each piglet, the BW and fecal dry matter were measured weekly, whereas the feed intake (FI) was measured daily. In addition, the FI and BW were used to calculate the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR). The latter three were calculated as the average of the whole experimental procedure from day 0 to 42.

Analysis of the fatty acid profile of abdominal adipose tissue

At the end of the experiment, six piglets per group were slaughtered to allow the collection of different samples to be used in parallel investigations. In the present work, approximately 50.0 g of abdominal adipose tissue was collected from between fifth and seventh ribs of the pork abdomen, packed in 50

mL of Falcon tubes, immediately frozen in liquid nitrogen, and then stored at −80°C until required for further analysis. Extraction and derivatization of the abdominal adipose tissue samples were performed in relation to 12 piglets according to the established laboratory protocols described by Bligh and Dyer (1959) and Bondia-Pons et al. (2007). The FAMEs were measured using a ThermoQuest Trace GC 2000 gas chromatograph (Thermo Scientific, Bremen, Germany) equipped with a flame ionization detector (Restek, PA, USA). Nonadecanoic acid (C19:0, 10 mg/mL of hexane) was used as the internal standard and nitrogen (N) as the carrier gas. The FAMEs were separated using a fused silica capillary column (Rt-2560, 100 m × 0.25 mm × 0.25 µm) according to the specified program: 70 °C for 5 min, increased by 2 °C/min until 240 °C, with a total chromatographic runtime of 120 min. The individual FAMEs were verified by comparing the peak retention times with standard mixtures (Supelco 37 FAME Mix, Bellefonte, PA, USA) and pure standard methyl esters obtained from Sigma-Aldrich (Saint Louis, MO, USA).

Blood collection and sample processing

At days 0, 21, and 42, 6 piglets from each dietary group were fasted overnight prior to blood collection. The blood was collected using vacutainers containing ethylenediaminetetraacetic acid (EDTA). To obtain the required plasma, the blood samples were centrifuged at 14,000 rpm for 10 minutes at room temperature. The plasma was then stored at -80 °C to allow for determination of the pattern of changes in the plasma metabolome using a mass spectrometry-based target metabolomic strategy. Prior to the analysis, the plasma samples were pre-processed to adapt them to the experimental conditions necessary for metabolomic investigation. More specifically, the protein concentration in each plasma sample was determined using a Nanodrop-1000 A280nm (Thermo Scientific). Then, 100 µL of each plasma sample was collected and mixed with 500 µL of extraction phase buffer (methanol:ethanol, 1:1 v/v). The samples were then vortexed and stored at -20 °C for 1 hour before being centrifuged at 14,800 rpm for 10 minutes. Next, 500 µL of the supernatant was collected and dried using N-flow. The dried extracts were then diluted in 50 µL methanol so that they were ready to be analyzed via ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS-MS) in both the positive and negative electrospray ionization (ESI) modes. Each sample was injected twice and 5 µL of each was used in both the positive and negative ESI modes. In the same analytical sequence of samples, a “wash” sample comprised of only the mobile phase A was injected. For the validation of the method, a reference pool sample was prepared by mixing 10 µL of each sample, whereas a blank sample was prepared as described above but using water rather than plasma.

Ultra-high-performance liquid chromatography and high-resolution tandem mass spectrometry (UHPLC/MS-MS) analysis

Untargeted UHPLC/MS-MS was also performed. All the samples were analyzed at UNITECH OMICs (University of Milan, Italy) using an ExionLC™ AD system (SCIEX) connected to a TripleTOF™ 6600 system (SCIEX) equipped with Turbo V™ ion source with an ESI probe. Chromatographic separation was achieved in a CORTECS UPLC T3 column (Waters)—150 mm (length) × 2.1 mm (ID) × 1.6 µm (particle size)—using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a flow rate of 400 µL/min. The column and autosampler temperatures were set at 40 °C and 7 °C, respectively. The sample injection volume was 5 µL. The MS spectra were collected over an m/z range of 50–1500 Da in positive/negative polarity when operating in information-dependent acquisition (IDA) mode. The collision energy was set at 30.

Statistical analysis and metabolomic data processing

The data concerning growth performance were analyzed using IBM SPSS Statistics v. 27 software (SPSS, Chicago, IL, USA). The Shapiro-Wilk test was used to test the normality of the distribution, and then the data were analyzed via ANOVA. The REPEATED statement was used for all the variables measured over time. Differences with a $P < 0.05$ were considered significant. MS-DIAL v. 4.24 software was used to process the UHPLC/MS-MS data (Tsugawa et al., 2015). Two databases were designed: i) MSMS-Public-Pos, for the positive ESI mode analysis; and ii) MSMS-Public-Neg, for the negative ESI mode analysis. At first, the data processing using MS-DIAL was performed by applying the Lowess normalization, which supports the Lowess algorithm and the "Blank filter" option, which filter the peaks present in the Blank sample. After this initial phase of normalization, the software generated 6124 IDs in ESI Pos and 10558 ISs in ESI Neg. The IDs were based on the m/z value (parent ion) detected and determined in high resolution. For the reference pool sample (n=7), only IDs with an average response area with a 30% lower CV% were considered and identified in all the technical replicates (n=7) of the reference pool samples, which obtained 4762 IDs in ESI Pos (known + unknown) and 752 IDs in Esi Neg (known + unknown). From the total IDs identified in both ESI Pos and ESI Neg, only those with a known MS-MS spectrum were selected, which resulted in 3867 IDs in ESI Pos and 432 IDs in ESI Neg. For each of these IDs, the mean of the areas of the technical replicates was calculated and the samples were classified by both dietary group (CTR, SA, SU) and time (T0, T1, T2). The peak list from MS-DIAL was then transferred to Excel to generate a matrix of m/z ratios, retention times, and peak intensities. MetaboAnalyst 5.0 software was used for the metabolomics analysis and interpretation of data (Pang et al., 2022). All the statistical analyses were performed using the same approach for both

the ESI Pos and ESI Neg data. Since the metabolomic analysis was semiquantitative, the concentrations mentioned here refer to the relative concentrations, as determined from the metabolites' peak area (arbitrary unit). The data were normalized considering the protein concentration prior to analysis (Supplementary Table S1). Any differences in the abundance of metabolites between the three dietary treatments and/or between the three different time points were assessed via ANOVA followed by post-hoc analysis (Kruskal-Wallis test and Fisher's least significant difference). For each treatment time, an unpaired analysis was performed to compare the dietary groups. Then, for each dietary group, a paired analysis was performed to compare the T0 and T2 treatment times. The *P* value threshold was set at $P < 0.05$. MetaboAnalyst 5.0 was also used to perform the sparse partial least squares-discriminant analysis (sPLS-DA), for which 10 variables per component were selected. The agglomerative hierarchical cluster analysis was performed based on Pearson's correlation as a similarity measure and Ward's linkage parameter as the clustering algorithm. To explore the correlations between the metabolomics features and metadata of interest (FI, BW, ADG, and FCR), the MetaboAnalyst 5.0 correlation analysis tool was used, with the specific metadata of interest chosen as the target and Pearson's r as the correlation measure.

Results

SA and SU FFPs did not impair the growth performance of growing piglets

All the piglets were in good health, and no morbidity or mortality was observed throughout the entire experimental trial. As reported in Table 2, there were no significant differences ($P > 0.05$) in terms of the BW, ADFI, ADG, and FCR between the piglets fed with SA and SU FFPs and the CTR group.

Table 2. Effect of FFPs on the growth performance of post-weaning piglets (overall mean days 0–42).

Parameter	CTR[1]	SA[2]	SU[3]	SEM[4]	*P* value
BW[5] (kg)	14.9	14.1	13.8	0.43	0.56
ADFI[6] (kg)	0.69	0.63	0.64	0.02	0.59
ADG[7] (kg)	0.47	0.45	0.44	0.01	0.62
FCR[8] (kg/kg)	1.49	1.48	1.50	0.02	0.96

[1] CTR = control diet.
[2] SA = 30% replacement of the control diet with salty FFPs.
[3] SU = 30% replacement of the control diet with sugary FFPs.
[4] SEM = standard error of the mean.
[5] BW = body weight.
[6] ADFI = average daily feed intake.
[7] ADG = average daily gain.
[8] FCR = feed conversion ratio.

SA and SU FFPs influenced the fatty acid profile of abdominal adipose tissue

The fatty acid profile of the piglets' abdominal adipose tissue (Table 3) showed a tendency (P = 0.06) toward a lower saturated fatty acid (SFA) content in the SA group than in the CTR and SU groups. An average significant (P < 0.001) increase of 17% in monounsaturated fatty acids (MUFA) was observed in the SA and SU groups when compared with the CTR group. This difference was probably driven by the significantly lower level of oleic acid (C18:1n9c) in the CTR group when compared with both the SA and SU groups. The level of polyunsaturated fatty acids (PUFA) was significantly reduced in the SU group when compared with the CTR group (-33%, P < 0.05). This trend with regard to PUFA was similarly observed for both the pooled n-6 and n-3 fatty acids and the single major n-6 (e.g. linoleic acid, C18:2n6c) and n-3 (e.g. linolenic acid, C18:3n3) species. Indeed, the SU group showed lower n-6 (-32%, P < 0.05) and n-3 (-43%, P < 0.01) levels than the CTR group, whereas the levels of PUFA, n-6, and n-3 were not significantly different between the SA and CTR groups (P > 0.05). Moreover, a significant increase (P < 0.001) in the n-6/n-3 ratio was observed in the SA (+14%) and SU (+18%) groups when compared with the CTR group.

Table 3. Fatty acid profiles of the abdominal adipose tissue samples from the three experimental groups. Data are expressed as the percentage (%) of the total fatty acids.

Fatty acid	**CTR[1]**	**SA[2]**	**SU[3]**	**SEM[4]**	***P* value**
C6:0	0.00	0.00	0.00	0.00	-
C8:0	0.01	0.01	0.01	0.00	0.58
C10:0	0.11	0.11	0.11	0.00	0.97
C11:0	0.00	0.00	0.00	0.00	-
C12:0	0.21	0.28	0.21	0.01	0.09
C13:0	0.00	0.00	0.00	0.00	-
C14:0	1.82	1.92	1.88	0.03	0.45
C14:1	0.03	0.03	0.03	0.00	0.31
C15:0	0.05	0.06	0.07	0.00	0.27
C15:1	0.00	0.00	0.00	0.00	-
C16:0	27.72	26.19	27.77	0.32	0.10
C16:1	2.85	2.98	2.70	0.13	0.75
C17:0	0.25	0.26	0.30	0.01	0.18
C17:1	0.00	0.00	0.00	0.00	-
C18:0	14.29	11.87	14.56	0.51	0.08
C18:1n9t	0.08	0.10	0.13	0.01	0.09
C18:1n9c	31.65^{a}	37.14^{b}	37.85^{b}	0.96	<0.001
C18:2n6t	0.00	0.00	0.00	0.00	-
C18:2n6c	17.37^{a}	15.88^{ab}	11.71^{b}	1.06	0.04
C20:0	0.20	0.18	0.21	0.01	0.69
C18:3n6	0.05	0.06	0.04	0.00	0.37
C20:1	0.57	0.55	0.62	0.03	0.69
C18:3n3	1.45^{a}	1.13^{ab}	0.79^{b}	0.10	0.004
C21:0	0.01	0.01	0.02	0.00	0.15
C20:2n6	0.53^{a}	0.42^{b}	0.35^{c}	0.02	<0.001

C22:0	0.02	0.03	0.05	0.01	0.05
C20:3n6	0.10	0.10	0.08	0.01	0.30
C22:1n9	0.01	0.01	0.01	0.00	0.36
C20:3n3	0.13[a]	0.09[b]	0.07[b]	0.01	<0.001
C20:4n6	0.29	0.35	0.27	0.02	0.26
C22:2	0.01	0.01	0.01	0.00	0.91
C24:0	0.01	0.01	0.01	0.00	0.19
C20:5n3	0.03	0.03	0.02	0.00	0.21
C24:1	0.01	0.01	0.01	0.00	0.84
C22:6n3	0.15	0.17	0.13	0.01	0.13
SFA[5]	44.70	40.94	45.19	0.77	0.06
MUFA[6]	35.20[a]	40.81[b]	41.34[b]	0.95	<0.001
PUFA[7]	20.10[a]	18.24[ab]	13.47[b]	1.21	0.03
n-6	17.81[a]	16.40[ab]	12.10[b]	1.08	0.04
n-3	1.76[a]	1.42[ab]	1.01[b]	0.12	0.004
n-6/n-3	10.12[a]	11.57[b]	11.98[b]	0.29	<0.001

Different letters in the same row (a, b, c) indicate statistically significant differences between groups ($P < 0.05$).

[1] CTR = control diet.
[2] SA = 30% replacement of the control diet with salty FFPs.
[3] SU = 30% replacement of the control diet with sugary FFPs.
[4] SEM = standard error of the mean.
[5] SFA = saturated fatty acids.
[6] MUFA = monounsaturated fatty acids.
[7] PUFA = polyunsaturated fatty acids.

SA and SU FFPs increased plasma caffeine and theobromine concentrations

The ANOVA showed that regardless of the time point, two features were significantly different ($P < 0.01$) between the different dietary groups. Details regarding the features identified via the one-way ANOVA and post-hoc analysis are summarized in Figure 1.

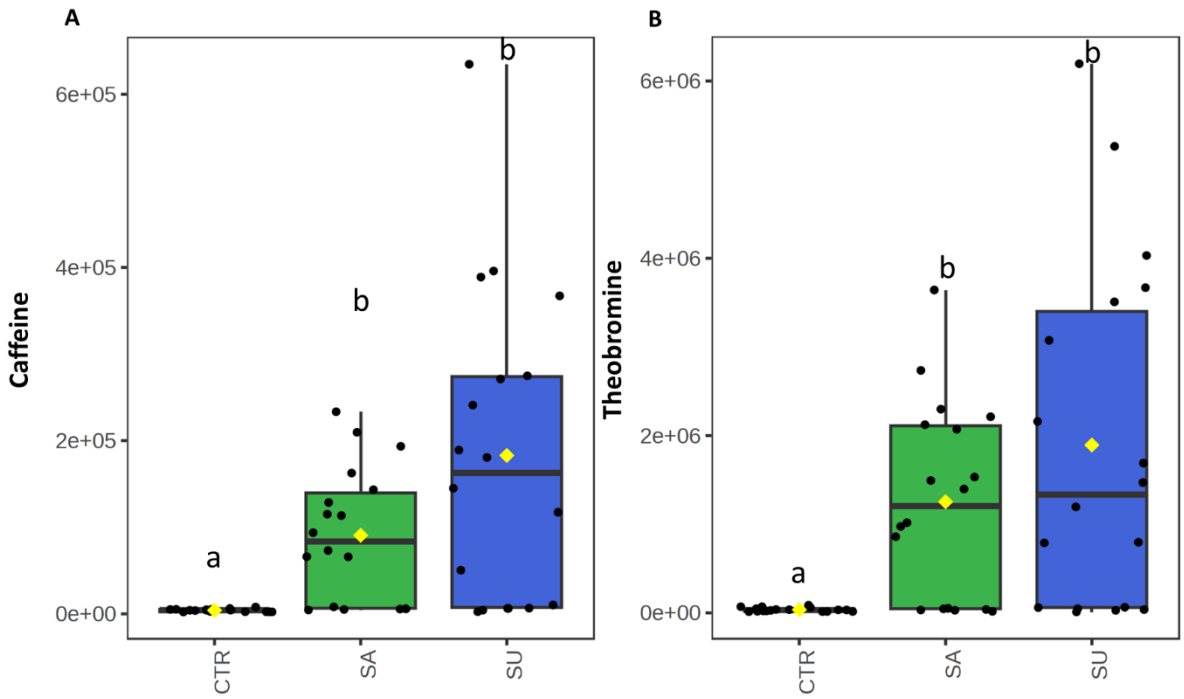

Figure 1. Caffeine and theobromine concentrations in the control (CTR), salty (SA), and sugary (SU) groups, irrespective of the time point. The values refer to arbitrary units, as determined from the metabolites' peak area.

The Kruskal-Wallis post-hoc test revealed that the caffeine and theobromine concentrations were increased ($P < 0.05$) in both the SA and SU groups when compared with the CTR group (Figure 1). The sPLS-DA analysis showed that the CTR group differed slightly from the SA and SU groups, while the SA and SU groups appeared to be more similar to each other (Figure 2).

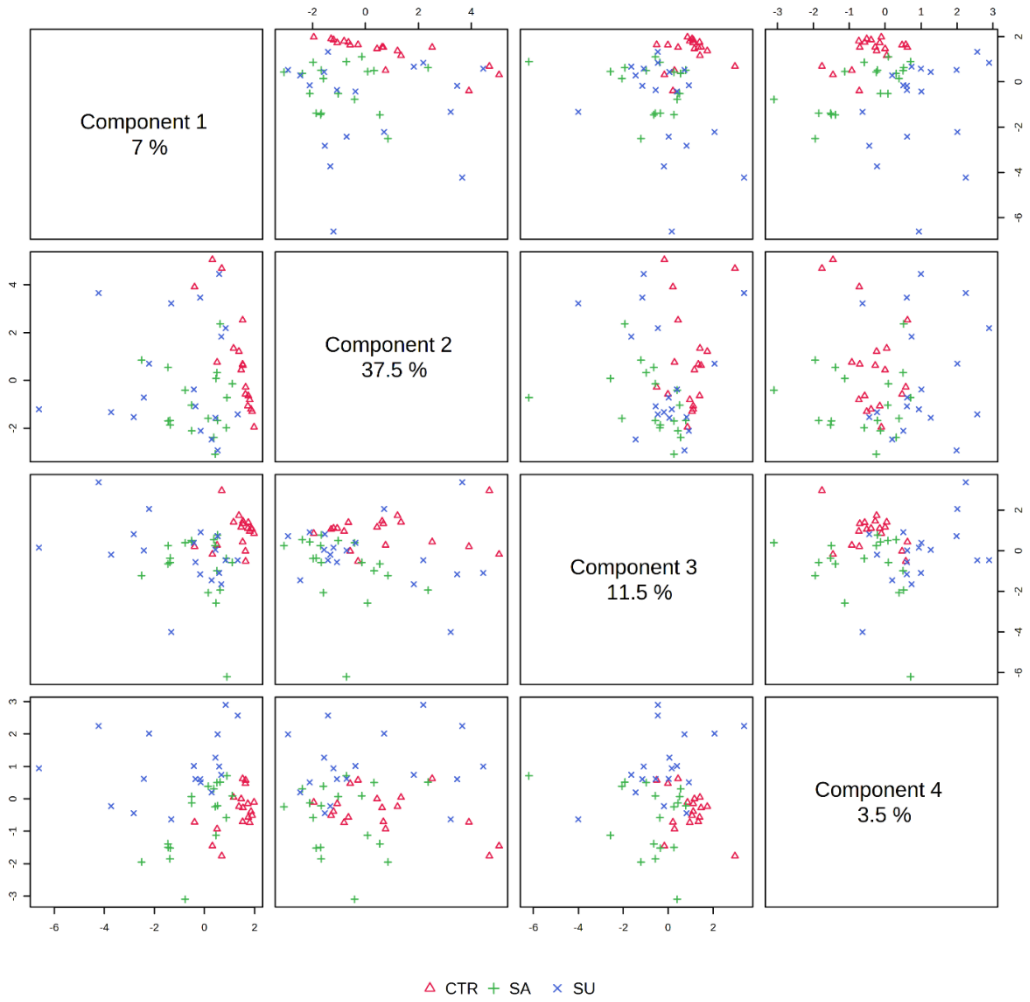

Figure 2. Pairwise score plots between the selected PCs. The variance of each PC is shown in the corresponding diagonal cell. CTR = control group; SA = salty FFP dietary group; SU = sugary FFP dietary group.

Metabolic features related to the length of exposure to the diets

A total of 36 metabolites were found to be affected by the piglets' feeding time, irrespective of the dietary treatment (Supplementary Table S2). Differences between the three different time points were also identified via the sPLS-DA analysis, in which the T0 cluster was separated from the T1 and T2 clusters (Figure 3).

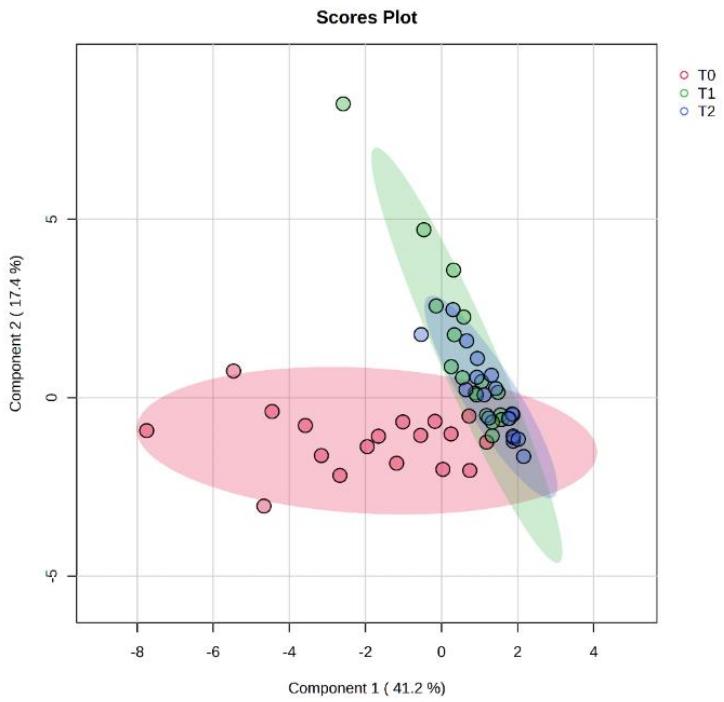

Figure 3. Multivariate supervised partial least square-discriminant analysis (PLS-DA) comparing the metabolomes of piglets at day 0 (T0), day 21 (T1), and day 42 (T2). The explained variances are shown in brackets.

Interaction between diet and age of the piglets

Considering the interaction between the time points and the dietary treatments, six metabolites were found to be significantly different between the three groups (Table 4).

Table 4. Significantly differently expressed metabolites considering the interaction time × dietary treatment (T×D) with $P < 0.05$. Data are presented as the mean. The means refer to arbitrary units, as determined from the metabolites' peak area.

Metabolite	CTR[1]			SA[2]			SU[3]			SEM[4]	*P* value		
	T0	T1	T2	T0	T1	T2	T0	T1	T2		T	D	T×D
Caffeine	4692	5067	2563	5824	173805	92630	6072	201399	341337	18169	<0.001	0.003	<0.001
Theobromine	59419	31125	15626	34919	2161476	1564156	40483	2506605	3133733	203210	<0.001	0.007	0.002
Proline_betaine	19041	15267	7493	17188	28119	13960	17258	13961	18982	1178	0.018	0.407	0.016
PC 32:0[5]	5556	8708	4250	5818	7171	2795	11093	4306	4779	468	0.001	0.667	0.031
Spermidine	16999	24668	11889	12484	16423	5915	16990	12061	12130	1125	0.001	0.568	0.056
L-Tryptophan	3375269	2198373	1111199	2038625	2150923	2006449	2981855	1741205	2937886	175637	0.007	0.789	0.042

[1] CTR = control diet.

[2] SA = 30% replacement of the control diet with salty FFPs.

[3] SU = 30% replacement of the control diet with sugary FFPs.

[4] SEM = standard error of the mean.

[5] PC 32:0 = dipalmitoyl-phosphatidylcholine.

Details regarding the Kruskal-Wallis post-hoc test comparison of the three dietary treatments at each time point are shown in Figure 4. At T0, the theobromine and caffeine concentrations were similar between the three groups; however, the concentrations were higher ($P < 0.05$) in both the SA and SU groups when compared with the CTR group at T1 and T2. The highest values were found at T2 for the SU group (Figure 4A and 5B). In both the CTR and SU groups, the concentration of proline-betaine was higher ($P < 0.05$) at T0 than at T2 or T1, although in the SA group, the highest value was found at T1 (Figure 4C). The concentration of the metabolite PC 32:0 was similar ($P > 0.05$) at day 42, despite the different ($P < 0.05$) starting values, with the SU group showing the highest concentration, followed by the SA and CTR groups (Figure 4D). The trend of spermidine was similar between the three groups, with the values decreasing ($P < 0.05$) at T2 when compared with those at T0 and T1 (Figure 4E). By contrast, the metabolism of L-tryptophan differed between the groups. More specifically, in the CTR group, the plasma concentration decreased ($P < 0.05$) over time, whereas in the SA group, it remained similar ($P > 0.05$) throughout the experiment. Conversely, in the SU group, the L-tryptophan concentration was higher ($P < 0.05$) at T0 when compared with T1 but similar ($P > 0.05$) to T2 (Figure 4F).

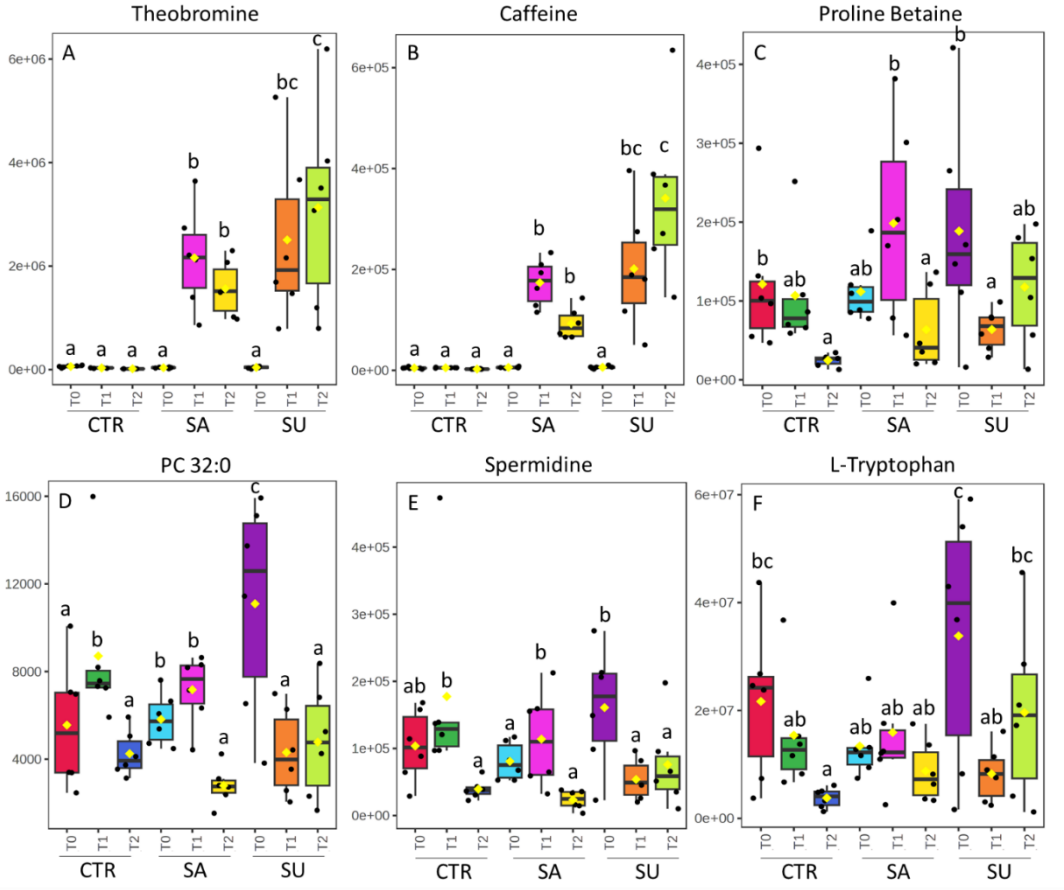

Figure 4. Concentrations of the compounds selected via ANOVA in the control (CTR), salty (SA), and sugary (SU) groups at time points 0 (T0), 1 (T1), and 2 (T2). The values refer to arbitrary units, as determined from the metabolites' peak area.

Correlation between the metabolome and growth performance traits

As expected, both theobromine and caffeine were found to positively correlate with the total FI (TFI), although no other metabolites were found to be positively correlated with the TFI (Figure 5A) or other growth performance traits, such as the ADG (Figure 5B) and BW (Figure 5C), of the piglets. By contrast, seven metabolites were found to positively correlate with the FCR—namely, betaine, L-methionine, N-acetyl tryptophan, apigenin-7-O-glucuronide, L-valine, spermine, and L-tryptophan (Figure 5D).

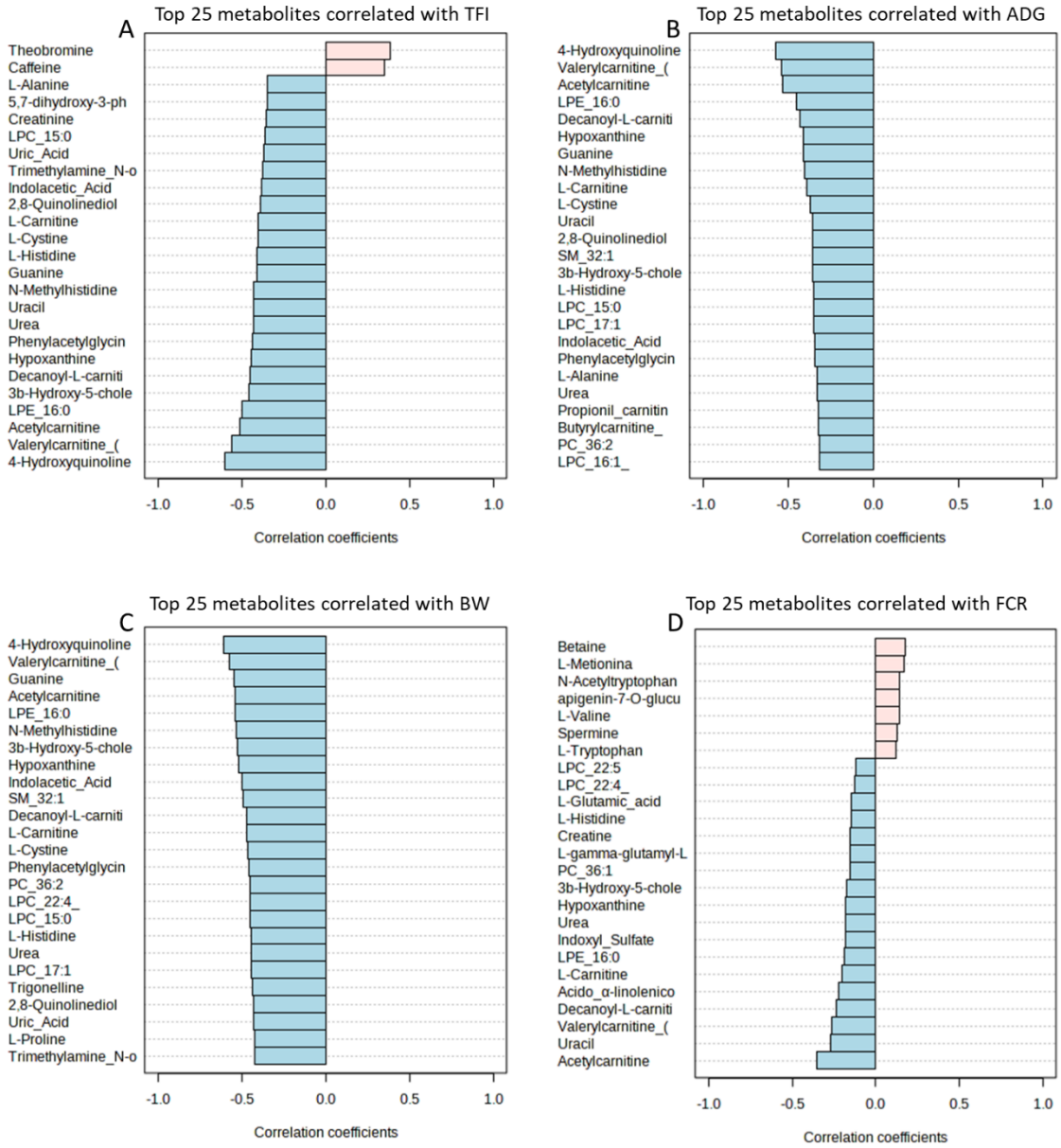

Figure 5. Top 25 metabolites correlated with the A) total feed intake (TFI), B) average daily gain (ADG), C) body weight (BW), and D) feed conversion ratio (FCR) of the piglets.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis performed exclusively at T2 revealed that when compared with the CTR group, the caffeine and tryptophan metabolism, together with the biosynthesis of the unsaturated fatty acid pathway, were affected by both the SA and SU diets. However, considering the Holm-adjusted *P* value reported in the column titled "*P* value," only the caffeine and glycerophospholipid metabolism in the SA group and the caffeine metabolism in the SU group were affected when compared with the CTR (Table 5).

Table 5. Statistical relevance list of the top pathways obtained via the KEGG metabolic pathway analysis based on the plasma metabolites of pigs fed sugary or salty FFPs compared with the standard diet.

	Pathway name	Hits	Total	%	*P* value	FDR[1]	Impact
CTR vs SA[2]							
	Caffeine metabolism	2	10	20	<0.001	<0.001	0.000
	Glycerophospholipid metabolism	2	36	6	<0.001	0.001	0.120
	Alpha-linolenic acid metabolism	2	13	15	0.002	0.028	0.333
	Beta-alanine metabolism	4	21	19	0.024	0.146	0.056
	Arachidonic acid metabolism	1	36	3	0.027	0.146	0.000
	Linoleic acid metabolism	1	5	20	0.027	0.146	0.000
	Tryptophan metabolism	2	41	5	0.046	0.195	0.143
	Biosynthesis of UFA[3]	1	36	3	0.048	0.195	0.000
CTR vs SU[4]							
	Caffeine metabolism	2	10	20	0.001	0.042	0.000
	Tryptophan metabolism	2	41	5	0.013	0.202	0.143
	Biosynthesis of UFA	1	36	3	0.018	0.201	0.000

[1] FDR = false discovery rate.
[2] CTR vs. SA = control versus salty feeding group.
[3] UFA = unsaturated fatty acids.
[4] CTR vs. SU = control versus sugary feeding group.

Discussion

This work extends previous studies in which the nutritional characterization of FFP and the effect of the dietary inclusion of FFP on both growth performance (Luciano et al., 2021; Mazzoleni et al., 2023b) and gut microbiota (Tretola et al., 2022) of post-weaning piglets and growing-to-finishing pigs were addressed. In this work, none of the indexes of growth performance (Table 2) was significantly altered in response to the SA and SU FFP diets, suggesting that their formulation met the nutritional requirements of the piglets. These results were in line with those reported by Luciano et al. (2021) and

Mazzoleni et al. (2023b). Both SA and SU FFP were nutritionally valid and safe, with no negative effect on the growth and well-being of the piglets. However, the results revealed some differences in the fatty acid profiles of the abdominal adipose tissues (Table 3) from the three different dietary groups (Table 1), which could be ascribed to the different fatty acid content of the experimental diets. The fatty acid profiles of the diets are representative of their composition, although that was not statistically tested. Still, similar trends in terms of the single fatty acids and pooled groups can be observed between the diets and the adipose tissues. The fatty acid metabolism and fat deposition in piglets attenuated the differences in the fatty acid profile from the experimental diets to the adipose tissues. The SFA in the adipose tissue showed a tendency toward a lower level in the SA group and a higher level in the SU group compared to CTR, which was comparable to the SFA level in the three diets. The MUFA level in the adipose tissue was higher in both SA and SU groups compared to CTR. However, whether in the SA and SU diets there was an average increase of +57% compared to CTR, the average increase in the adipose tissue was +17%. The same trend was observed for oleic acid, which increased +60% in the SA and SU diets compared to CTR diet but only +18% in the adipose tissues of SA and SU groups. These results indicated that piglets were able to mitigate and rebalance the large differences in MUFA level from the diets to the adipose tissue. It has been reported that the fat deposition of each specific fatty acid decreases as its inclusion in the diet increases (Duran-Montgé et al., 2008), thus explaining what observed for oleic acid. However, this mechanism was not verified in the present study. For PUFA, the trend was similar, as the lower content (-27%) in the SA diet compared to CTR diet was reduced to -9% in the respective adipose tissue, whereas the decreased PUFA level of -44% in the SU diet compared to CTR diet was reduced to -33% in the adipose tissue.

Further confirmation came from the values of the major n-6 and n-3 fatty acids in the diets and in the adipose tissues (Duran-Montgé et al., 2008; Wood et al., 2008)—namely, linoleic and linolenic acid—because the lowering trend from the CTR diet to the SA and SU diets was similar, although the differences among the groups were reduced to the extent that there was no significant difference between the CTR and SA diets. Both SA and SU diets were formulated using processed and ultra-processed food, which are known to be more energy-dense and include more fat, sugar, and sodium than raw food, which might impact their metabolic fate (Rauber et al., 2015). The PUFA content of animals' adipose tissue is strictly related to the PUFA content of the feed source (Riley et al., 2000; Flis et al., 2007). The PUFA level was already reduced in the FFP diets, especially in the SU diet, because of the lower incidence of PUFA-rich selected ingredients (e.g., soybean oil). This might have induced a carryover effect in the abdominal fatty acid profile of the SU-fed piglets. For the n-6/n-3 ratio, the increasing trend from the CTR to the SA and SU groups was similar between the diets and the adipose tissue. Swine usually have a high n-6/n-3 ratio due to the diets applied in pig production, usually based on corn, sunflower, and soybean vegetable oils, which are all rich in n-6 PUFA. However, a low n-6/n-

3 ratio in the range of 5:1–3:1 in pork meat has been suggested to ensure better meat quality and provide greater health benefits to animals and humans (Fanalli et al., 2022). Here, the increased n-6/n-3 ratio possibly induced by FFP deserves further investigation to understand the exact mechanisms underlying the observed effects, especially considering the age of the animal and the length of the dietary treatment. To summarize, SA group showed higher MUFA level and n-6/n-3 ratio than CTR, whereas SU group showed higher MUFA level and n-6/n-3 ratio but lower PUFA level than CTR (Table 3).

The inclusion of FFP was further evaluated moving from a "macro" to a "micro" perspective. Here, metabolomics was used to assess the impact of FFP on plasma metabolome and to observe which metabolites and metabolic pathways were differentially modulated by FFP. This is the first study in which an untargeted metabolomics approach was applied to study the potential perturbation of plasma metabolome in swine in response to the dietary inclusion of feed and/or food by-products. So far, few studies applied metabolomics in piglets. In previous studies, the main differentially expressed metabolites between control and experimental diets were fatty acids, triglycerides, phosphatidylcholine, amino acids, betaine, choline, cholesterol, bile acids (and conjugates), and glucose (Hanhineva et al., 2013; Nielsen et al., 2014; Sun et al., 2014; Dannenberger et al., 2016). Here, the 30% inclusion of SA and SU FFP resulted in a limited perturbation of plasma metabolome. Six metabolites were differentially modulated in response to SA and SU FFP diets and time points: caffeine, theobromine, proline-betaine, dipalmitoyl-phosphatidylcholine, spermidine, and L-tryptophan (Table 4). The effect of time (Supplementary Table S2 and Fig. 3) was stronger than the effect of the dietary treatments (Fig. 1). Indeed, 36 metabolites were differentially expressed when considering the 3 time points, whereas only 2 metabolites were differentially expressed across the 3 dietary treatments.

Caffeine and theobromine were the only two differentially expressed metabolites, even when considering the dietary groups separately (Fig. 1). This finding is confirmed by the P values of T and D of caffeine and theobromine shown in Table 4. Overall, these two metabolites appear to be the most differentially expressed between CTR and both SA and SU groups. The fact that the levels of both metabolites were higher at T1 and T2 for the SA and SU groups (Fig. 4) may be directly related to the experimental feeding trial, during which the content of caffeine and theobromine accumulated in piglets, particularly in their plasma. Caffeine and theobromine are two metabolites found in cocoa beans, coffee beans, and chocolate-based products (Zoumas et al., 1980). It is possible that the FFP diet included chocolate-based products that might explain the higher level of caffeine and theobromine at T1 and T2 in the FFP groups compared to CTR. In human liver, caffeine is metabolized by cytochrome CYP1A2 to theobromine, paraxanthine, and theophylline (Harpaz et al., 2017). Caffeine was associated with thermogenesis and lipid oxidation, indicating the effect of caffeine on the regulation of total body fat (Harpaz et al., 2017). In our study, caffeine and theobromine were

positively correlated with TFI (Fig. 5), likely due to their positive effect on the regulation of both lipid metabolism and BW. However, the "metabolic impact" of caffeine and theobromine was limited, considering the KEGG metabolic pathway analysis (Table 5). Caffeine metabolism and glycerophospholipid metabolism were the only two significant metabolic pathways. However, caffeine metabolism had a null impact (0.000), suggesting that despite its significance, caffeine did not particularly affect other metabolites or metabolic pathways.

In addition, a number of other metabolites differed between CTR and FFP groups (Table 4). Proline-betaine, also known as stachydrine, is a secondary metabolite of proline usually found in all living organisms and certain crops, such as soy and triticale (HMDB, 2006). The highest proline-betaine value was observed at T0 for the CTR and SU groups (but not for the SA group), probably because it was contained in some of the feed ingredients administered to piglets, for example cereals present in both CTR and FFP diets at different levels of inclusion. PC 32:0, also known as dipalmitoyl-phosphatidylcholine, is a glycerophospholipid and the main component of lung surfactant. PC 32:0 was found to be involved in glycerophospholipid metabolism, one of the two significant pathways identified by the KEGG pathway analysis (Table 5). In general, phospholipids and lipids are prone to differential expression in pigs following experimental dietary treatments. It was observed that the expression of phospholipids differed in pigs following the administration of n-3 and n-6 fatty acids (Dannenberger et al., 2016) and a high-fat diet (Hanhineva et al., 2013). In this study, both SA and SU diets affected lipid deposition in abdominal adipose tissue in terms of MUFA, PUFA, and n-6/n-3 ratio, although the exact mechanism was not defined. Dietary intervention with different levels and sources of lipids and energy can modulate the fatty acid composition of the pork carcass. Lipid digestibility is affected by the molecular structure of fatty acids, degree of saturation, length of the carbon chain, and position of the single fatty acids in the triglyceride molecule. For example, dietary unsaturated lipids are more digestible than saturated lipids in pigs due to the greater accessibility of lipase to triglycerides (Fanalli et al., 2022). Thus, the availability of these lipids in the experimental diets might have influenced the trafficking of lipids from the feed to the adipose tissue, and consequently, its composition.

Spermidine is a polyamine with a high content in soybean (Sagara et al., 2020) and a presence in sow's milk. Fig. 4 highlights the higher level of spermidine at T0 for all three dietary groups, which suggests that spermidine was probably present in the diets. Spermidine is known to positively modulate cell proliferation, migration, and inflammatory response in porcine intestinal epithelial cells (IPEC-J2 cell line) (Wei et al., 2022). Additionally, oral supplementation with spermine and spermidine in pre-weaning piglets was found to promote intestinal physiology and development in terms of an increased intestinal absorptive area (van Wettere et al., 2016).

Tryptophan is an aromatic amino acid and an essential nutrient for pigs (NRC, 1998; Sève, 1999). Like lipids, amino acids are subject to variations regarding the response to dietary treatments. A higher level of amino acids was observed in pigs fed with a high-fat and high-fiber diet (Jégou et al., 2015) and in piglets fed with a standard diet supplemented with insect meal (Meyer et al., 2020). In this study, tryptophan was found to play a physiological role in feed intake and growth performance, as evidenced by the positive correlation between tryptophan and FCR (Fig. 5). However, the possible interaction between energy sources and processed food such as ex-foods, bakery meals, and FFP merits further investigation, as suggested by Stein et al. (2023). In their study, the authors observed a variation in the standard ileal digestibility of amino acids after testing the intake of 11 different sources of bakery meals as feed ingredients for pigs. This result was correlated with differences in the raw food sources used in the bakery meal blends, despite the apparent efficiency of the bakery meal processors in providing final products with similar chemical compositions. In addition, the different procedures applied during the processing of raw foods or bakery meals might have affected the ileal digestibility of the amino acids (Stein et al., 2023).

Overall, no substantial differences were observed between SA and SU diets. Therefore, the effect of FFP might be related to their nutritional composition rather than to their specific dietary components, as these diets were designed to be both iso-energetic and iso-nitrogenous.

Conclusions

The inclusion of SA and SU FFP at a level of 30% in the diet of post-weaning piglets had no effect on growth performance of piglets. The fatty acid profile of abdominal adipose tissue indicated that piglets were able to rebalance the fatty acid profile of the experimental diets and attenuate the differences between the three dietary groups. The impact of both SA and SU FFP diets on the plasma metabolome and related metabolic pathways was limited to a restricted group of metabolites, with caffeine and theobromine being the two most differentially expressed metabolites. Despite the well-known effects of caffeine and theobromine on lipid metabolism, their metabolic impact was not defined. Therefore, the dietary inclusion of SA and SU FFP did not cause any detrimental alteration of lipid metabolism and plasma metabolome in post-weaning piglets, thus increasing the reliability of FFP as alternative and sustainable feed ingredients. Further work could involve adult pigs and longer feeding trials with FFP in order to increase the knowledge about the potential perturbation of the metabolism and physiology of pigs fed FFP and to further validate the safe use of FFP as sustainable feed ingredients.

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Supplementary Tables

Supplementary Table S1
Protein concentration (mg/mL) of plasma samples collected at day 0 (T0), day 21 (T1) and day 42 (T2).

	Protein concentration		
Sample ID	**T0**	**T1**	**T2**
CTR[1] 1	0.09	0.16	0.24
CTR 2	0.16	0.16	0.37
CTR 3	0.11	0.17	0.33
CTR 4	0.15	0.10	0.27
CTR 5	0.24	0.18	0.34
CTR 6	0.28	0.18	0.32
SA[2] 1	0.20	0.20	0.37
SA 2	0.13	0.14	0.29
SA 3	0.15	0.14	0.38
SA 4	0.13	0.10	0.27
SA 5	0.20	0.25	0.19
SA 6	0.15	0.15	0.16
SU[3] 1	0.07	0.16	0.13
SU 2	0.08	0.24	0.49
SU 3	0.15	0.22	0.13
SU 4	0.10	0.23	0.17
SU 5	0.32	0.38	0.27
SU 6	0.09	0.21	0.16

[1] CTR = control diet.
[2] SA = salty FFP experimental diet.
[3] SU = sugary FFP experimental diet.

Supplementary Table S2
Compound selected by ANOVA with *P*-value threshold 0.05 depending on the time point.

Compounds	F value	P value	FDR[1]	Fisher's LSD[2]	
4-Hydroxyquinoline	14.27	<0.0001	0.001	T0 vs T1	T0 vs T2
LPC[3] 15:0	14.17	<0.0001	0.001	T0 vs T1	T0 vs T2
LPC 17:1	12.94	<0.0001	0.001	T0 vs T1	T0 vs T2
LPE[4] 18:1.1	10.82	0.0001	0.002	T0 vs T1	T0 vs T2
Guanine	10.45	0.0002	0.003	T0 vs T1	T0 vs T2
Valeryl carnitine (C5)	9.946	0.0002	0.003	T0 vs T1	T0 vs T2
Indol-acetic acid	9.668	0.0003	0.003	T0 vs T1	T0 vs T2
Trimethylamine N-oxide	9.183	0.0004	0.004	T0 vs T1	T0 vs T2
3b-Hydroxy-5-cholenoic acid	8.776	0.0005	0.005	T0 vs T1	T0 vs T2
Propionyl-L-carnitine	8.638	0.0006	0.005	T0 vs T1	T0 vs T2
L-Proline	8.341	0.0007	0.006	T0 vs T1	T0 vs T2
N-Methyl-histidine	7.445	0.0014	0.011	T0 vs T1	T0 vs T2
4-Hydroxyhippuric acid	7.414	0.0015	0.011	T0 vs T1	T0 vs T2
Uric Acid	6.961	0.0021	0.015	T0 vs T1	T0 vs T2
3,5-Dimethoxycinnamic acid	6.597	0.0028	0.017	T0 vs T1	T0 vs T2
Trigonelline	6.583	0.0028	0.017	T0 vs T1	T0 vs T2
Histrelin acetate	6.567	0.0029	0.017	T0 vs T1	T0 vs T2
Urea	6.357	0.0034	0.018	T0 vs T1	T0 vs T2
Hypoxanthine	6.315	0.0035	0.018	T0 vs T2	
N6-Acetyl-L-lysine	6.281	0.0036	0.018	T0 vs T1	T0 vs T2
Acetyl-L-carnitine	6.067	0.0043	0.020	T0 vs T2	T1 vs T2
Indoleacetic acid	5.917	0.0049	0.022	T0 vs T1	T0 vs T2
LPE 16:0	5.826	0.0052	0.023	T0 vs T1	T0 vs T2
L-Cystine	5.559	0.0065	0.027	T0 vs T1	T0 vs T2
Uracil	5.336	0.0078	0.029	T0 vs T2	
L-Histidine	5.336	0.0078	0.029	T0 vs T1	T0 vs T2
LPC 22:4	5.310	0.0081	0.029	T0 vs T1	T0 vs T2
Creatinine	5.279	0.0082	0.029	T0 vs T1	T0 vs T2
Phenylacetyl glycine	5.265	0.0083	0.029	T0 vs T1	T0 vs T2
Theobromine	5.153	0.0092	0.031	T1 vs T0	T2 vs T0
Salicylic acid	4.952	0.0108	0.034	T2 vs T0	
Decanoyl-L-carnitine	4.949	0.0108	0.034	T0 vs T2	
Caffeine	4.859	0.0117	0.036	T1 vs T0	T2 vs T0
L-Carnitine	4.732	0.0130	0.038	T0 vs T1	T0 vs T2
L-gamma-glutamyl-L-leucine	4.631	0.0142	0.039	T0 vs T1	T0 vs T2
PC 36:2	4.356	0.0179	0.049	T0 vs T1	T0 vs T2

Data are presented as F value and P-value. Significant pairwise comparisons are reported by Fisher's LSD.

[1] FDR = false discovery rate

[2] LSD = least significant difference.

[3] LPC = Lyso-phosphatidylcholine

[4] LPE = Lyso-phosphatidylethanolamine

Section A – Article 2

Impact of the dietary inclusion of salty and sugary former food products on the liver and plasma profile of pigs through OMICS approaches

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Abstract

Replacing cereals with food leftovers could reduce feed-food competition and keep nutrients and energy in the food chain. Former food products (FFPs) are industrial food leftovers no more intended for human but still suitable as alternative and sustainable feedstuffs for monogastric. In this study, omics approaches were applied to evaluate the impact of dietary FFPs on pig liver proteome and plasma peptidome. Thirty-six Swiss Large White male castrated pigs were randomly assigned to three dietary treatments [control (CTR), 30% CTR replaced with salty FFP (SA), 30% CTR replaced with sugary FFP (SU)] from the start of the growing phase (22.4 ± 1.7 kg) until slaughtering (110 ± 3 kg). The low number of differentially regulated proteins in each comparison matrix (SA/SU vs. CTR) and the lack of metabolic interaction indicated a marginal impact on hepatic lipid metabolism. The plasma peptidomics investigation showed low variability between the peptidome of the three dietary groups and identified three possible bioactive peptides in the SA group associated with anti-hypertension and vascular homeostasis regulation. To conclude, the limited modulation of liver proteome and plasma peptidome by the SA and SU diets strenghtened the idea of reusing FFPs as feed ingredients to make pig production more sustainable.

Introduction

The higher the demand for animal source food, the higher the use of cereal crops for pig nutrition, exacerbating feed-food competition (Pinotti et al., 2021). The research for alternative and sustainable feedstuffs could decrease the environmental impact of pig production (Pinotti et al., 2023a; Govoni et al., 2022). Food leftovers have a lower environmental impact than the original product but are still

nutritionally valid (Andretta et al., 2021). Former food products (FFPs) are food leftovers dropping out from the production of ready-to-eat products such as snacks, chips, biscuits, and bread, originally intended for human consumption but no more suitable for that due to technical or logistical defects (Pinotti et al., 2021). Also, since FFPs originate from processed and ultra-processed food, they contain compounds from specific food ingredients such as cocoa, coffee, and chocolate (EFSA, 2008).

The FFPs were used to partially replace conventional cereals for post-weaning piglets, growing, and finishing pigs because of the high energy, simple sugars and highly-digestible starch content of FFPs (Pinotti et al., 2023b). This is a sustainable way to recycle food leftovers and keep their nutritional value in the feed-food chain. The FFPs were used as blend (Tretola et al., 2019; Luciano et al., 2022a) or divided into salty FFPs (SA) and sugary FFPs (SU) (Luciano et al., 2021). The SA and SU FFPs were formulated to be isoenergetic but evaluated separately to discriminate the effect of their ingredients on the animal. Replacing conventional cereals up to 30% with FFPs as blend did not impair the growth performance of post-weaning piglets (Tretola et al., 2019; Luciano et al., 2022a). Further research was conducted by using SA and SU FFPs diets, and neither dietary treatments used to replace conventional cereals up to 30% altered the growth performance and the feeding behaviour of post-weaning piglets (Luciano et al., 2021). Interestingly, a higher level of coffee- and cocoa-related metabolites, such as caffeine and theobromine, was observed in the plasma of post-weaning piglets fed FFPs diets (Pinotti et al., 2023c), as similarly observed in human (Su et al., 2023).

This study follows previous work of Mazzoleni et al. (2023), in which SA and SU FFPs diets did not impair the growth performance, feeding behaviour and carcass composition of adult pigs. Furthermore, no significant alteration was observed in the level of a panel of serum metabolites related to liver disease and hepatic fatty acids accumulation (Wang et al., 2017), such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, total bilirubin and cholesterol, albumin, and globulins (Mazzoleni et al., 2023). Despite no relevant alteration was observed, our interest was to further evaluate the potential impact of the FFPs diets on the metabolism of pigs. First, the liver was targeted because it is the major metabolic centre in the organism involved in body energy regulation and metabolism of nutrients and exogenous compounds. The effect of the diet is reflected on the hepatic pathways involving amino acids (Eugenio et al., 2023) and fatty acids (Szostak et al., 2016; Hodson et al., 2020). Then, plasma was targeted to observe the impact of experimental diets on the physiology of the organism (Bovo et al., 2016). Therefore, we decided to apply shotgun label-free quantitative proteomics and peptidomics to investigate how the dietary inclusion of SA and SU FFPs could affect the pig physiology in terms of liver proteome and plasma peptidome profiles. So far, proteomics was rarely applied to evaluate alternative feedstuffs for pigs. Instead, peptidomics is a tool often used for identifying diet-related biomarkers or for detecting bioactive peptides (Dallas et al., 2015; Dupont, 2017). The coupling of these two omics approaches is considered an opportunity to enlarge the

amount of data available and to obtain a broader picture of the metabolic and physiological state of the animal (Cunningham et al., 2012; Di Meo et al., 2016).

The digestion rate of starch is known to affect the regulation of blood glucose and insulin levels and the lipid metabolism in liver (Jun et al., 2010; Yin et al., 2021). Then, the amount of dietary fat content is related to altered liver functions, such as liver lipid accumulation. In growing pigs fed a high-fat diet (301 vs. 44 g/kg dry matter (DM) of standard diet), there was an increased level of serum lipids but no evidence of liver lipid accumulation, while the proteins involved in fatty acid oxidation were upregulated (Sejersen et al., 2013). In addition, the fatty acid profile affects liver lipid metabolism. Pigs at 60 kg body weight (BW) were fed until slaughtering (100 kg BW) with diets containing seven dietary oil and fat sources. The authors observed that several lipogenic genes in liver were downregulated by fish oil inclusion (high poly-unsaturated fatty acids (PUFA) content) but upregulated by high-oleic sunflower oil (high mono-unsaturated fatty acids (MUFA) content), suggesting the central role of fatty acid saturation in the regulation of liver lipogenesis in pigs (Duran-Montgé et al., 2009). Also, caffeine and theobromine are biotransformed in liver, and a stimulating effect on hepatic lipid oxidation together with a reduced expression of lipogenic genes (lowering the risk of non-alcoholic fatty liver disease) was observed in mice after caffeine (Sinha et al., 2014) and theobromine (Wei et al., 2021) supplementation. However, theobromine was considered an undesirable compound in feed (EFSA, 2008), therefore its biotransformation along with its effect on hepatic lipid metabolism must be further addressed.

Concomitantly, the plasma peptidome of the same animals was evaluated to disclose if SA and SU diets could modulate the peptidomic profile with a particular focus on possible bioactive peptides. It is well known that various raw food materials contain bioactive peptides that exert a specific physiological function in tissues and fluids of living organisms (Lafarga and Hayes, 2014; Nongonierma and Fitzgerald, 2015; Cerrato et al., 2021; Caira et al., 2022). In parallel, gastrointestinal digestion of dietary proteins generates a huge number of peptides that become bioactive once released by proteolysis in the gut (Chakrabarti et al., 2018; Mora et al., 2018). Peptidomics approaches have been introduced recently in research concerning animal feeding. However, while dietary exogenous proteins and peptides released in the gut during digestion have been investigated extensively, the possibility that non-dietary proteins that are also present in the digestive tract may give rise to endogenous bioactive peptides has only recently received attention (Dave et al., 2016). In a study conducted on pigs fed a casein-based diet, up to 80% of endogenous proteins was digested and reabsorbed by the end of the small intestine into the bloodstream (Souffrant et al., 1993). Moreover, Dave et al. (2014) demonstrated that large numbers of bioactive peptides exist within the amino acid sequences of endogenous proteins, and they may be cleavable by digestive enzymes. Therefore, it is likely that these proteins, as well as dietary proteins, can be a source of latent bioactive peptides (from 2 to greater

than 40 amino acids long) that, when released during digestion in the gastrointestinal tract, can act as modulators of various physiological functions (Meisel, 1997; Rutherfurd-Markwick and Moughan, 2005; Shahidi and Zhong, 2008). Despite the biological activity of these endogenous peptides is still little studied, there is evidence that some of these peptides possess a range of effects including antihypertensive, cholesterol-lowering, antioxidant, anticancer, immunomodulatory, antimicrobial, opioid, antiobesity, and mineral binding effects (Shahidi and Zhong, 2008).

In this study, label-free mass spectrometry-based omics approaches were applied to the liver proteome and plasma peptidome of pigs fed diets with 30% dietary inclusion of SA and SU FFPs to replace conventional cereals. The aim was to observe how FFPs modulate the liver proteome and related metabolic pathways and to identify all endogenous plasma peptides, with particular reference to the corresponding bioactive ones.

Material and methods

Reagents

Purified and sterilized bi-distilled water with the Milli-Q™ system (Millipore, Bedford, MA, USA) was used to prepare the buffers or for other applications that required it. The following reagents were used for tissue homogenisation: SDS (sodium dodecyl sulphate), supplied by Bio-Rad (Hercules, CA, USA), NaCl (sodium chloride) and $MgCl_2$ (magnesium chloride), from Fluka Chemical (Ronkonkoma, NY, USA), TEAB (triethylammonium bromide) and Benzonase® Nuclease, purchased from Sigma-Aldrich (Milan, Italy) and, finally, cOmplete™ protease inhibitor cocktail, purchased from Roche Diagnostics GmbH (Mannheim, Germany). The protein concentration was performed using the BCA kit (Sigma-Aldrich, Milan, Italy).

For the proteolytic digestion of protein extracts, solutions containing TCEP (tris(2-carboxyethyl) phosphine), IAA (iodoacetamide) and AMBIC (ammonium bicarbonate) supplied by the company Sigma-Aldrich (Milan, Italy) were prepared; other reagents used during proteolytic digestion were: H_3PO_4 (phosphoric acid, Fluka Chemical, Ronkonkoma, NY, USA), MeOH (methanol, Sigma-Aldrich, Milan, Italy) and TEAB (see above), while trypsin (sequencing-grade trypsin, Roche, Mannheim, Germany) was chosen as the proteolytic enzyme. S-TRAP spin-columns, purchased from Protifi (Huntington, NY, USA), were used for maximising efficiency of protein digestion. The ultrapure formic acid (FA) and acetonitrile (ACN) used in the LC-MS analysis were supplied by Sigma-Aldrich™ (Milan, Italy), as well as Amicon Ultra-0.5 mL centrifugal filters (MWCO 10K) and trifluoroacetic acid (TFA) used for the high molecular weight protein depletion in peptidomics. Plasma samples cleanup, recovery, and concentration were performed using Zip-Tip C18 Millipore (Billerica, MA, USA).

Experimental design

The in vivo trial complied with the ARRIVE guidelines and was authorized by the Swiss Federal Committee for Animal Care and Use (authorization number: 2021-35-FR). The in vivo trial was performed at the Agroscope research unit located in Posieux, Switzerland. All methods were performed in accordance with the relevant guidelines and regulations. Thirty-six Swiss Large White male castrated pigs (22.4 ± 1.7 kg initial BW) were assigned to three isoenergetic and isonitrogenous dietary treatments at the beginning of the growing period: 1) control diet (conventional cereals, CTR), 2) replacement of 30% CTR with salty FFPs (SA), and 3) replacement of 30% CTR with sugary FFPs (SU). The dietary treatments lasted until the pigs reached the required BW for slaughtering (110 ± 3 kg final BW) (Mazzoleni et al., 2023). The diets were characterized by Mazzoleni et al. (2023). Briefly, the three diets in the finishing phase had a similar content of crude protein and crude fibre. The SA diet had approximately double the sodium content compared to the SU and CTR diets, whereas the SU diet had a higher crude fat content (59.0 g/kg DM) than SA diet (53.0 g/kg DM) and CTR diet (45.0 g/kg DM). The SU diet had a higher saturated fatty acids (SFA) content (20.0 g/kg DM) than SA diet (12.0 g/kg DM) and CTR diet (16.0 g/kg DM), and the SA diet had a higher MUFA content (29.0 g/kg DM) compared to SU diet (25.0 g/kg DM) and CTR diet (14.0 g/kg DM). Instead, the content of PUFA was similar for all the diets.

Slaughter procedure and liver and plasma sampling

The pigs were slaughtered at the Agroscope research slaughterhouse after being stunned with CO_2 and then exsanguinated, scalded, dehaired, and eviscerated. Blood was sampled directly during bleeding after CO_2 stunning using blood collection tubes with tri-potassium K3EDTA (Vacuette®; Greiner Bio-One GmbH, Kremsmuenster, Austria), which were stored upside down at room temperature for 1 h prior to processing. The Vacuette® tubes were then centrifuged for 15 min at 3000 x g and subsequently for 2 min at 4000 x g to isolate plasma. Two aliquots of plasma were stored at -20 °C in Eppendorf tubes. The liver was removed from each pig carcass and a sample of liver tissue was collected from the half distal part of the right lateral lobe. All samples were individually packed in aluminium foil and immediately snap-frozen in liquid nitrogen. They were further refrigerated at -80 °C until tissue homogenization for proteomic analysis.

Label-free quantitative (LFQ) proteomics

Here below, a scheme showing the experimental workflow of the label-free quantitative proteomics analysis on liver tissue samples is reported (Fig. 1).

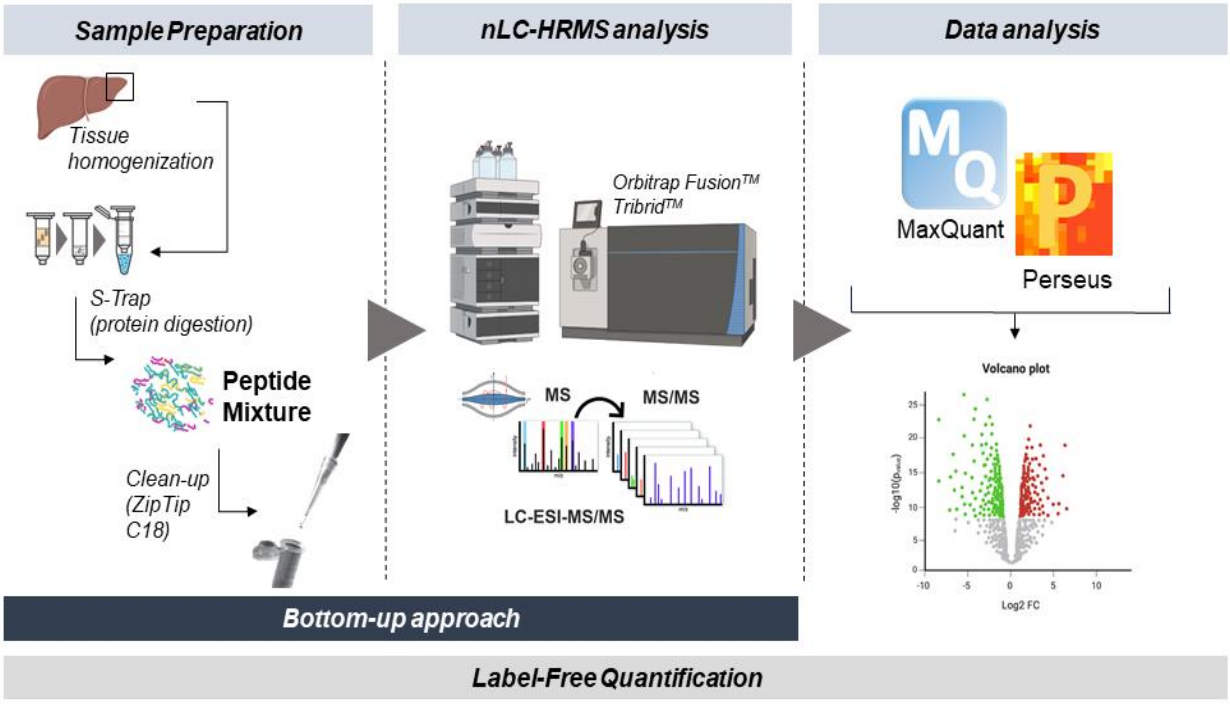


Figure 1. Overview of the experimental workflow applied for the proteomics analysis of pigs' liver tissue samples.

Sample preparation

Tissue homogenization and animal sorting

Two portions for each animal of liver tissue (~200 mg each) were randomly collected using a sterile scalpel and transferred into micro-beads (Zirconium Bead 1.5 mm) pre-filled homogenizer tubes, along with 1 mL of lysis buffer (about 20% w/v). The tissue homogenization required a harsh lysis buffer to dissolve hard-to-solubilize proteins, compatible with S-Trap devices used later for proteolysis: SDS 5%, TEAB 50 mM, NaCl 50 mM, $MgCl_2$ 5 mM, *Benzonase® Nuclease* 500 U/mL, of *cOmplete™ cocktail* (1 tablet/10 mL). A high-energy benchtop homogenizer (BeadBug™ microtube homogenizer), compatible with low working temperatures (4 °C), designed to promote rapid tissue disintegration through constant high-velocity impact of hardened microspheres was used. In detail, 4 cycles of homogenization were conducted at 4000 rpm for 2 minutes each. Samples were then centrifuged at 10,000 rpm for 30 minutes at 4 °C, and the supernatant transferred into a new Eppendorf for the protein concentration assessment carried out using the BCA kit.

Since the label-free quantitative proteomics approach is more prone to errors introduced by measurement conditions than methods involving labeling, one of the main limitations related to the application of label-free methods was the total number of samples that could be analyzed. Therefore, given the impossibility of examining all 36 animals (12 for each experimental group), 6 animals per

group were selected following a total randomization approach (Fig. 2). The codes were entered into an open-source algorithm (https://www.random.org/lists/) allowing the elements of a list to be randomly ordered (Haahr, 2010). The first six codes per group were selected for further experiments.

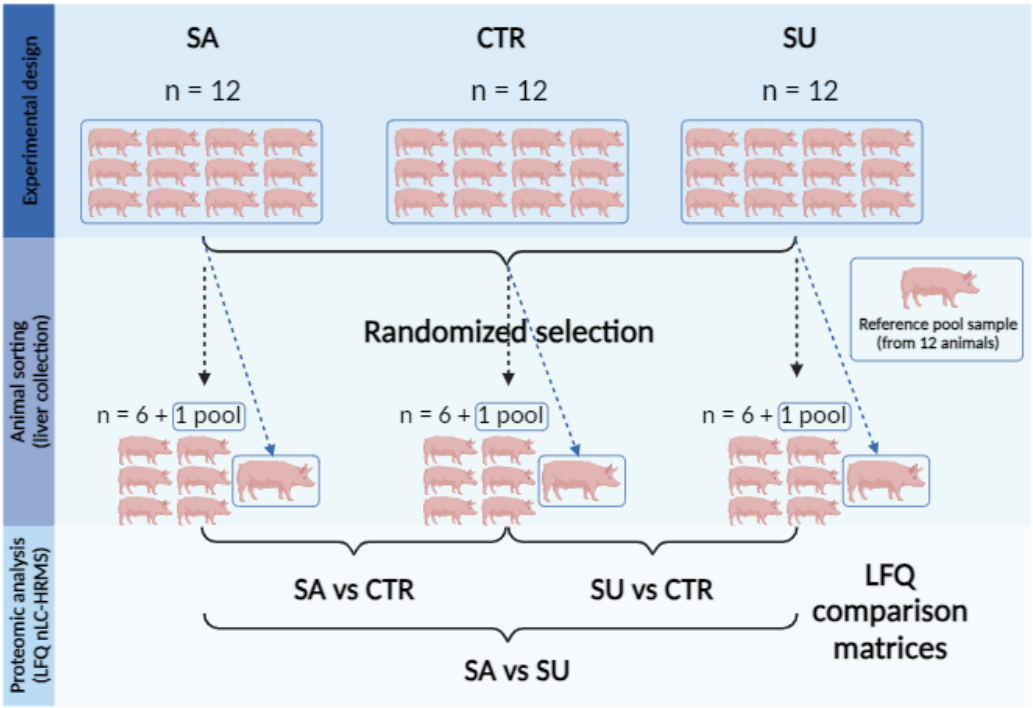


Figure 2. Scheme of the proteomics study design.

In addition to the 6 selected samples, 3 pools obtained by combining the 12 biological samples from each group, taking the same amount of protein from each (BCA assay measurement), were also prepared and analyzed separately to be used as reference samples for the animal reproducibility evaluation.

Proteolytic digestion using spin-column S-TRAP

An adequate amount of protein extract (30 µg) was processed following an *in-house* well-optimized protocol for tryptic digestion (Ferrario et al., 2022), based on the usage of S-TRAP spin-columns (Protifi, Huntington, New York, USA) to guarantee excellent yield of enzymatic hydrolysis and efficient removal of reagents or impurities from the digestion process (clean-up process). According to Ferrario et al. (2022), the collected peptic mixtures obtained were then dried under vacuum in the SpeedVac (Eppendorf s.r.l.) at a working temperature of 37 °C, and stored at -80 °C until analysis.

nLC-HRMS analysis: Orbitrap Fusion Tribrid Mass Spectrometer

The nLC-HRMS (*nano Liquiq Chromatography-High Resolution Mass Spectrometry*) method already optimized in our laboratories involves the use of an analytical platform consisting of a Dionex Ultimate 3000 nano-LC chromatograph (Sunnyvale, CA, USA), coupled to the OrbitrapFusion Tribrid high-resolution mass spectrometer (Thermo Scientific, Bremen, Germany). Each sample was injected in triplicate and analyzed as reported by Ferrario et al. (2022). Between each run, stationary phase washes were carried out by injecting 100% ACN to prevent carry-over.

Data analysis

The acquired experimental data were firstly processed by means of MaxQuant version 1.6.0 (Max Plank Institute of Biochemistry, Germany). The Andromeda search engine, implemented within the software itself, allowed the identification of proteins by searching a database, namely that of *Sus scrofa* (Taxonomy ID: 9823). The MaxLFQ algorithm (version 1.6.2.3, Max Planck Institute of Biochemistry, Martinsried, Germany) was employed for quantification, considering only so-called unique razor peptides. To ensure accurate identification and quantification of protein mixtures, certain input information regarding the sample preparation method were provided to the software, namely the protease used (trypsin) and the maximum number of missed cleavages (two) allowed. In addition, the match-between-run option was enabled, which was useful for increasing the number of peptides identified and thus the coverage of the proteome in question. A tolerance limit of 5 ppm was set for the identification of precursor ions, consistent with the performance of the high-resolution instrument used. Finally, chemical modifications of some amino acid residues due to sample processing were included, such as the carbamidomethylation of the thiol groups of cysteines as a fixed/static modification, whereas particularly frequent variable modifications were oxidation of methionine and acetylation at the N-terminus of the protein.

Perseus (version 1.6.1. 43; Max Planck Institute of Biochemistry, Martinsried, Germany) was used for facilitating the handling and interpretation of large and complex data matrices derived from MaxQuant, through statistical reworking: LFQ intensity values were first converted to a base-two logarithmic scale and then filtered for significance (Benjamini-Hochberg corrected two-sample t-test with a threshold value of 0.05 False Discovery Rate - FDR). Three comparison matrices were then built for calculating log2 Fold-Change (FC) values (or difference or ratio), indicating the different expression level of individual gene products in the different experimental conditions. As a method of visualizing the results, graphical representations known as Volcano Plots were used, with on the x-axis log2 FC values of a given protein species between two experimental conditions and on the y-axis the -log p-value identifying the significance level of the assignment. Finally, Perseus also allows the reproducibility of biological and technical replicates to be assessed by calculating the Pearson

correlation factor. The information about significantly up- and down-regulated proteins was then used to study the modulated pathways in the different experimental groups. Specifically, the String software was used for functional analysis to search for potential interactions between significantly down-regulated (log2 FC < -0.57) and significantly up-regulated (log2 FC > 0.57) proteins.

Label-free quantitative peptidomics

Experimental design and sample preparation

A shotgun label-free quantitative peptidomic approach was applied to investigate the plasma peptidome of all the thirty-six pigs involved in the study (12 for each experimental group). The workflow included peptide enrichment by ultrafiltration, protein precipitation followed by LC-ESI-MS/MS analysis, as well as identification, label-free quantitation and bioinformatic analysis to identify differential peptides in the three dietary groups. The experimental protocol used in this study is schematically summarized in Figure 3.

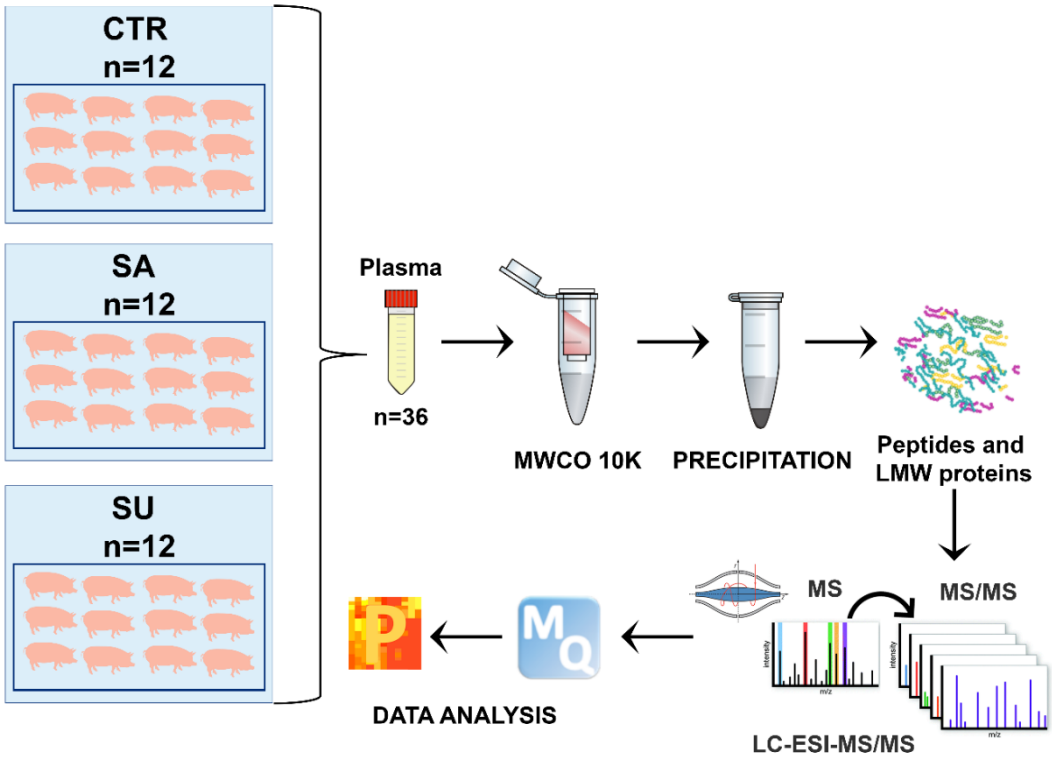


Figure 3. Shotgun label-free quantitative peptidomic analysis. Overview of the workflow applied for the analysis of peptides on plasma of pigs fed with conventional diet (CTR), salty (SA) and sugary (SU) FFP diets.

In detail, plasma was isolated from blood samples and then aliquots of 500 µL of plasma were diluted with 32% (v/v) acetic acid and ultra-filtered using Amicon Ultra-0.5 mL centrifugal filters (MWCO 10K)

for high molecular weight protein depletion (Aletti et al., 2016; Giromini et al., 2019; Maffioli et al., 2020). The flow through was then precipitated with two volumes of cold acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) and centrifuged at 13200 rpm for 30 min at 4 °C to remove residual proteins. The supernatant containing peptides and low molecular weight proteins was collected, dried, dissolved in 1% (v/v) formic acid and desalted (Coccetti et al., 2008) before mass spectrometric (MS) analysis without any previous digestion.

Nano-LC-ESI-MS/MS analysis: Mass Spectrometry-Based Shotgun Peptidomics

Nano-HPLC coupled to MS/MS analysis was performed on all the 36 plasma samples (one for each animal involved in the trial) using a Dionex UltiMate 3000 directly connected to an LTQ Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray ion source, as described for the proteomic approach.

Data analysis

The acquired raw files were subjected to data analysis using MaxQuant software as like as for the proteomics investigation and as described in Toni et al. (2019), setting the enzyme specificity as unspecific (Aletti et al., 2016; Giromini et al., 2019; Maffioli et al., 2020). The mass spectrometry raw data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD047855. Peptides identified by MaxQuant were analyzed by the Perseus software (version 1.5.5.3). Hits to the reverse database were eliminated and the LFQ intensities were converted to a log scale (log2). Only peptides present and quantified in at least 70% biological replicates were considered as positively identified in a group (Maffioli et al., 2020). Principal Component Analysis (PCA) was generated during Perseus analysis, with the resulting datasets. The individual variability of the biological replicates was evaluated by comparing plasma samples belonging to the same experimental group in terms of number of identified peptides, sum of LFQ signal intensity and Pearson correlation coefficient values, calculated using the log2 LFQ intensity. Specific comparisons were carried out, namely CTR vs. SA vs. SU, CTR vs. SA, CTR vs. SU and SA vs. SU. A Student's t-test (FDR ≤ 0.05) was carried out to identify peptides differentially present among the different conditions. Peptides were considered to be differentially present if they were present only in one condition or showed significant t-test difference.

All peptides were searched in the Structurally Annotated Therapeutic Peptides database (SATPdb) (Singh et al., 2016) and in the Food-Derived Bioactive Peptides database (DFBP) (Qin et al., 2020) to find potentially bioactive peptides. The search was performed applying an "IF" nested function to a

matrix that compared the sequence of each peptide found with those of the database (Microsoft Excel 2018, version 16.16.27; Microsoft Corp., Redmond, WA) (Giromini et al., 2019).

Results and Discussion

Hepatic protein profile

The reproducibility of the biological and technical replicates was confirmed by the calculation of Pearson's linear correlation factor, which exceeded 0.98 for all experimental conditions, compared in pairs (Supplementary Figure S1). From a technical point of view, the proteomic analysis allowed for the identification and quantification of 2881 proteins by merging all experimental conditions. Then, for each observation, the extent of the protein abundance variation (log2 FC) was evaluated (Table 1).

Table 1. Technical parameters of quantification of the proteomics analysis.

	SA vs. CTR	**SU vs. CTR**	**SA vs. SU**
Significantly modulated proteins (n)	54	47	24
Log2 Fold-change > 0.5 (n)	9	14	5
Log2 Fold-change < -0.5 (n)	11	10	7

For each comparison matrix, the number of significantly quantified proteins and the number of significantly modulated proteins, both in terms of up-regulation and down-regulation, were reported. Proteins were considered significantly modulated if $P < 0.05$.

Figure 4 shows the Volcano Plots of the distribution of log2 FC values versus -LogP values, indicative of the significance of the calculation, where significantly up-regulated gene products are on the right side and significantly down-regulated ones on the left side of the graph.

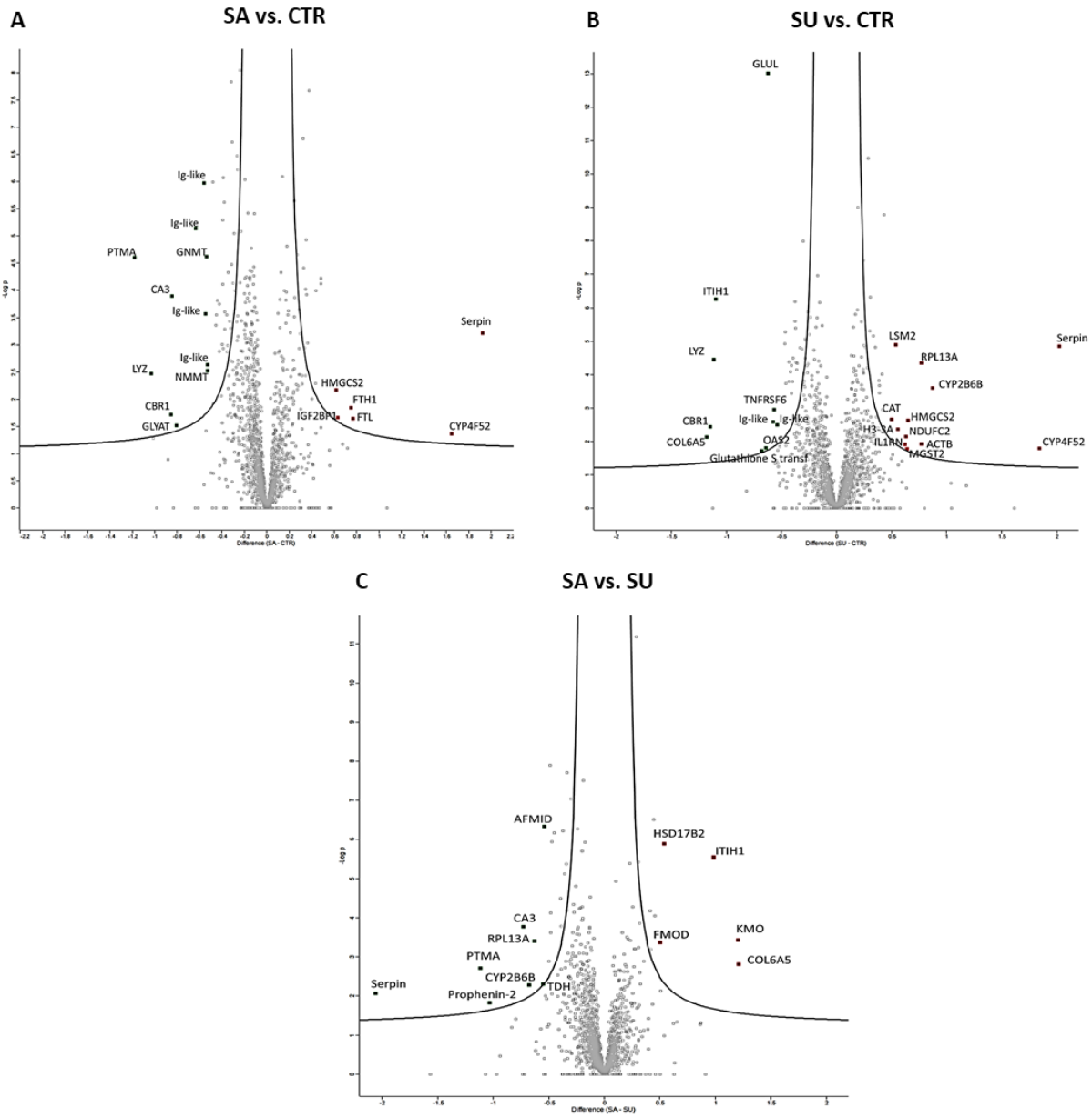


Figure 4. Volcano Plots derived from the LFQ analyses of SA vs. CTR (A), SU vs. CTR (B), SA vs. SU (C) comparison matrices.

A total of 125 proteins was significantly modulated considering the three comparison matrices (SA vs. CTR, SU vs. CTR, and SA vs. SU). Of this, 28 proteins were upregulated (log2 FC > 0.5) and 28 were downregulated (log2 FC < -0.5). Functional enrichment of significantly modulated proteins and thus gene annotation-based interactome analysis were performed. In this case, the String software was used for searching potential interactions among significantly up-regulated and significantly down-regulated proteins. The results showed that the minimal modulation exerted by the SA and SU dietary treatments led to no correlation between the modulated proteins. Despite this result, a further data

exploration was done manually, investigating individual proteins to obtain a picture of the most interesting biological information that emerged from the proteomics results and the literature.

Proteome modulation induced by the salty diet (SA vs. CTR)

Table 2 summarizes the most relevant significantly modulated gene products in the SA group compared to the CTR group, together with the log2 FC values and a panel of values indicating the animal reproducibility, namely the number of animals confirming each evidence (if the value was confirmed in ≥2 technical replicates, the biological replicate was considered valid; identifications absent in the pool and in less or equal to 3 biological replicates were excluded).

Table 2. Most relevant significantly modulated proteins in the SA vs. CTR comparison matrix. Negative values of log2 FC indicate the corresponding gene products' down-regulation, while positive values the gene products' up-regulation. The "animal reproducibility" columns indicate the number of animals per experimental group that confirms the evidence (up/down-regulation).

Quantitative Analysis	**Functional Evaluation**		**Animal Reproducibility**	
Log2 FC	**Gene Name**	**Protein Name**	**SA**	**CTR**
-1.031	LYZ	Lysozyme C-3	4	5
-0.970	CA3	Carbonic anhydrase 3	7	7
-0.804	GLYAT	Glycine N-acyltransferase-like protein	7	7
-0.659	UniProtKB unreviewed (TrEMBL)	Ig-like domain-containing protein	7	7
-0.651	UniProtKB unreviewed (TrEMBL)	Ig-like domain-containing protein	7	7
-0.573	NNMT	Nicotinamide N-methyltransferase	7	6
-0.572	LOC100156325	Serpin domain-containing protein	6	5
0.616	HMGCS2	Hydroxymethylglutaryl-CoA synthase, mitochondrial	7	7
0.716	GSTA4	Glutathione transferase	5	4
0.765	FTL	Ferritin	7	7
0.953	H3-3A	Histone H3	3	3
0.991	FTH1	Ferritin	7	7
1.278	H3-C1	Histone H2A/H2B/H3 domain-containing protein	6	5

Among the most interesting findings regarding lipid metabolism there are the downregulation of carbonic anhydrase (CA3) and nicotinamide N-methyltransferase (NNMT), and the up-regulation of

mitochondrial hydroxymethylglutaryl-CoA synthase (HMGCS2) in the SA group compared to the CTR group. Then, other upregulated gene products of interest were also FTL and FTH1, which encode for the light chain and heavy chain of the ferritin protein, respectively, and glutathione transferase GSTA4, which conjugates glutathione to numerous endogenous and exogenous electrophiles. The last but not the least important evidence concerned the upregulation of histone proteins (H3-3A and H3-C1).

Proteome modulation induced by the sugary diet (SU vs. CTR)

Table 3 summarizes the most relevant significantly modulated gene products following SU diet feeding compared to the CTR, along with log2 FC values and a panel of values indicating the reproducibility of the animals.

Table 3. Most relevant significantly modulated proteins in the SU vs. CTR comparison matrix. Negative values of log2 FC indicate the corresponding gene products' down-regulation, while positive values the gene products' up-regulation. The "animal reproducibility" column indicates the number of animals per experimental group that confirms the evidence (up/down-regulation).

Quantitative Analysis	**Functional Evaluation**		**Animal Reproducibility**	
Log2 FC	**Gene Name**	**Protein Description**	**SU**	**CTR**
-1.659	COL6A5	Collagen type VI alpha 5 chain	5	7
-1.244	LYZ	Lysozyme C-3	5	5
-0.970	ITIH1	Inter-alpha-trypsin inhibitor heavy chain 1	7	7
-0.728	TNFRSF6	FAS-associated death domain protein	5	4
-0.675	LOC100526118	Glutathione S-transferase	6	6
-0.652	GLUL	Glutamine synthetase	7	7
-0.642	OAS2	2'-5'-Oligoadenylate Synthetase 2	6	5
-0.641	FMO4	Flavin-containing monooxygenase	4	5
-0.573	UniProtKB unreviewed (TrEMBL)	Ig-like domain-containing protein	6	7
0.575	LSM2	U6 snRNA-associated Sm-like protein LSm2	4	4
0.630	NDUFC2	NADH dehydrogenase (ubiquinone) 1 subunit C2	3	3
0.648	HMGCS2	Hydroxymethylglutaryl-CoA synthase, mitochondrial	7	7
0.724	GRK3	G protein-coupled receptor kinase	3	3
0.769	ACTB	Actin B	6	7
1.012	H3-3A	Histone H3	6	5

Here, the trend of HMGCS2 upregulation induced by the SU diet compared to CTR was superimposable to that observed for the SA, whereas no other gene product related to lipid metabolism was observed.

In addition, as shown for the SA vs. CTR comparison, the upregulation of histone protein H3 was also significant for the SU diet. Then, the upregulation of actin together with the downregulation of collagen type 6 (alpha chain 5) and inter-alpha-trypsin inhibitor heavy chain 1 (ITIH1) were observed in the SU group compare to CTR.

Proteome modulation induced by the salty diet compared to the sugary diet (SA vs. SU)

Finally, Table 4 summarizes the most relevant gene products found to be significantly modulated by comparing the intake of the SA diet with the SU diet. The purpose was to confirm the experimental evidence already provided by the corresponding comparison matrices compared to the CTR.

Table 4. Most relevant significantly modulated proteins in the SA vs. SU comparison matrix. Negative values of log2 FC indicate the corresponding gene products' down-regulation, while positive values the gene products' up-regulation. The "animal reproducibility" column indicates the number of animals per experimental group that confirms the evidence (up/down-regulation).

Quantitative Analysis	**Functional Evaluation**		**Animal Reproducibility**	
Log2 FC	**Gene Name**	**Protein Description**	**SA**	**SU**
-2.119	LOC106504547	Serpin domain-containing protein	5	4
-0.810	CYP2B6B	Unspecific monooxygenase	3	3
-0.776	CA3	Carbonic anhydrase 3	7	7
-0.752	TDH	L-threonine 3-dehydrogenase, mitochondrial	7	7
-0.631	RPL13A	60S ribosomal protein L13a	4	3
0.644	FAH	Fumarylacetoacetase	7	7
1.020	ITIH1	Inter-alpha-trypsin inhibitor heavy chain 1	7	7
1.465	KMO	Kynurenine 3-monooxygenase	4	3
1.644	COL6A5	Collagen type VI alpha 5 chain	5	4

The analysis confirmed that the downregulation of CA3 was specific to the SA diet, as well as the downregulation of ITIH1 and COL6A5 upon the SU diet intake confirmed to be specific for the SU diet. The absence of modulation of HMGCS2 and H3-3A also confirmed their dependence on the intake of both SA and SU diets compared to CTR. Interestingly, the SA diet mediated the upregulation of the Kynurenine 3-monooxygenase (KMO), whose modulation was not significant in the SA vs. CTR comparison, but in this case the significant upregulation indicated that the SA diet had a higher KMO level compared to the SU diet. Therefore, the SU diet might have a lower KMO level than the CTR diet, otherwise the KMO level should have been significantly different also in the case of the SA vs. CTR

comparison. Figure 5 shows this hypothetical comparison of the KMO level in the three dietary groups considering the level of significance.

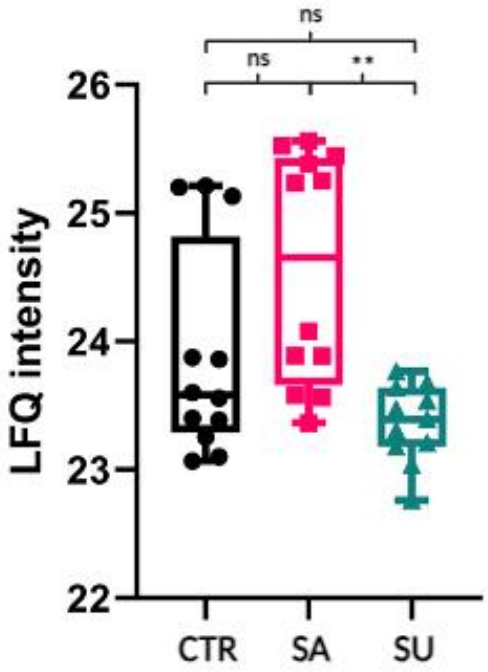


Figure 5. Graphical representation of the distribution of LFQ intensity values recorded for KMO in the CTR, SA and SU feeding treatment groups, and related significance. ns = non-significant; ** = P < 0.01.

Plasma peptidomic profile

Considering as positively identified all the peptides present in at least 70 % of each data set, 84, 98, and 89 peptides were detected in CTR, SA and SU group, respectively, for a total of 122 identified peptides.

Evaluation of the intra-group variability and comparison of the plasma peptidome

The individual variability of the biological replicates, evaluated by comparing plasma samples belonging to the same experimental group in terms of number of identified peptides and sum of LFQ signal intensity demonstrated a very low variability, most often less than 10% (Table 5).

Table 5. Number of identified proteins, sum of the LFQ intensity signal and variance percentage values for each biological replicate belonging to a group (CTR, SA or SU).

Diet	Sample	Number of identified peptides	Variability % (number of identified peptides)	LFQ signal intensity	Variability % (LFQ signal intensity)
CTR	1	110	8.9	4.05E+10	29.9
	2	108	8.8	8.76E+09	6.5

	3	54	4.4	1.42E+09	1.0
	4	83	6.7	2.08E+09	1.5
	5	119	9.7	9.94E+09	7.3
	6	108	8.8	6.22E+09	4.6
	7	108	8.8	1.40E+10	10.4
	8	122	9.9	5.73E+09	4.2
	9	140	11.4	2.46E+10	18.2
	10	94	7.6	6.62E+09	4.9
	11	88	7.1	4.23E+09	3.1
	12	98	8.0	1.11E+10	8.2
SA	1	132	9.1	1.57E+10	9.5
	2	120	8.3	3.39E+09	2.0
	3	148	10.2	1.61E+10	9.7
	4	142	9.8	7.86E+09	4.7
	5	117	8.1	6.08E+09	3.7
	6	101	7.0	1.30E+10	7.9
	7	131	9.1	4.94E+09	3.0
	8	118	8.2	2.50E+10	15.1
	9	108	7.5	2.98E+10	18.0
	10	109	7.5	9.36E+09	5.6
	11	123	8.5	2.04E+10	12.3
	12	97	6.7	1.41E+10	8.5
SU	1	136	9.6	9.52E+09	3.4
	2	140	9.9	1.15E+10	4.1
	3	117	8.3	8.73E+10	31.0
	4	139	9.8	1.59E+10	5.7
	5	88	6.2	9.78E+09	3.5
	6	107	7.6	8.03E+09	2.9
	7	112	16.4	9.43E+09	6.8
	8	115	8.1	6.11E+09	2.2
	9	122	8.6	6.27E+10	22.2
	10	100	7.1	1.56E+10	5.5
	11	99	7.0	2.59E+10	9.2
	12	137	9.7	2.00E+10	7.1

The Pearson's correlation coefficient values confirm that there was a strong correlation between datasets of the same group (CTR, SA or SU group), without any outlier sample (Supplementary Tables S1-S3).

A principal component analysis (PCA) was carried out on the peptidome of all thirty-six samples, suggesting a differential clustering of the three groups (CTR, SA and SU) (Figure 6A).

The three-way comparison between the peptidome of plasma samples obtained from pigs of all the three dietary groups allowed to identify peptides in common and those that were exclusively present in one group. In particular, 59 peptides were present in all groups, 11 were exclusively identified in CTR, 17 in SA and 4 in SU group (Figure 6B).

All these peptides were produced by the proteolysis of 25 plasma proteins (Figure 6C), of which 68% (n=17) common to CTR, SA and SU plasma. Only an uncharacterized protein was exclusively identified in CTR and 4 proteins in SA group, namely: heparan sulfate proteoglycan 2 (HSPG2), poly(A) binding protein cytoplasmic 4 (PABPC4), vanin 3 (VNN3) and legumain (LGMN).

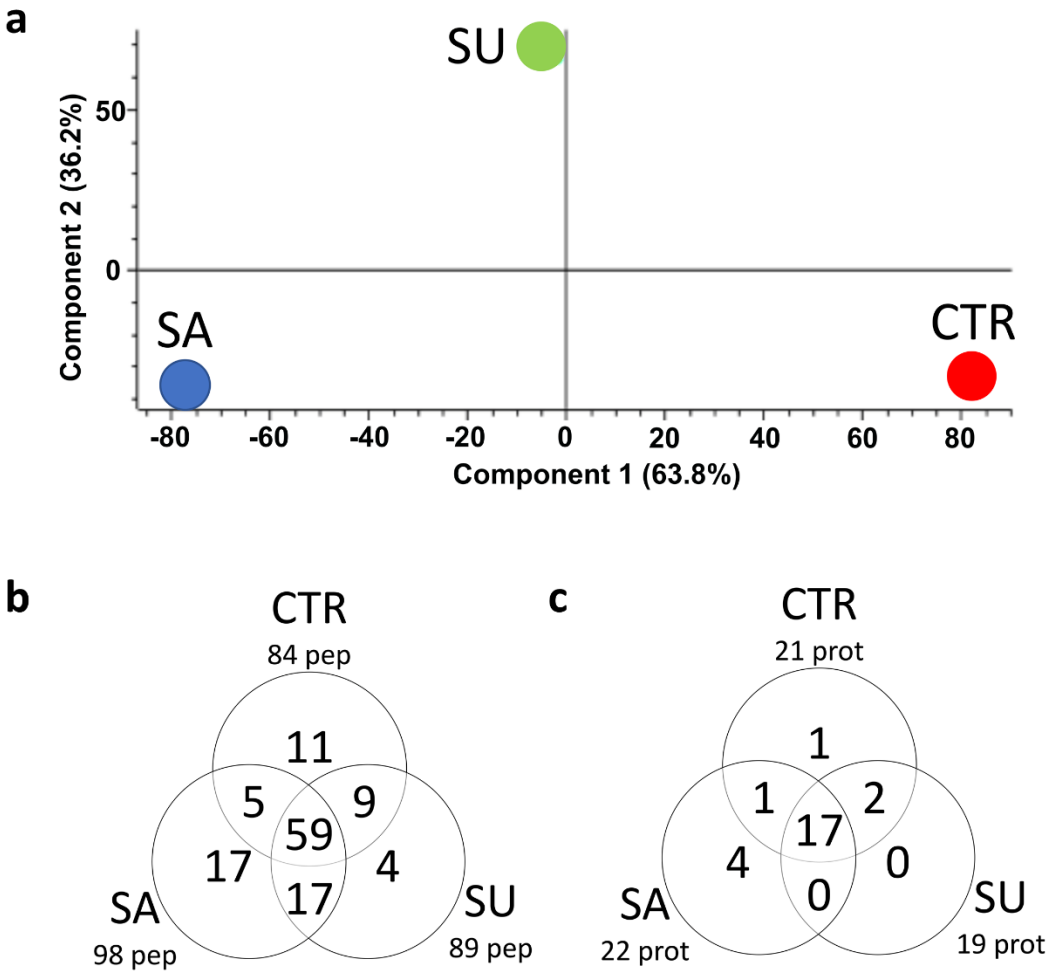


Figure 6. Schematic representation of peptidomic results. Legend: PCA analysis of the peptidome after grouping and averaging quantitative data related to peptides in CTR (red), SA (blue) and SU (green) group (A), Venn diagram of all peptides identified in plasma samples from the comparison CTR vs. SA vs. SU (B), and Venn diagram of all the proteins of which produced by the proteolysis that generated peptides identified in plasma samples from the comparison CTR vs. SA vs. SU (C).

Search for bioactive peptides

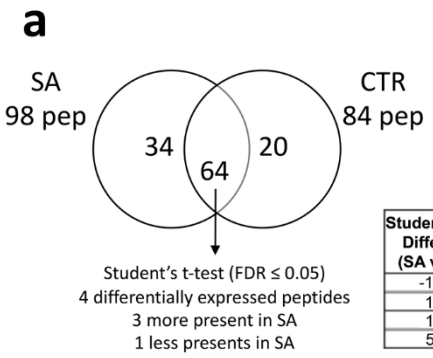
The search of potentially bioactive peptides was carried out in SATPdb on all data sets from CTR, SA and SU groups. Interestingly, three peptides with antihypertensive activity were exclusively identified in the SA group (Table 5).

Table 6. List of potentially anti-hypertensive peptides found in plasma samples from SA group. In bold the sequence stretches corresponding to the SATPdb peptides with anti-hypertensive activity.

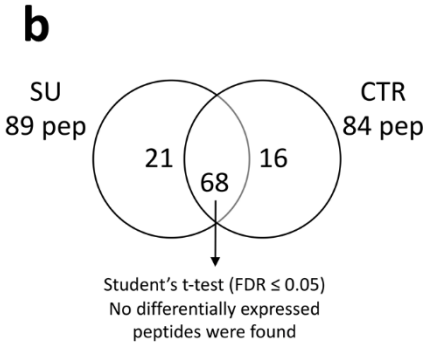
Database SATPdb sequence	Sequence identified in SA peptides	Protein of origin	Gene
LSLP	LRAYDG**LSLP**EDAETISAGRAG	A0A286ZHV7	HSPG2
RALP	NQYMQRVAGM**RALP**ANAILNQFQ	A0A8W4FKU3	PABPC4
VFER	ALYGRVFERDPPRLGQGPGQVQ	F1S3Q8	VNN3

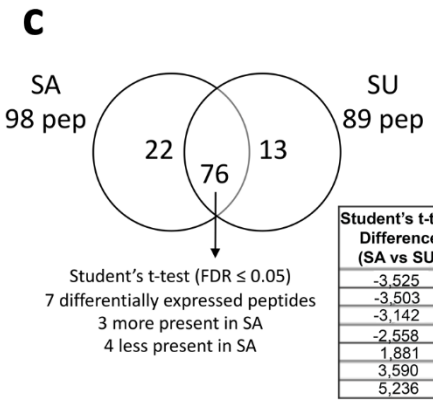
According to the SATPdb database, the peptides LRAYDGLSLPEDAETISAGRAG and ALYGRVFERDPPRLGQGPGQVQ contained a sequence stretch corresponding to the SATPdb peptides with anti-hypertensive activity. They originated from the proteins heparan sulfate proteoglycan 2 (HSPG2) and vanin-3 (VNN3), respectively. In addition, the peptide NQYMQRVAGMRALPANAILNQFQ originated from cytoplasmic poly(A) binding protein 4 (PABPC4).

Specific analyses, carried out by two-way comparisons between the peptidome of plasma samples obtained from piglets fed with three different diets (SA vs. CTR, SU vs. CTR, and SA vs. SU), allowed to identify peptides in common and those that were exclusively present in one group. A Student's t-test (FDR ≤ 0.05) was carried out to identify peptides differentially present among the different conditions. Peptides were significantly different if they were identified only in one condition or showed significant t-test difference (FDR ≤ 0.05) (Fig. 3). According to our results, 20 and 16 peptides were exclusively expressed in CTR in the comparisons SA vs. CTR and SU vs. CTR (respectively), then 34 and 22 peptides were exclusively expressed in SA in the comparisons SA vs. CTR, and SA vs. SU (respectively), whereas 21 and 13 peptides were exclusively expressed in SU in the comparisons SU vs. CTR, and SA vs. SU (respectively). However, no potentially bioactive peptides were identified by searching in SATPdb and DFBP database on peptides that showed significant t-test difference (FDR ≤ 0.05), according to the results described above.



Student's t-test Difference (SA vs CTR)	Sequence	Leading razor protein	Gene name
-1,050	ATASRGAPQARAPQG	P20305	GSN
1,432	TASRGAPQARAPQGR	P20305	GSN
1,477	DKGETGEQGDRGIK	A0A287BLD2	COL1A1
5,958	LWESASLLR	A0A5G2QME3	C3





Student's t-test Difference (SA vs SU)	Sequence	Leading razor protein	Gene name
-3,525	KETQTGPYTLSF	A0A287BJ64	AZGP1
-3,503	SAPVRHRILWESASLLR	A0A5G2QME3	C3
-3,142	AIDYDEDEDGRPKVHVD	A0A5G2R564	FGB
-2,558	ATASRGAPQARAPQGRVSPMRPSTM	P20305	GSN
1,881	TASRGAPQARAPQGR	P20305	GSN
3,590	ILWESASLLR	A0A5G2QME3	C3
5,236	LWESASLLR	A0A5G2QME3	C3

Figure 7. Schematic representation of the peptidomics two-way comparisons results. Venn diagram of all peptides identified in plasma samples from the comparison SA vs. CTR (A), SU vs. CTR (B), and SA vs. SU (C). Tables report the list of peptides that showed significant t-test difference (FDR ≤ 0.05).

Discussion

This study followed previous research about the use of FFPs as alternative and sustainable feedstuffs for pigs. After observing that SA and SU FFPs diets were not detrimental for the growth and feeding behavior of piglets and pigs, we decided to apply omics approaches to further evaluate the impact of FFPs by focusing on liver proteome and plasma peptidome.

A label-free quantitative proteomics strategy was applied on liver tissue samples with the aim of assessing the impact of the SA and SU diets on the modulation of liver function and of any relevant cellular pathway. Specifically, the experimental conditions tested were compared with each other in

order to obtain a comprehensive picture elucidating the extent of liver proteome modulation by the salty diet (SA vs. CTR), as well as by the sugary diet (SU vs. CTR) and by comparing the two experimental diets (SA vs. SU). As seen above, the results of the functional analysis only showed a minimal modulation exerted by the SA and SU diets on liver proteome and any significant correlation between the modulated proteins was detected. However, we decided to further explore the proteomics results by observing and discussing the biological role of the most relevant modulated proteins.

The SA vs. CTR comparison (Table 2) and the SU vs. CTR comparison (Table 3) pointed out the modulation of several proteins involved in hepatic lipid metabolism. Specifically, compared to CTR group, the SA diet led to the downregulation of CA3 and NNMT and the up-regulation of HMGCS2, whereas the SU diet only caused the upregulation of HMGCS2 (similarly to the SA diet). The CA3 protein catalyzes the reversible CO_2 hydration/dehydration reaction, which is necessary for the synthesis of long chain fatty acids, requiring bicarbonate. Nicotinamide N-methyltransferase (NNMT) catalyzes the methylation of nicotinamide and it is involved in hepatic lipid metabolism and energy homeostasis (Liang et al., 2023). Besides, HMGCS2 is the rate-limiting enzyme in the ketogenesis pathway (a biochemical process by which organisms produce ketone bodies by cleaving fatty acids and ketogenic amino acids). A dysregulated ketogenesis is associated with fatty liver development (Asif et al., 2022). Such differentially expressed proteins might suggest an altered lipid metabolic activity induced by the higher crude fat content and the different fatty acid profile of the SA and SU diets compared to the CTR diet, in particular a higher MUFA content for both SA and SU diets and a lower SFA content for the SA diet compared to CTR (Mazzoleni et al., 2023). It was observed that the SA and SU FFPs diets modulated the synthesis of fatty acids and the related acidic profile of adipose tissue (Luciano et al., 2022b), and this could be related to alterations in lipogenesis/lipolysis pathways in liver. The upregulation of NNMT was responsible for PPARγ transactivation and related lipid accumulation in a model of mouse liver cells challenged with palmitic acid, a SFA known to be positively correlated to hepatic lipotoxicity (Song et al., 2023). The low SFA content of the SA diet could be related to the downregulation of NNMT, whereas the higher MUFA content of both SA and SU diets could be related to the upregulation of HMGCS2. To summarize, we hypothesized that the downregulated CA3 reduced lipogenesis and that lower NNMT level and the upregulated HMGCS2 led to reduced lipid accumulation in liver.

In addition, the upregulation of FTL, FTH1, and GSTA4 in the SA group was observed (Table 2). The FTL and FTH1 proteins are both involved in the storage of iron in a non-toxic form. The heavy chain (FTH1) also possesses ferroxidase activity (Ali et al., 2021). The glutathione transferase GSTA4 and in general GST genes are known to be upregulated in response to oxidative stress (Nebert and Vasiliou, 2004). If the upregulation of proteins involved in iron storage could be related to increased dietary iron intake, the overexpression of GSTA4 could indicate a cellular response to oxidative stress. Instead, glutathione

S-transferase was found to be downregulated by the SU diet (Table 3), suggesting that the primary antioxidant defense was activated to an even lesser extent than in the CTR condition. However, in the present study any marker of oxidative stress was measured.

Then, both SA and SU diets led to the upregulation of histone proteins compared to CTR (Tables 2 and 3), which are considered an early response to cellular stress and occurred before the release of markers of stress including heat shock proteins. The post-translational modifications of histone H3 work as epigenetic regulators of gene transcription, influencing chromatin structure and providing binding sites for many transcription factors, thereby regulating various cellular functions, such as gene expression, cell cycle, replication, and stress-induced DNA repair. However, the upregulation of histone proteins was not correlated with any metabolic pathway involved in cellular stress. In the SU group, a hypothetical reorganization of the cell structure could be suggested by the modulation of actin, collagen type 6 (alpha chain 5) and inter-alpha-trypsin inhibitor heavy chain 1 (ITIH1) (Table 3), which is mainly involved in stabilizing the extracellular matrix by binding with hyaluronic acid.

Finally, the upregulation of KMO, a mitochondrial enzyme involved in the kynurenine pathway (KP) of the tryptophan degradation, was observed in the SA group compared to SU group (Table 4). The KMO controls the synthesis of several kynurenine metabolites, including 3-hydroxykynurenine (3-HK), quinolinic acid (QUIN) and kynurenicacid (KYNA), as well as anthranilic acid. These bioactive metabolites are associated with inflammatory conditions (Wu et al., 2022). A positive correlation was observed between the increased hepatic fatty acid oxidation and tryptophan metabolites concentration, such as the KMO-mediated kynurenine metabolites cited above, in rat serum (Lindquist et al., 2020). Here, we hypothesized that HMGCS2 upregulation mediated by both SA and SU diets was correlated with reduced lipid accumulation in liver, but the SA diet also led to CA3 and NNMT downregulation. Therefore, the alteration of lipid metabolism caused by the SA diet might explain the significant upregulation of KMO compared to the SU diet, although no measurement of kynurenine metabolites was performed.

The peptidomics analysis showed low intra-group variability (around 10% in all cases) (Table 5), whereas the PCA showed a differential clustering of the CTR, SA, and SU groups. Around 70% of plasma proteins from which the detected peptides were derived was shared by the three dietary groups, but four proteins were specific of the SA group. Heparan sulfate proteoglycan 2 (HSPG2) is a core protein to which three long chains of glycosaminoglycans are attached. This protein binds to and cross-links many extracellular matrix components and cell-surface molecules, as laminin, prolargin, collagen type IV, and transthyretin, playing an essential role in multiple biological activities, such as helping to maintain the endothelial barrier function. It is a potent inhibitor of smooth muscle cell proliferation and is thus thought to help maintain vascular homeostasis (Pretorius et al., 2022). Vanin genes (VNN) are clustered and encode isoforms of pantetheinase, whicho hidrolyzes pantetheine into pantothenic

acid (vitamin B5), and cysteamine (CysH, 2-aminoethanethiol), a sulfhydryl compound which is in equilibrium with its oxidized form cystamine (CysN). The role of vanins in the regulation of mucosal inflammation may contribute to structural damage of the intestinal mucosa (Jansen et al., 2009; Mariani et al., 2017). In particular, vanin-1 and vanin-2 are ectoenzymes (GPI)-linked anchored to the cell surface, expressed by different organs among which the intestinal tract. They have pantetheinase enzymatic activity, but the activity of vanin-2 is weaker than that of vanin-1. Vanin-3, which generated a peptide exclusively identified in SA group, by lacking the GPI-anchoring consensus, encodes a secreted truncated protein whose expression is induced by oxidative stress (Kaskow et al., 2012). Legumains (LGMN) are cysteine protease with a receptor modulating effect on integrin αvβ3 that alters the downstream signaling cascades in vascular smooth muscle cells (Solberg et al., 2022). Also in this case, the presence of peptides originated by its degradation may be relatable with the SA diet since this endogenous modulator of integrin triggers vascular degeneration, dissection, and rupture.

Altogether, the peptidomics analysis did not highlight significant differences between the three diets, substantially confirming what was highlighted by the proteomic analysis with the only exception of the four proteins exclusively identified in the SA group, whose described activity was related to keep vascular homeostasis and counteract inflammation, oxidative stress and vascular degeneration.

Of the three peptides exclusively identified in the SA group (Table 5), the peptides LRAYDGLSLPEDAETISAGRAG and ALYGRVFERDPPRLGQGPGQVQ originated from the proteins HSPG2 and VNN3, respectively, whose involvement in helping maintain vascular homeostasis and in oxidative stress response was discussed above. In addition, the peptide NQYMQRVAGMRALPANAILNQFQ originated from the protein PABPC4, whose involvement in the anti-hypertensive response is not reported in the literature, but possesses the RALP sequence whose anti-hypertensive and renin-inhibitory activities are described in both the SATPdb and the DFBP database. Altogether, the peptidomic results suggested that the SA diet, with about 2-fold higher sodium content compared to CTR and SU diets (3.2 g/kg DM in SA diet vs. 1.7 g/kg DM in both SU and CTR diets) (Mazzoleni et al., 2023), could have triggered the activation of adaptive physiological mechanisms through which pigs tried to counteract any damage that could have been caused by the content of sodium in the SA diet, generating potentially anti-hypertensive plasma peptides from proteins involved in helping maintain vascular homeostasis and oxidative stress response. The presence of an increased number of peptides with biological activity in the plasma of SA-fed pigs was interesting, even though the bioavailability of peptides has been often questioned (Foltz et al., 2010; Miner-Williams et al., 2014).

Conclusions

The proteomics investigation identified a low number of differentially modulated proteins in each comparison matrix, indicating a limited impact on liver function. The functional analysis confirmed that the significantly modulated proteins led to any relevant metabolic correlation for the activation of specific pathways. The literature investigation showed a partially similar modulation of lipid metabolism in liver in response to the SA and SU diets, with reduced lipogenesis and increased lipid oxidation. The SA diet was also associated with a potential increase in oxidative stress, even though no marker of oxidative stress was measured. Both SA and SU diets induced dysregulation of histone protein H3, attributable to a possible remodulation of cellular structure. In any case, the limited number of significantly modulated proteins and the absence of relevant metabolic pathways modulated led us to conclude that both the SA and SU diets did not markedly alter the liver function.

The peptidomics analysis identified no relevant differences between the three dietary groups, with the only exception of the endogenous peptides originated by four proteins exclusively identified in the SA dietary group whose activity was related to keep vascular homeostasis and counteract inflammation, oxidative stress and vascular degeneration. Interestingly, our data also demonstrated the presence of three peptides with antihypertensive activity and biological activity related to the maintenance of vascular homeostasis, identified exclusively in the SA group whose bioavailability and effectiveness were not investigated.

To summarize, the metabolic impact of SA and SU FFPs diets in pigs was limited and very few liver proteins and plasma peptides were differentially expressed between the control and the experimental dietary groups. Altogether, these findings provided further evidence of the validity of reusing FFPs in pig nutrition, to keep their nutritional value in the food chain and reduce the competition between feed and food.

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Supplementary Tables

Supplementary Table S1

Pearson correlation of peptides intensities in plasma of pigs fed with CTR diet.

	CTR-1	CTR-2	CTR-3	CTR-4	CTR-5	CTR-6	CTR-7	CTR-8	CTR-9	CTR-10	CTR-11	CTR-12
CTR-1	1	0,358	0,655	0,667	0,694	0,750	0,808	0,640	0,764	0,782	0,767	0,781
CTR-2	0,358	1	0,610	0,479	0,616	0,574	0,224	0,740	0,565	0,365	0,301	0,202
CTR-3	0,655	0,610	1	0,858	0,711	0,832	0,660	0,648	0,750	0,669	0,724	0,723
CTR-4	0,667	0,479	0,858	1	0,758	0,812	0,662	0,539	0,788	0,625	0,656	0,674
CTR-5	0,694	0,616	0,711	0,758	1	0,883	0,752	0,623	0,880	0,722	0,719	0,700
CTR-6	0,750	0,574	0,832	0,812	0,883	1	0,791	0,667	0,869	0,830	0,802	0,779
CTR-7	0,808	0,224	0,660	0,662	0,752	0,791	1	0,433	0,816	0,941	0,905	0,939
CTR-8	0,640	0,740	0,648	0,539	0,623	0,667	0,433	1	0,583	0,564	0,581	0,435
CTR-9	0,764	0,565	0,750	0,788	0,880	0,869	0,816	0,583	1	0,784	0,770	0,779
CTR-10	0,782	0,365	0,669	0,625	0,722	0,830	0,941	0,564	0,784	1	0,926	0,936
CTR-11	0,767	0,301	0,724	0,656	0,719	0,802	0,905	0,581	0,770	0,926	1	0,947
CTR-12	0,781	0,202	0,723	0,674	0,700	0,779	0,939	0,435	0,779	0,936	0,947	1

Supplementary Table S2

Pearson correlation of peptides intensities in plasma of pigs fed with SA FFP diet.

	SA-1	SA-2	SA-3	SA-4	SA-5	SA-6	SA-7	SA-8	SA-9	SA-10	SA-11	SA-12
SA-1	1	0,817	0,772	0,796	0,589	0,650	0,666	0,609	0,447	0,792	0,618	0,612
SA-2	0,817	1	0,767	0,828	0,674	0,472	0,770	0,582	0,481	0,846	0,539	0,492
SA-3	0,772	0,767	1	0,899	0,719	0,759	0,772	0,724	0,624	0,817	0,674	0,624
SA-4	0,796	0,828	0,899	1	0,752	0,690	0,814	0,715	0,615	0,839	0,650	0,587
SA-5	0,589	0,674	0,719	0,752	1	0,730	0,858	0,813	0,744	0,859	0,832	0,749
SA-6	0,650	0,472	0,759	0,690	0,730	1	0,646	0,879	0,712	0,772	0,866	0,951
SA-7	0,666	0,770	0,772	0,814	0,858	0,646	1	0,732	0,633	0,880	0,735	0,624
SA-8	0,609	0,582	0,724	0,715	0,813	0,879	0,732	1	0,781	0,853	0,950	0,912
SA-9	0,447	0,481	0,624	0,615	0,744	0,712	0,633	0,781	1	0,606	0,819	0,744
SA-10	0,792	0,846	0,817	0,839	0,859	0,772	0,880	0,853	0,606	1	0,841	0,785
SA-11	0,618	0,539	0,674	0,650	0,832	0,866	0,735	0,950	0,819	0,841	1	0,935
SA-12	0,612	0,492	0,624	0,587	0,749	0,951	0,624	0,912	0,744	0,785	0,935	1

Supplementary Table S3

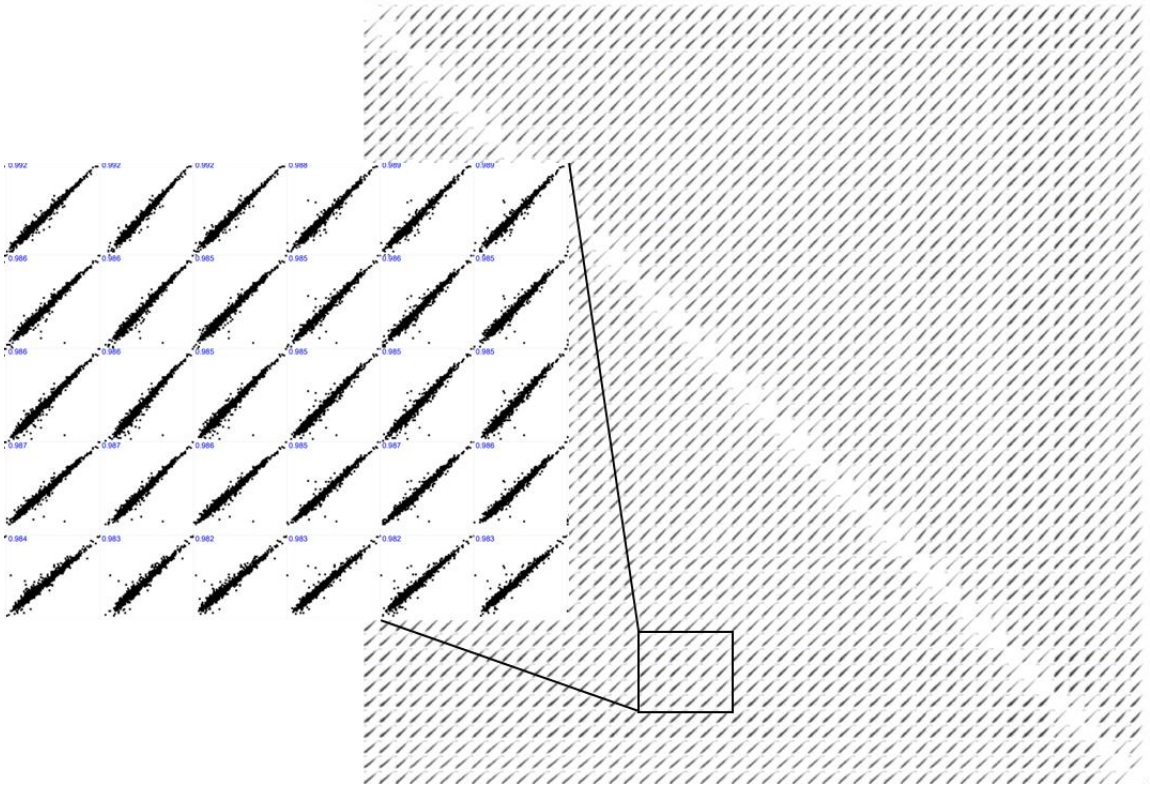
Pearson correlation of peptides intensities in plasma of pigs fed with SU FFP diet.

	SU-1	SU-2	SU-3	SU-4	SU-5	SU-6	SU-7	SU-8	SU-9	SU-10	SU-11	SU-12
SU-1	1	0,855	0,525	0,889	0,613	0,725	0,738	0,667	0,519	0,765	0,527	0,868
SU-2	0,855	1	0,601	0,863	0,632	0,727	0,773	0,607	0,539	0,663	0,576	0,891
SU-3	0,525	0,601	1	0,564	0,866	0,736	0,750	0,837	0,898	0,907	0,912	0,626
SU-4	0,889	0,863	0,564	1	0,660	0,827	0,787	0,725	0,598	0,752	0,616	0,865
SU-5	0,613	0,632	0,866	0,660	1	0,794	0,798	0,846	0,788	0,845	0,884	0,717
SU-6	0,725	0,727	0,736	0,827	0,794	1	0,913	0,907	0,654	0,767	0,688	0,767
SU-7	0,738	0,773	0,750	0,787	0,798	0,913	1	0,868	0,715	0,786	0,708	0,820
SU-8	0,667	0,607	0,837	0,725	0,846	0,907	0,868	1	0,747	0,869	0,746	0,650
SU-9	0,519	0,539	0,898	0,598	0,788	0,654	0,715	0,747	1	0,811	0,913	0,627
SU-10	0,765	0,663	0,907	0,752	0,845	0,767	0,786	0,869	0,811	1	0,881	0,657
SU-11	0,527	0,576	0,912	0,616	0,884	0,688	0,708	0,746	0,913	0,881	1	0,698
SU-12	0,868	0,891	0,626	0,865	0,717	0,767	0,820	0,650	0,627	0,657	0,698	1

Supplementary Figures

Supplementary Figure S1

Multi-scatter plot depicting the distribution of LFQ intensities between two conditions (3 biological replicates for 3 technical replicates).



Section B – Article 1

Effect of ellagic and gallic acid on the mitigation of methane production and ammonia formation in an *in vitro* model of short-term rumen fermentation

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Abstract

Ruminant production is an important source of animal proteins for human nutrition. However, ruminants contribute to about 30% of anthropogenic methane (CH_4) emissions worldwide. The reduction of CH_4 emissions could represent an important strategy against climate warming. Tannins can play an important role in the mitigation of CH_4 emissions from ruminants. However, their mode of action is not yet well known. Thus, the present study aimed to gain a better understanding of the effect of ellagic acid (EA) and gallic acid (GA) on rumen fermentation using a model of short-term *in vitro* rumen fermentation. The basal diet (hay) was supplemented with EA and GA in five treatments (mg/g dry matter): i) EA 75, ii) EA 150, iii) GA 75, iv) GA 150 and v) EA 75 + GA 75. After a 24 h incubation, pH, ammonia formation, gas production, short-chain fatty acids (SCFA), *in vitro* organic matter digestibility (IVOMD) and the microbial count were assessed. Total gas production and digestible organic matter (dOM) were decreased after all the treatments, except for GA 75. The treatments EA 150 and EA+GA significantly decreased CH_4 production per unit of dietary DM, dOM, CO_2 and SCFA. Ammonia production was significantly decreased by EA 150 and EA+GA. EA and GA differently affected the relative abundance of selected microbial species in rumen microbiota. To conclude, EA 150 and EA+GA exerted a significant effect on the reduction of CH_4 emissions and ammonia formation, but affecting also the rumen digestibility of the diet and the total SCFA production, whereas EA 75 and GA 75 were not effective as EA 150 and EA+GA on CH_4 and ammonia, but were less detrimental on feed digestibility and SCFA. Further studies are needed to determine whether the beneficial and detrimental effects of tannins on rumen fermentation can be dissociated.

Introduction

Livestock production is the source of animal proteins and plays an essential role in human nutrition worldwide. Through the enteric fermentation processes occurring in rumen, ruminants can efficiently convert low-quality and human-indigestible plant polysaccharides into energy and human-edible food, such as meat and milk (Shabat et al., 2016). It is also known that enteric fermentation leads to the physiological CH_4 production, making ruminants the main responsible for greenhouse gas (GHG) emissions among farm animals (Jackson et al., 2021; Vargas et al., 2022). One of the strategies to reduce CH_4 emissions and improve the environmental sustainability of ruminant production relies on tannins as a dietary supplement (Martin et al., 2010; Hassan et al., 2020). Tannins are plant polyphenol secondary metabolites with well-known antimicrobial, anti-inflammatory and antioxidant effects (Huang et al., 2018). Tannins are known to affect rumen fermentation processes through: i) mitigation of CH_4 emissions from enteric fermentation; ii) reduction of ammonia formation; iii) alteration of the rumen degradability of feed, in particular of proteins; and iv) modulation of the rumen microbiota (Jayanegara et al., 2012; Aboagye and Beauchemin, 2019; Vasta et al., 2019; Hassan et al., 2020). Tannins can reduce CH_4 emissions by directly inhibiting methanogenic archaea or indirectly modulating the action of methanogen-associated protozoa and fibre-degrading bacteria, for example by increasing the abundance of the bacterial group *Prevotella*, known to be a competitive H_2 sink for methanogens (Aboagye and Beauchemin, 2019; Aguilar-Marin et al., 2020). Moreover, they can bind dietary proteins and carbohydrates. The formation of protein–tannin complexes protect proteins from rumen degradation, thus leading to higher nitrogen (N) utilisation (Yanza et al., 2021). This results in reduced ruminal ammonia formation and a shift in N excretion from urine to faeces, thus reducing the emission of nitric oxide, which is mainly excreted by urine (Grainger et al., 2009; Aboagye et al., 2018). However, the binding of tannins with carbohydrates (e.g. plant fibres) reduces feed palatability and then feed intake. The effect of tannins varies according to the dosage and the animal species considered. The balance between the beneficial and detrimental effects of tannins on rumen fermentation depends mainly on the dosage used but also on the diet composition, the animal species and the source of supplementation (Vasta et al., 2019). The potential of tannins to mitigate CH_4 emissions and ammonia formation in ruminants has been demonstrated both *in vitro* (Getachew et al., 2008; Wei et al., 2018; Terranova et al, 2020; Giller et al., 2021; Foggi et al., 2022) and *in vivo* (Aboagye et al., 2018; Aboagye and Beauchemin, 2019). Most of the studies did not involve the use of single tannin molecules but rather the use of plants or tannin-containing forages, such as woody and herbaceous plants (Terranova et al., 2018; Terranova et al., 2020), *Stevia rebaudiana Bertoni* (Sarnataro et al., 2020), fruit and vegetable pomaces (Giller et al., 2021), quebracho (Getachew et al., 2008; Foggi et al., 2022) and mimosa and chestnut extracts (Bhatta et al., 2009; Hassanat and Benchaar, 2012; Foggi et al., 2022). The two main subgroups of tannins are condensed tannins (CT) and hydrolysable tannins (HT). CT are

polymers of flavan-3-ol subunits, whereas HT are water-soluble molecules composed of a glucose core esterified with gallic acid (GA) or ellagic acid (EA). The EA and GA components of HT are the potential bioactive molecules that likely exert the effect on rumen fermentation (Lotfi, 2020). It has been observed that HT are more effective than CT in decreasing CH_4 emissions while maintaining the digestibility of nutrients (Jayanegara et al., 2015; Yanza et al., 2021). Currently, the main knowledge gap about the effect of EA and GA in rumen is their detailed mode of action, their modulating effect on rumen microorganisms, and subsequently their metabolic fate in rumen. Therefore, we decided to investigate the effect of EA and GA, alone or in combination in their pure form, on the modulation of rumen microbes and rumen fermentation in a short-term *in vitro* simulation of rumen fermentation using the Hohenheim gas test (HGT). The EA and GA molecules were chosen as major components of HT. Also, EA-secondary metabolites, such as urolithins, are considered to be the responsible molecules exerting the modulating effect in the rumen. The doses of supplementation were chosen because of i) the significant results of *in vitro* studies showing reduced CH_4 emissions up to 15–20% DM of tannins inclusion (Getachew et al., 2007; Bhatta et al., 2009; Hassanat and Benchaar, 2012; Terranova et al., 2018; Terranova et al., 2020; Giller et al., 2021) and ii) the average high level of HT (around 60–80% DM) in plant extracts such as chestnut, quebracho or mimosa (Hassanat and Benchaar, 2012; Min et al., 2015; Lotfi, 2020). The aim of the study was multiple: first, to observe at what extent these high doses of supplementation could positively influence rumen fermentation and rumen microbes, in particular regarding CH_4 emissions, without deeply altering the rumen degradability of feed; second, to assess how EA and GA could interact in their modulating effect on rumen fermentation; last, to observe how EA and GA could modulate a selected panel of rumen microbes.

Materials and methods

Incubated materials

Rye grass-based hay (first cut, > 90% grass content) was used as a control and basal diet for the incubation of the supplements. It was ground using a centrifugal mill (Model ZM 200, Retsch GmbH, Hann, Germany) to pass through a 1 mm sieve. The chemical composition of the hay was (g/kg DM) 925 organic matter (OM), 75.1 ash, 112 crude protein (CP), 540 neutral detergent fibre (NDF), 295 acid detergent fibre (ADF) and 24.1 ether extract (EE). The EA and GA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity level was ≥ 95% for EA and ≥ 98.5% for GA. For incubation, 200 mg DM of hay were used and supplemented with EA and GA in pure form in the following concentrations (solid phase). Five different treatments were evaluated in this study (mg/g of DM): i) EA 75, ii) EA 150, iii) GA 75, iv) GA 150 and v) EA 75 + GA 75. Rumen fluid (pure) and rumen fluid with hay were added as a blank and a control.

Animal ethics

Animal manipulation was performed according to the Swiss guidelines for animal welfare procedure and was approved by the animal ethics committee of the Cantonal Veterinary Office of Zurich with approval number ZH113/18.

Rumen fluid collection and in vitro incubation

Animals were fed a total mixed ration (TMR). Each animal was fed with 17.8 kg/DM/day. The TMR was composed of (% DM) grass silage (48%), maize silage (20%), sugar beet pulp (17%), hay (8%), concentrate (8%) and mineral supplement (0.2%). The experiment was conducted during October and November 2021. For each run, rumen fluid was collected directly before the morning feeding from one of four in total fistulated, lactating Original Brown Swiss cows. The pH of the freshly taken rumen fluids always ranged between 5.9 and 6.9. Within 1 h of collection, the rumen fluid was transported in a preheated thermos flask to the laboratory and filtered through four layers of gauze. Two samples of pure rumen fluid were collected to measure pH and ammonia and to perform a microbial count (bacteria and protozoa). Then, rumen fluid was mixed with a preheated (39°C) reduced buffer solution in a 1:2 ratio according to the protocol of Menke and Steingass (1988). Scaled glass syringes with two outlets, one for rumen fluid and one for gas analysis, were the experimental units used for incubation (Soliva and Hess, 2007). The glass syringes were filled with the solid phase and then with 30 ml of the rumen fluid–buffer solution (Suppl. Figure 1). The solid phase was composed of the basal diet alone (control) or the basal diet in combination with EA and GA alone or combined. Additionally, a standard hay (purchased from the Institute of Animal Nutrition, University of Hohenheim, Stuttgart, Germany) and blanks (rumen fluid only) were incubated.

Sample collection and analysis

Syringes were incubated for 24 h at 39°C. After 24 h, the syringes were taken out of the incubator and the volume of total gas production was recorded. Fermentation was stopped by removing the liquid phase and leaving the gas phase inside the syringes for later analysis. Ammonia and pH were measured in the liquid phase samples using a potentiometer (ammonia: model 713, Metrohm, Herisau, Switzerland; pH: model 913; Metrohm, Herisau, Switzerland) equipped with electrodes. Then, 4 ml of liquid phase per syringe were mixed with H_2SO_4 50% (m/v) to stabilise the samples and frozen at –20°C for later SCFA analysis. For microbial count, the liquid phase samples were mixed with a solution of 6% formaldehyde in a 1:1 ratio for protozoa and 4% formaldehyde in a 1:100 ratio for bacteria. Gas samples of 150 µl were collected using a Hamilton syringe and injected in a gas chromatograph (GC-

TCD 6890 N, Agilent Technologies, Wilmington, NC, USA) equipped with a thermal conductivity detector to measure the CH_4 and CO_2 concentrations. The SCFA profile in rumen fluid samples was determined by HPLC. Samples were filtered and analysed for total SCFA production using a liquid chromatography (Ultimate 3000, Thermo Fisher Scientific, Reinach, Switzerland) with an exchange ion column (Nucleogel ION 300 OA 300 x 7.8 mm) and equipped with a refractive index detector (RefractoMax 521, Thermo Fisher Scientific, Reinach, Switzerland). To calculate the IVOMD, we used Menke and Steingass's (1988) standard equation: IVOMD (g/kg) = 14.88 + 0.8893 × total gas production (ml/200 mg DM) + 0.0448 × crude protein (g/kg DM) + 0.0651 × ash (g/kg DM). In total, four runs were performed, testing each of the treatments in triplicate (n = 12 replicates per treatment).

Real-time qPCR analysis

The liquid phase samples collected after the incubation were kept at –80°C for further DNA extraction and quantitative PCR (qPCR). The DNA was extracted using QIAMP Fast DNA Stool Mini Kit (Qiagen, Hombrechtikon, Switzerland), as described by Böttger et al. (2019), with minor modifications. In brief, 2 ml of liquid phase samples were centrifuged at 6500 × g for 30 min at 4°C. Then, the pellet was resuspended in 1.5 ml of Inhibitex buffer (provided with the kit) and heated at 90°C for 5 min. The tubes were allowed to return to room temperature before 15 s vortexing and further centrifugation at 16,000 × g for 1 min. Later, 200 µL of the supernatant were used for DNA extraction following the kit's procedure. The concentration of the DNA extracts was measured with spectrophotometry by using NanoDrop 1000 (Witec AG, Luzern, Switzerland). The quality of the extracted DNA was assessed by capillary electrophoresis using Fragment Analyzer (Agilent technologies, Basel, Switzerland). Before the qPCR, the DNA extracts were diluted at a final concentration of 4 ng/µl with RNase-free water. A reference sample was generated using a mixture of DNA derived from five different random samples. The real-time qPCR was performed to measure the relative abundance of several microbial species and groups commonly present in rumen fluid (i.e. bacteria, archaea) and important for the rumen fermentation processes (Tapio et al., 2017; Lan and Yang, 2019). The PCR was performed using the KAPA SYBR FAST Universal Kit (Roche, Basel, Switzerland). The primers (Table 1) were used at a final concentration of 200 nmol/l with a PCRmax real-time PCR device (PCRmax, Staffordshire, UK). The amplification profile included an activation step of 5 min at 95°C followed by 40 cycles of a two-step amplification step (5 s at 95°C and 20 s at 60°C). The percentage of each rumen microbe considered in relation to the total bacterial 16S ribosomal DNA (determined by amplification with 16v3 primers) was calculated for the reference sample using the formula previously described (Brinkhaus et al., 2016). For all the other samples, an induction fold was calculated relative to the abundance in the reference sample using a ΔΔCt method with efficiency correction (Pfaffl, 2001) and the EcoStudy software

(PCRmax, Staffordshire, UK). The induction fold was then multiplied by the percentage calculated for the reference sample.

Table 1. List of primers used for real-time qPCR.

Target	Primers	Sequences (5'-3')	Size (bp)
16v3	Forward	CCTACGGGAGGCAGCAG	193
	Reverse	ATTACCGCGGCTGCTGG	
Butyrivibrio fibrisolvens	Forward	ACCGCATAAGCGCACGGA	65
	Reverse	CGGGTCCATCTTGTACCGATAAAT	
Fibrinobacter succinogenes	Forward	GTTCGGAATTACTGGGCGTAAA	121
	Reverse	CGCCGTCCCCTGAACTATC	
Ruminococcus albus	Forward	CCCTAAAAGCAGTCTTAGTTCG	176
	Reverse	CCTCCTTGCGGTTAGAACA	
Ruminococcus flavefaciens	Forward	TGTCCCAGTTCAGATTGCAG	171
	Reverse	GGCGTCCTCATTGCTGTTAG	
Selenomonas ruminantium	Forward	TGCTAATACCGAATGTTG	237
	Reverse	GCTTTCGCCCATTGCGGA	
Prevotella	Forward	CCAGCCAAGTAGCGTGCA	152
	Reverse	TGGACCTTCCGTATTACCGC	
Methanobrevibacter	Forward	TATTCACCGCGCGATTGTGAC	190
	Reverse	ACGACGGTAGGTCCGTATGC	

Note: 16v3: V3 region of 16S rRNA.

Statistical analysis

Data were analysed by ANOVA with RStudio software (version 4.0.5) using linear mixed-effects regression (Lme4) models (Bates et al., 2014). Residuals were checked for normality and homoscedasticity. If the data were not normally distributed, they were analysed with the non-parametric Kruskal–Wallis test. Multiple comparisons were performed using the pairwise Wilcoxon comparison post hoc test with the Benjamini–Hochberg *P*-value correction method. The effect of the treatment was used as a fixed factor, whereas the effect of the run was used as a random factor. Differences were considered significant if $P<0.05$. Data were reported as least squares means and pooled standard error of the mean (SEM).

Results

Effects on rumen fermentation parameters

The pH of the liquid phase samples after incubation was on average 7 ± 0.6 with no significant differences between the control and treatments ($P>0.05$, data not shown). As reported in Table 2, total gas production per unit of dietary DM, IVOMD and the amount of OM digested in 24 h (dOM) were

decreased by 10% on average with all the treatments (*P*<0.001), except for GA 75. The gas chromatography results showed that CH_4 production per unit of dietary DM significantly decreased with EA 150 (–20%, *P*<0.001) and EA+GA (–25%, *P*<0.001) treatments. These two treatments significantly reduced CH_4 production per unit of dOM (both –15%, *P*<0.001) and CH_4-to-SCFA ratio (both –25%, *P*<0.001). CO_2 production per unit of dietary DM significantly decreased (*P*<0.001) with all the treatments (except for GA 75), but the rate of decrease was lower if compared to the decrease of CH_4 production. The latter was also confirmed by the results of the CH_4/CO_2 ratio (*P*<0.001). Ammonia production per unit of dietary DM significantly decreased after the treatments EA 150 (–13%, *P*<0.001) and EA+GA (–20%, *P*<0.001). As seen above, EA 150 and EA+GA were the most effective treatments for all results of the study.

Table 2. Comparison of selected rumen fermentation parameters between control and treatments.

Item	CTR	EA 75	EA 150	GA 75	GA 150	EA+GA	SEM	*P*-value
Total gas/DM (ml/g)	52.3^{c}	46.5^{ab}	45.3^{a}	51.1^{c}	47.5^{b}	46.6^{ab}	0.7	$2.2*10^{-16}$
IVOMD (g/kg)	63.5^{c}	58.4^{ab}	57.3^{a}	62.5^{c}	59.3^{b}	58.5^{ab}	0.6	$2.2*10^{-16}$
dOM (mg/24 h)	117.5^{c}	108.0^{ab}	106.1^{a}	115.6^{c}	109.7^{b}	108.1^{ab}	1.1	$2.2*10^{-16}$
CH_4/DM (ml/g)	30.6^{d}	26.7^{b}	24.5^{a}	29.1^{c}	25.9^{b}	23.7^{a}	2.2	$2.2*10^{-16}$
CO_2/DM (ml/g)	170.4^{bc}	162.5^{ab}	155.8^{a}	176.3^{c}	161.1^{a}	157.1^{a}	10.0	$2.2*10^{-16}$
CH_4/dOM (ml/g)	66.4^{d}	59.7^{b}	56.9^{a}	61.3^{c}	59.2^{b}	55.9^{a}	2.6	$5.9*10^{-4}$
CH_4/CO_2 (ml/l)	178.6^{d}	164.1^{c}	156.5^{b}	164.5^{c}	160.1^{c}	151.6^{a}	8.4	$2.2*10^{-16}$
CH_4/SCFA (mmol/ml)	110.2^{d}	90.8^{b}	84.6^{a}	99.4^{c}	90.6^{b}	83.9^{a}	3.8	$2.2*10^{-16}$
Ammonia (mmol/l)	11.7^{c}	12.1^{c}	10.1^{a}	12.3^{c}	10.9^{b}	9.5^{a}	1.9	$2.2*10^{-16}$

Note: Values are presented as mean and standard error of the mean (SEM). Numbers with different superscripts (^{a,b,c,d}) in a row differ significantly. CTR = control; DM = dry matter; EA = ellagic acid; GA = gallic acid; IVOMD = *in vitro* organic matter digestibility; dOM = digestible organic matter.

Total SCFA production decreased by approximately 10% with all the treatments (*P*<0.001). However, total SCFA production decreased to a lesser extent than CH_4/DM, as can be inferred by the production of CH_4 per moles of total SCFA produced (CH_4/SCFA, Table 2), which was decreased by 24% (*P*<0.001) by EA 150 and EA+GA. Furthermore, slight differences were observed for the ruminal SCFA profile, excluding acetic, isovaleric and valeric acid (*P*>0.05) (Table 3).

Table 3. Comparison of total SCFA production and of the relative abundance of single SCFA species.

Item (n_i/n_{tot})	CTR	EA 75	EA 150	GA 75	GA 150	EA+GA	SEM	*P*-value
Total SCFA	99.98^{c}	92.99^{b}	86.93^{a}	93.02^{b}	86.94^{a}	86.95^{a}	0.02	$1.9*10^{-11}$
Acetic acid	64.94	65.09	65.06	65.96	65.78	65.88	0.86	$4.9*10^{-1}$
Propionic acid	20.44^{c}	20.22^{bc}	20.41^{c}	19.64^{a}	20.09^{abc}	19.80^{ab}	0.30	$9.2*10^{-8}$
Isobutyric acid	0.89^{c}	0.84^{ab}	0.82^{a}	0.85^{b}	0.84^{ab}	0.81^{a}	0.11	$2.2*10^{-16}$
Butyric acid	10.72^{ab}	10.94^{c}	10.87^{bc}	10.61^{a}	10.61^{a}	10.77^{ab}	0.45	$2.4*10^{-12}$
Isovaleric acid	1.48	1.37	1.32	1.41	1.40	1.32	0.27	$2.4*10^{-1}$
Valeric acid	1.53	1.53	1.54	1.70	1.71	1.51	0.08	$8.6*10^{-2}$

Note: Values are expressed as molar fraction (n_i/n_{tot}) = mol single gas/mol total SCFA. Values are presented as mean and standard error of the mean (SEM). Numbers with different superscripts (^{a,b,c,d}) in a row differ significantly. CTR = control; EA = ellagic acid; GA = gallic acid; SCFA = short chain fatty acids.

Effects on selected rumen microbes

The treatments did not affect the bacterial and the protozoal count (*P*>0.05) (Suppl. Figure 2). The results of the quantitative real-time qPCR showed that EA and GA differently modulated the relative abundance of the bacterial species and groups evaluated. Indeed, *Butyrivibrio fibrisolvens* and *Ruminococcus albus* are two cellulolytic bacteria, and their relative abundance was not significantly modulated by the treatments with EA and GA (*P*>0.05). Instead, there were significant differences in the relative abundance of the cellulolytic *Ruminococcus flavefaciens*, the hemicellulolytic *Prevotella* and the amylolytic *Selenomonas ruminantium* between the control and treatments. In particular, EA 75 significantly decreased the abundance of *R. flavefacie*ns, whereas EA 150 significantly increased the abundance of *S. ruminantium* and of the bacterial group *Prevotella.* Furthermore, the relative abundance of the methanogenic archaeal group Methanob*revibacter* was not significantly altered by the treatments (*P*>0.05).

Table 4. Relative abundance of rumen microbes in rumen fluid after *in vitro* fermentation.

Rumen microbes (mRNA induction fold)	Treatment						SEM	*P*-value
	CTR	EA 75	EA 150	GA 75	GA 150	EA+GA		
Butyrivibrio fibrisolvens	1.00	0.87	0.85	0.92	0.88	0.97	0.07	$2.7*10^{-2}$
Fibrobacter succinogenes	1.00^{ab}	0.91^{ab}	1.07^{b}	0.84^{a}	0.98^{ab}	0.92^{ab}	0.14	$1.1*10^{-2}$
Ruminococcus albus	1.00	0.91	0.95	0.92	1.01	0.94	0.50	$3.6*10^{-1}$
Ruminococcus flavefaciens	1.00^{b}	0.77^{a}	0.84^{ab}	0.84^{ab}	1.00^{b}	0.98^{ab}	0.11	$6.0*10^{-4}$
Selenomonas ruminantium	1.00^{a}	1.45^{ab}	1.60^{b}	1.36^{ab}	1.24^{ab}	1.29^{ab}	0.45	$1.7*10^{-3}$
Prevotella	1.00^{a}	1.08^{ab}	1.15^{b}	1.04^{ab}	0.98^{a}	1.06^{ab}	0.10	$1.3*10^{-2}$
Methanobrevibacter	1.00	1.04	1.01	1.05	1.13	1.23	0.31	$5.2*10^{-2}$

Note: CTR = control; EA = ellagic acid; GA = gallic acid. Numbers with different superscripts (^{a,b,c,d}) in a row differ significantly.

Discussion

Tannins can reduce the environmental impact of ruminant production in terms of CH_4 emissions and ammonia production (Orzuna-Orzuna et al., 2021). However, the impact of tannins on feed intake and nutrient utilisation is well known (Yanza et al., 2021). In this study, we assessed the effect of EA and GA on rumen fermentation using HGT. This is a valid method to determine the effect of plant extracts

or single molecules on rumen fermentation and offers a higher level of standardisation and reproducibility when compared to the *in vivo* condition (Jayanegara et al., 2012). Furthermore, since the duration of the experimental trial is 24 h, HGT is considered a short-term and fast approach, if compared to other long-term rumen simulation techniques, such as Rusitec (Deitmers et al., 2022). Here, we used single molecules of tannins rather than tannin plant extracts because plant extracts contain different tannin molecules, thus making difficult to assess which component exerts a specific effect. In this study, EA and GA alone or in combination at high doses were used to simulate a diet including natural extracts rich in hydrolysable tannins.

Gas production, digestibility of the diet and SCFA production

All the treatments, except for GA 75, decreased total gas production. The main reduction was exerted on CH_4 rather than CO_2, as observed with the CH_4/CO_2 ratio (Table 2). EA 150 and EA+GA exerted the most effective reduction of CH_4 production per unit of DM, dOM and total SCFA, whereas GA alone exerted a weaker effect than EA. So far, few studies have reported the effective CH_4-mitigating activity of EA (Wei et al., 2018), whereas the literature on GA is more consistent. Aboagye et al. (2019) reported a mild CH_4 decrease exerted by GA 15 mg/g DM when supplemented to beef cattle fed with a standard diet containing alfalfa silage. Wei et al. (2019) observed that adding GA up to 40 mg/g DM linearly decreased CH_4 production in a short-term *in vitro* rumen fermentation, whereas the GA effect was weaker when performing a long-term *in vitro* fermentation. Significant results were also observed using tannin plant extracts. Bhatta et al. (2009) observed reduced CH_4 emissions using chestnut, quebracho and mimosa extracts (alone or in combination) supplemented up to 250 mg/g DM. Similar results were also obtained by Hassanat and Benchaar (2012), who reported that quebracho extract supplemented at 100, 150 and 200 mg/g DM decreased CH_4 emissions by 23%, 34% and 40%, respectively, when compared to the control. Furthermore, Foggi et al. (2022) observed a reduced CH_4 production up to 15 mg/g DM by using chestnut and quebracho extracts, alone or in combination, at 20 mg/g DM. With the exception of the total SCFA and CO_2, in our study, EA 15 was more effective than GA 15 for all the parameters evaluated, whereas the co-treatment likely improved the effect of GA in terms of total gas production, CH_4 emissions and ammonia formation (Table 2). In a single study (Lotfi et al., 2020), it was reported that EA can be converted to GA in the rumen. Since EA can be potentially converted into GA, it can be hypothesized that GA is the responsible of the effect on rumen fermentation, even though overall action of EA is more effective than GA. In any case, the metabolic pathway linking EA and GA should be further investigated. The lower level of ammonia formation could be related to the complex formation between tannins and proteins, subtracting N from rumen degradation and increasing its ritention (Aboagye and Beauchemin, 2019). In Terranova et al. (2018),

several plant substrates supplemented at 167 mg/g DM reduced both CH_4 emissions and ammonia formation using HGT. The reduced CH_4 emissions and ammonia formation were also observed when tannin-rich plant extracts were *in vitro* supplemented to a high-forage and a high-concentrate diet (Jayanegara et al., 2020). The significant reduction of ammonia formation observed in this study (Table 2) could be also given by the average low CP content of hay used as a standard diet.

Concomitantly, tannin supplementation also influences the fermentability of a standard diet with potential consequences for SCFA production as well (Aboagye and Beauchemin, 2019). In this study, EA and GA caused an 10% average reduction of total SCFA production (Table 3). Furthermore, the CH_4/SCFA ratio was significantly reduced (Table 2). As discussed for the CH_4/CO_2 ratio, the percentage reduction was lower for CH_4 than for total SCFA. Several plant extracts supplemented at 167 mg/g DM showed an average 10% reduced total SCFA production (Terranova et al., 2018). Tannin plant extracts supplemented at around 80 mg/g DM reduced the production of total SCFA by 14% (Jayanegara et al., 2015). When HT-source plant extracts were used as supplements, there was a negative correlation between increasing the HT-extract concentration and the total SCFA production (Bhatta et al., 2009; Hassanat and Benchaar, 2013; Foggi et al., 2022). Interestingly, Getachew et al. (2008) observed that GA supplemented at 50 and 100 mg/g DM did not impair the total SCFA production.

Furthermore, only GA 75 did not impair dOM and IVOMD (Table 3). The reduced digestibility of the organic matter could derive from the binding between tannins and macromolecules, such dietary carbohydrates, thus subtracting them from the degradation in rumen. In all the studies cited above, there was a linear correlation between a reduction in gas production, dOM, IVOMD (or *in vitro* dry matter digestibility), CH_4 emissions and total SCFA production. Hence, tannin supplementation influences rumen fermentation with a cascade effect. This explains why GA 75 did not impair dOM and IVOMD, since this treatment was less effective for the other parameters as well. Total SCFA production and OM digestibility are related to diet digestibility and nutrient availability. Therefore, their reduced level in response to EA and GA could partially explain the mitigation of CH_4 production observed in this study.

Microbial count and quantification of selected rumen bacteria

The bacterial and protozoal count did not reveal any quantitative differences between the control and the treatments (Suppl. Figure 2). This is in line with the literature, where it has been reported that the variation of protozoa abundance is not associated with CH_4 emissions (Guyader et al., 2014) and the level of dietary tannins (Jayanegara et al., 2012). However, some studies have reported reduced CH_4

emissions and a reduced level of protozoa after *in vitro* supplementation with dietary tannins, thus underlining the "subordinate" role of protozoa in methanogenesis, along with the major role of methanogen archaea (Bhatta et al., 2009; Morgavi et al., 2010; Sarnataro et al., 2020). In our study, a similar total microbial count between the control and the treatments also confirmed that the concentrations used were not toxic for the ruminal bacterial community. Despite there being no quantitative difference, the relative abundance of *R. flavefaciens, S. ruminantium* and *Prevotella* was modulated by EA and GA (Table 4). Surprisingly, only EA 75 decreased the abundance of *R. flavefaciens*. Contrastingly, a higher concentration of EA not only did not affect *R. flavefaciens* but also increased the relative abundance of *S. ruminantium* and *Prevotella*. A higher abundance of *Prevotella* was correlated with lower CH_4 emissions *in vivo* (Aguilar-Marin et al., 2020). It is possible that propionate, the main fermentation product of *Prevotella*, is an H_2 sink and can compete with methanogen archaea for the use of H_2 (Aguilar-Marin et al., 2020; Pereira et al., 2022). H_2 is used by methanogens to reduce CO_2 to CH_4 (Aboagye and Beauchemin, 2019). *R. flavefaciens* is one of the predominant cellulolytic rumen bacteria (Miron et al., 2001), and its ability to produce acetic and formic acids, H_2 and CO_2 is well known (Latham and Wolin, 1977). Therefore, the reduced CH_4 production in EA groups could be explained by the increased abundance of the hemicellulolytic *Prevotella* and decreased abundance of the cellulolytic *R. flavefaciens*. Even if not significant, a numerical difference in the relative abundance of *R. flavefaciens* was observed between the control and EA 15. Moreover, EA showed a stronger modulating effect on the ruminal bacterial community than GA.

Tannins can interact with the extracellular enzymes secreted and the cell walls of bacteria, leading to membrane disruption, detrimental effects on microbial metabolism and deprivation of substrates for microbial growth (Patra and Saxena, 2011). However, several species of tannin-tolerating bacteria have been identified (Patra and Saxena, 2011). The increased abundance of *S. ruminantium* in the EA 150 group could be explained by the metabolic characteristics of such bacteria. Members of *S. ruminantium* species are able to grow on tannic acid or condensed tannin as a sole energy source but cannot grow on GA as an energy source (Skene and Brooker, 1995). This could explain why the abundance of the microbial species and groups only increased in the EA group and not in GA-treated samples. *S. ruminantium* mainly ferments carbohydrates to lactate, propionate, acetate and CO_2. Its ability to produce H_2 seems limited, but it can strongly increase in the presence of methanogenic archaea (Scheifinger et al., 1975). Similar to *Prevotella*, *S. ruminantium* was associated with a higher production of propionate (Asanuma et al., 1999). Also, *Prevotella* species have been found to tolerate the presence of tannins in the culture medium (Patra and Saxena, 2009), justifying the effects of EA on *Prevotella* abundance. Even in short-term *in vitro* trials, the contrasting effects of tannins against bacteria differ for their ability to tolerate the presence of these phenolic compounds.

EA and GA did not significantly alter the relative abundance of *Methanobrevibacter,* one of the major methanogen archaea present in rumen (Table 4). Therefore, the obtained results suggested that a reduction in CH_4 production was achieved by modulating the relative abundance of fibre-degrading bacteria, such as *R. flavefaciens, Prevotella* and *S. ruminantium*, rather than a direct modulation of *Methanobrevibacter*. Indeed, fibre-degrading bacteria are H_2 producers; therefore, they indirectly sustain the methanogenic process because methanogens use H_2 to reduce CO_2 to CH_4 (Aboagye and Beauchemin, 2019). It is possible that EA and GA impaired the action of fibre-degrading bacteria, thus reducing CH_4 production.

Given the complexity of the rumen microbial community, it is necessary to further investigate the effects of EA and GA on the molecular processes occurring during rumen fermentation. In addition, it would be interesting to test if in a long-term *in vitro* rumen fermentation, a shift in the rumen microbiota from low to high tannin-tolerant bacteria could result in a more adapted ecosystem able to limit the detrimental effects of EA and GA observed in this study.

Conclusion

The EA 150 and EA+GA treatments altered the relative abundance of selected rumen microbial species and groups, lowering CH_4 production and ammonia formation but also affecting the total SCFA production and the *in vitro* digestibility of OM. The novelty of this work stands in how EA and GA influenced rumen fermentation. The effects of EA and GA were similar at 75 mg/g DM, but at 150 mg/g DM EA showed a stronger effect than GA. Furthermore, the EA+GA treatment showed similar effect to EA 150, suggesting a potential mutual effect of EA and GA that need to be further investigated. To conclude, the reduced CH_4 production was a consequence of the altered mechanisms of H_2 production by EA and GA. These results could lead the way to further analyses on the long-term effects of EA and GA on rumen fermentation. Further, a metagenomic investigation of the perturbation of the rumen microbial community by EA and GA could deepen and strengthen our current findings, to assess whether the decreased CH_4 emissions and ammonia formation and the decreased digestibility could be dissociated.

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Section B – Article 2

Hydrolysable tannin metabolites modulate rumen microbiota and enteric methane production *in vitro*

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Abstract

Ruminant production must meet the growing demand for animal-source food while reducing the impact on the environment. The dietary supplementation with tannins can mitigate methane production by affecting enteric fermentation. Therefore, we investigated the effect of gallic acid (GA) and ellagic acid (EA) on rumen microbiota by using them as supplements of a control diet (CTR, 10 g DM/day) in an 8-fermenter rumen simulation system (Rusitec) for 10 days. Three experimental conditions were investigated (mg/g DM): i) GA 75, ii) EA 75, iii) EA 75 + GA 75. Total gas production was not significantly altered, whereas EA and EA+GA (EA+) reduced daily methane production along with total SCFA production and nutrient degradation. The EA+ treatments reduced the abundance of the fibrolytic bacteria *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* as well as increased the nitrate-reducing *Selenomonas ruminantium*. Also, The EA+ treatments altered the level of urolithins A and B in rumen. High-throughput sequencing showed that EA+ treatments reduced bacterial richness, evenness, and diversity, whereas a limited impact was observed on archaea. The EA+ treatments altered the taxonomic composition of microbial rumen communities differently compared to EA- treatments (CTR and GA). Bacterial communities were dominated by *Megasphera elsdenii* after EA+ treatments, while archaeal communities were always dominated by the *Methanomethylophilaceae* family. Therefore, the modulation of rumen microbiota by EA+ treatments led to a decreased methane production, with GA being less effective on methane. Nevertheless, GA showed a lower impairing effect on SCFA production nutrient degradation in rumen than EA. We concluded that the dietary supplementation with tannins is a valid option to affect rumen

microbiota and in turn enteric fermentation, but caution must be paid to the extent of supplementation, in order to reduce the detrimental effect of tannins.

Introduction

Ruminants are able to convert low-quality plant fibres into energy and proteins through the enteric fermentation processes exerted by the microbial community in the rumen. The enteric fermentation of organic matter (OM) also causes the emission of greenhouse gases (GHG), of which methane (CH_4), carbon dioxide (CO_2), and hydrogen (H_2) are the main ones (Gerber et al., 2013; Lan and Yang, 2019). Most of the attention is given to CH_4 because it has a shorter atmospheric lifespan but a higher global warming potential than CO_2 (Grossi et al, 2019). The share of CH_4 emitted from the livestock sector contributes 30% to global anthropogenic CH_4 emissions, 17% to global food system GHG emissions, and 5% to global GHG emissions. Of the global livestock CH_4 emissions, 88% is contributed by enteric fermentation (Arndt et al., 2022). Strategies to reduce CH_4 emission from enteric fermentation are needed for a more sustainable livestock production. The CH_4 is a physiological product of rumen methanogenic archaea, therefore the mitigating strategies should modulate rumen microbiota and in turn enteric CH_4 by targeting the CH_4 production without impairing the energy production and the well-being of the animal (Beauchemin et al., 2022). One strategy is the use of tannins as dietary supplements, because they are known to affect enteric fermentation in rumen (Aboagye and Beauchemin, 2019).

Tannins are plant secondary metabolites able to bind macromolecules, such as dietary proteins and microbial enzymes. They can reduce CH_4 production by directly targeting the methanogenic pathways of archaea or indirectly by affecting the action of feed fermentation by other rumen microorganisms, such as bacteria and protozoa, whose H_2 production is essential for the methanogen-mediated CH_4 production (Cardoso-Gutierrez et al., 2021). If properly balanced, tannins can reduce CH_4 emissions and ammonia (NH_3) formation, while avoiding the detrimental effects of tannins on the fermentation activity of rumen microbiota (Hassan et al., 2020). Tannins are classified as condensed tannins (CT) and hydrolysable tannins (HT). The CT have a higher molecular weight than HT, increasing their ability to bind macromolecules compared to HT (Aboagye and Beauchemin, 2019). Tannin-containing plant extracts such as chestnut and quebracho were used for the in vitro screening of the modulating effect of tannins on rumen microbial fermentation (Foggi et al., 2022; Battelli et al., 2023). So far, the mode of action at molecular level of individual tannin components in rumen has never been fully reported, and notably the specific effects of different tannins need to be further investigated.

In the present study, we focused on individual tannin molecules, in order to better characterize the potential effect of each tannin molecule tested. In a previous study, we tested the effects of two individual HT metabolites, ellagic (EA) and gallic (GA) acid alone or in combination (EA+GA) using a 24-hour Hohenheim Gas Test (HGT) rumen fermentation trial. The treatment with EA 75 mg/g DM, more than the GA 75 mg/g DM, reduced CH_4 production per unit of dietary DM, digestible OM (dOM), CO_2 and SCFA and NH_3 formation but at the same time lowered the nutrient degradation in rumen and the production of SCFA. The combined treatment EA+GA (both at 75 mg/g DM) significantly reduced CH4 production but also the rumen feed degradation (Manoni et al., 2023). Here, the next step was to observe the effect of the individual and combined treatment of EA and GA on rumen microbiota, because a modulation of the community structure of the microbiota determines an alteration of feed fermentation processes and consequently of gas production. To address this, we used an in vitro model called rumen simulation technique (Rusitec). This is a well-established, continuous and standardized in vitro model useful for setting up experiments operating for more than 24 hours and lasting for several days (in our case 10 days). In comparison to Hohenheim Gas Test, it allows for a better characterization of the persistency and consistency of the investigated effects (García-González et al., 2010). If coupled with high-throughput sequencing, the Rusitec allows to get a detailed overview about the kinetics of alteration of the different rumen microbial populations (Wetzels et al., 2018). In relation to rumen microbiota, some studies reported that the interaction between HT and rumen microbiota leads to the production of HT-secondary metabolites through the action of rumen microbes. An example are urolithins, associated with the metabolism of ellagitannins. These metabolites are considered as potential executors of the action usually ascribed to tannins at rumen level, but there has not yet been clear evidence for this (González-Barrio et al., 2012; Espìn et al., 2013; Lotfi, 2020).

Therefore, the aim of this study was to investigate the effect of EA and GA by applying a 10-day Rusitec trial and focusing on: i) the modulation of the community structure of rumen bacteria and archaea, ii) the potential influence of tannins on the rumen microbial metabolism, in particular CH_4, NH_3, SCFA, and nutrient degradation, iii) the correlation between gas production and specific rumen microbial populations, and iv) the production of HT-secondary metabolites named urolithin A (UroA) and urolithin B (UroB) all over the 10 days of experiment.

Materials and methods

Experimental design, reagents and incubated materials

Rusitec, described in detail by Soliva and Hess (Soliva and Hess, 2007), was used for the incubation of three treatments and one control in three consecutive experimental runs. Each run lasted for 10 days,

with day 1 to day 5 to let the system reach a steady-state condition, and day 6 to day 10 for sampling and data collection. A basal diet consisting of 10 g dry matter (DM) of ryegrass hay and barley concentrate (7.5:2.5 ratio) was added to each fermenter every day. The ryegrass hay was ground to pass through a 5 mm sieve, whereas the barley concentrate was ground to a particle size of 1 mm using a centrifugal mill (Model ZM 200, Retsch GmbH, Hann, Germany). The nutrient composition of the basal diet was (g/kg DM) 964 organic matter (OM), 36 ash, 109 crude protein (CP), 376 crude fibre (CF), 751 neutral detergent fibre (NDF), 417 acid detergent fibre (ADF), and 19 ether extracts (EE). The basal diet, without any supplementation, was used as the control substrate (CTR). For the treatments, the CTR was supplemented with gallic acid (GA) and ellagic acid (EA), alone or in combination. GA and EA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity level was ≥ 98.5% for GA and ≥ 95% for EA. The four treatments were i) the basal diet alone as control (CTR) and the CTR supplemented with 75 mg/g DM of ii) GA, iii) EA or iv) both EA and GA (EA+GA).

Rumen fluid collection

Animal manipulations were performed according to the Swiss guidelines for animal welfare (Cantonal Veterinary Office of Zurich; approval number ZH113/18). Cows were fed with 17.8 kg DM/day of a total mixed ration (TMR) composed of (% DM) grass silage (48%), maize silage (20%), sugar beet pulp (17%), hay (8%), concentrate (8%) and mineral supplement (0.2%). For each run, rumen fluid was collected before the morning feeding from one of three fistulated, lactating Original Brown Swiss cows. The pH of the freshly taken rumen fluids ranged between 5.9 and 6.7. Preheated glass bottles with water at 40 °C were used to keep the rumen fluid warm during transport. The inoculation took place within 2 h after rumen fluid collection. Then, the rumen fluid was strained through four layers of medicinal gauze (pore size 1 mm) before the transfer into the fermenters.

Operation of the Rusitec

The Rusitec consisted of eight 1 L fermenters. At the beginning of each of the three runs, the fermenters were filled with 800 ml of strained rumen fluid, collected from one of the three different cows involved in the study (Soliva and Hess, 2007), and 100 ml of artificial saliva, which was composed of 9.80 g/L $NaHCO_3$; 4.67 g/L $Na_2HPO_4 \times 2H_2O$; 0.47 g/L NaCl; 0.57 g/L KCl; 0.05 G/L $CaCl_2 \times 2H_2O$; 0.13 g/L $MgCl_2 \times 2H_2O$ (McDougall, 1948). The components of artificial saliva were dissolved in distilled water. The fermenters were located into a heated water bath maintained at 39.5 °C. The incubation fluid in the fermenters was slowly moved up and down by an electric motor (six times per minute). The diet (CTR, GA, EA, EA+GA) was added daily to each fermenter into nylon bags (70 × 140 mm, pore size 100 μm). Each of the four treatments was allocated in duplicate to the eight fermenters in a completely

randomized design. The experiment was repeated in three different runs. On day 1, two feed bags were administered to each fermenter, one containing the respective experimental diet and one containing about 40 g fresh matter of solid rumen content. On day 2, each fermenter was opened and the bag with fresh matter was removed, squeezed and washed in artificial saliva. The liquid fractions of the washings were returned to each fermenter, and the removed bag was then replaced with a new bag containing the experimental diet, for a total of two bags per fermenter. Each feed bag was incubated for 48 h and then substituted with a new one containing the same diet. Each fermenter was flushed with N_2 gas for 3 min to maintain anaerobic conditions after the daily substitution of the feed bags. The flow of artificial saliva to the fermenters was continuous and about 400 ml per day, resulting in a dilution rate of the incubation fluid of about 40% per day. The overflown incubation fluid was collected in glass flasks and immediately frozen at -20 °C to stop fermentation. A representative summary of the experimental set-up and the sampling scheme was reported in the Supplementary Figure S1.

Sample collection and laboratory analyses

Feed samples were lyophilized (Delta 1-24 LSC, Christ, Osterode, Germany) and ground (Brabender mill with titanium blades, Brabender, Duisburg, Germany). Dry matter and ash contents were measured gravimetrically by oven drying (prepASH 229, Precisa, Dietikon, Switzerland) for 3 h at 105 °C and subsequently incinerating at 550 °C for 4h. The difference between DM and ash was defined as OM. The contents of NDF and ADF (NDF: method ISO 16472:2006; ADF: ISO 13906:2008) were determined using Fibertherm (Gerhardt, Königswinter, Germany). The total N content of the feed samples were determined using the Kjeldahl method (AOAC International, 1995; method 988.05). To calculate CP contents, the N content was multiplied by 6.25. Ether extract content was analyzed by extraction following hydrolysis (ISO 6492:1999).

Every day, 3 h before the replacement of the feed bags, 10 mL of incubation fluid were collected directly from each fermenter. These samples were analysed for pH, redox potential and NH3 using a potentiometer (pH: model 913, Metrohm; redox: model 632, Metrohm; NH3: model 713, Metrohm, Herisau, Switzerland) equipped with electrodes. Additional samples were mixed with H2SO4 50% (m/v) to stabilise the samples and frozen at -20 °C for SCFA analysis, as described in Manoni et al. (2023). Furthermore, the samples were used for microbial counting at the microscope. Before counting, the samples were diluted 1:11 and 1:100 for protozoa and bacteria with Hayem solution, respectively. Protozoa were counted using a Neubauer haemocytometer (0.100 mm depth, Blau-Brand), whereas bacteria were counted using a Bürker haemocytometer (0.02 mm depth, Blau-Brand, Wertheim, Germany). On day 2, day 6 and day 10, two additional samples of 10 mL were collected from each

fermenter and stored at -80 °C. One sample was used for DNA extraction, PCR and high-throughput sequencing (HTS). The other sample was used to measure the level of urolithin A (UroA) and urolithin B (UroB), two major secondary metabolites of EA produced by the rumen microbial fermentation (Lotfi et al., 2020). The days of sampling were chosen to have a picture of the variation occurring in the microbial community and the related level of secondary metabolites produced (i.e. urolithin A and B) in the initial (day 2), middle (day 6), and final stage (day 10) of Rusitec incubation.

The daily overflow of incubation fluid from each fermenter was recorded, to adjust the flow rate of artificial saliva among fermenters in real-time. Fermentation gas was collected in gas-tight aluminium bags (TECOBAG 8 L, PETP/ AL/PE: 12/12/75 quality; Tesseraux Container GmbH, Bürstadt, Germany). Gas production was analysed every day by collecting the gas samples from the bags and injecting into a gas chromatograph (GC-TCD 6890 N, Agilent Technologies, Wilmington, NC, USA), as described in Manoni et al. (2023). The total amount of fermentation gas produced was quantified by the water displacement technique (Soliva and Hess, 2007). The feed bags removed every 48 h from the fermenters were washed 30 min with cold water and without detergent in a domestic washing machine, squeezed and stored at -20°C. The feed bags were lyophilised for 48 h, allowed to air dry for 24 h and weighed. Later, the feed residues contained in the bags from day 6 to day 10 were mixed, ground to pass a 0.5 mm sieve and analysed for their analytical contents as previously described for the feed samples. The analytical composition was then used to determine the degradation of the feed components, calculated as the amount of material disappeared from the feed bag after 48 h of incubation. The apparent degradation was expressed in percentage as the ratio of g degraded/g incubated feed.

Measurement of urolithin A and urolithin B

Sample extraction

All chemicals, reagents, as well as the UroA and UroB standards were of analytical grade and purchased from Merck (Darmstadt, Germany). The samples were extracted according to the protocol of Garcia-Villalba et al. (2017). Briefly, an aliquot of 2 mL incubation fluid was mixed with 5 mL of ethyl acetate acidified with 1.5% formic acid. The mixture was vortexed for 2 min and centrifuged at 2500 g for 10 min. The organic phase was separated and evaporated by a vacuum rotary evaporator (Heidolph, Schwabach, Germany) at 35 °C. The dry sample was then re-dissolved in 200 µL of 0.1% formic acid in water: methanol (90:10) and 5 µL of 100 µg mL-1 of internal standard (6,7-dihydroxycoumarin) was added. Then, the extract was diluted 100 times before injection in the UPLC-HRMS system.

UPLC-HRMS analysis

The analysis was carried out by an UPLC-HRMS system made by a Vanquish device (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Thermo Orbitrap™ Exploris 120 (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a heated electrospray ionization (HESI) source. A Raptor ARC-18 5 µm, 150 × 2.1 mm column (Restek, Bellefonte, PA, USA) was used for the chromatographic separation. Mobile phases A (0.1% aqueous formic acid) and B (MeOH) were mixed during the gradient, which started with 5% B kept for 1 min, increasing to 95% in 7 min and remaining until the 11th min. After 0.5 min, the initial conditions were re-established until the 15th. The flow was set at 0.3 mL min^{-1}. With regard to the detector, the capillary and vaporizer temperatures were set at 330 and 280 °C, respectively, the sheath and auxiliary gas at 35 and 15 arbitrary units (AU) and the electrospray voltage at 3.50 kV in negative mode. The full-scan (FS) acquisition was combined with parallel reaction monitoring (PRM) mode for the confirmatory response based on an inclusion list. The FS worked with a resolution of 60,000 FWHM, a scan range of 150–400 m/z, a standard automatic gain control (AGC), an RF lens % of 70 and an automatic maximum injection time. The PRM acquisition operated at 15,000 FWHM, with a standard AGC target, an automatic maximum injection time and scan range mode and an isolation window of 1 m/z. Fragmentation of the precursors was optimized with a two-step normalized collision energy (40 and 60 eV). The precursor of UroA was the ion at 227.0350 m/z and that of UroB at 211.0401 m/z, instead the main fragments were at 159.0449 and 167.0501 m/z for UroA and UroB, respectively. The software used was XcaliburTM 4.5 (Thermo Fisher Scientific, Waltham, MA, USA). The limit of quantification (LOQ) was 5 ng mL^{-1}.

Real-time qPCR

A panel comprehending the major rumen bacteria involved in the enteric fermentation of dietary polysaccharides and the major methanogenic archaea was explored by quantitative PCR (qPCR) (Morgavi et al., 2010; Chaucheyras-Durand and Ossa, 2014). The incubation fluid samples collected at day 2, day 6 and day 10 were kept at –80 °C for the subsequent DNA extraction and qPCR. The DNA was extracted using QIAMP Fast DNA Stool Mini Kit (Qiagen, Hombrechtikon, Switzerland), following the method reported in Böttger et al. (2019). Briefly, 2 ml of incubation fluid samples were centrifuged at 6500 × g for 30 min at 4 °C to collect and resuspend the pellet using the Inhibitex buffer. This solution was heated at 90 °C for 5 min. After vortexing and centrifuging at 16,000 × g for 1 min, 200 µL of the supernatant were collected to measure the concentration of DNA using the NanoDrop 1000 spectrophotometer (Witec AG, Luzern, Switzerland), and the length of the extracted DNA fragments was measured with capillary electrophoresis with a QSep100 device (Bioptic, New Taipei City, Taiwan). Then, the real-time qPCR was performed as reported in detail in Manoni et al. (2023). The relative

abundance was measured in relation to the abundance of the total bacterial 16S ribosomal DNA, used as reference sample and measured by the amplification with the 16v3 primers, as previously described (Pfaffl, 2001; Brinkhaus et al., 2016).

High-throughput sequencing

DNA extracts of rumen fluids, as well as of samples from days 2, 6, and 10, were used for the assessment of bacterial and archaeal communities using high-throughput sequencing. Variable regions 3 and 4 of the ribosomal RNA gene sequence were amplified using primers 341F (5'-CCTAYGGGDBGCWSCAG-3') and 806R (5'- GGACTACNVGGGTHTCTAAT-3') (Frey et al. 2016) for bacteria, as well as Arch349F (5'- GYGCASCAGKCGMGAAW-3') and SSU666ArR (5'-HGCYTTCGCCACHGGTRG-3') (Bahram et al., 2019) for archaea at a concentration of 1 µM in PCR reactions of 25 µl. TruSeq adapter sequences were appended to the primers to allow for subsequent sequencing library construction. Three PCR reactions with 12 ng of DNA in each were made for all samples and the two markers. These PCR triplicates were pooled prior to sequencing. PCR consisted of an initial denaturation step at 95 °C for 2 minutes, followed by cycles of denaturation at 94 °C for 40 s, annealing of bacteria or archaea PCR-primer pairs at 56 °C or 66 °C for 40 s, and an elongation step at 72 °C for 1 min. A final elongation step was performed at 72 °C for 10 minutes. Amplifications were made with 25 cycles for bacterial and 40 cycles for archaeal markers. Library preparation and NextSeq Illumina sequencing were performed at the Functional Genomics Centre of the University of Zürich. Raw amplicon sequences were quality filtered and grouped into amplicon sequence variants (ASVs) using a pipeline largely based on vsearch (Herzog et al., 2019), which included several filtering steps such as primer pruning, removal of sequences with a maximum expected error bigger than 1, chimera removal, and target verification using metaxa version 2.2.3 (Bengtsson-Palme et al., 2015). For the taxonomic assignment, ASVs were compared to release 214 of the genome taxonomy database (Parks et al. 2021) using an RDP classifier implemented in mothur version 1.47.0 (Schloss et al., 2009). Non-target sequences, i.e., non-bacterial or non-archaeal, as well as chloroplast or mitochondrial sequences, were removed prior to further analyses.

Statistical analysis

The results were analyzed by repeated measures ANOVA using linear mixed-effects regression models (Lmer) implemented in Rstudio (version 4.0.5). All models contained the treatment and the day as fixed effects, while the run and the fermenters were considered as random effects. For pairwise comparisons, a modified Tukey test for multiple comparisons of means, the Sidak function was

performed. Statistical means and standard error of the means (SEM) were calculated with the lsmeans function from the package emmeans. Residuals of Lmer models were checked for normality and homoscedasticity. If those conditions were not respected, the independent value was transformed or analysed with the package nparLD (Noguchi et al., 2012), useful for the analysis of longitudinal data in factorial experiments. The main packages used were: lme4 (V. 1.1-27.1) (Bates and Maechler, 2009), lsmeans (V. 2.30-0) (Lenth and Lenth, 2018), multcomp (V. 1.4-18) (Hothorn et al., 2016), and nparLD (V. 4.2.2) (Noguchi et al., 2012). For the analysis of high-throughput sequencing data, the distributions of ASV richness, Simpson evenness, and inverse Simpson indices of bacterial and archaeal communities were first evaluated for outliers and normality using functions identify_outlier and shapiro_test of the R package rstatix version 0.7.2 (Kassambara, 2023). These checks revealed that the assumptions for ANOVA were met. To test differences of these bacterial and archaeal diversity measures among treatments and over time, we used a two-way repeated measures ANOVA (function anova_test in rstatix) followed by pairwise t-tests with Benjamini-Hochberg p-value adjustments (function pairwise_t_test in rstatix). Unconstrained ordination using nonmetric multidimensional scaling (NMDS) implemented in the function metaMDS of the R package vegan version 2.6.4 (Oksanen et al. 2022). Venn diagrams were drawn using R package ggVennDiagram version 1.2.2 (Gao et al., 2021).

Results

Gas production and ammonia formation

Total gas production was not significantly altered by any treatment ($P>0.05$, Table 1). However, the average daily CH_4 production was significantly reduced by EA (-45%, $P<0.001$) and EA+GA (-60%, $P<0.001$) if compared to CTR, whereas GA had no effect ($P>0.05$, Table 1). The average daily CO_2 production followed the same trend of CH_4, with a significant reduction exerted by EA (-20%, $P<0.01$) and EA+GA (-14%, P<0,01). The reduction rate was higher for CH_4 than CO_2, as shown by the CH_4/CO_2 ratio ($P<0.001$, Table 1). Moreover, the average daily H_2 production was not significantly altered by the treatments ($P>0.05$). Instead, NH_3 formation was significantly reduced by all the treatments (Table 1), mainly by EA and EA+GA (-46% and -56% respectively, $P<0.001$) and to a lesser extent also by GA (-19%, $P<0.001$). The mean pH value during the 5 days of measurement was 6.9 for the CTR and GA treatment and was slightly increased to 7.0 and 7.1 in the EA+GA and EA treatments (data not shown). Figure 1 reported the measurements of specific parameters from days 6 to 10 that revealed treatment effects of variable strengths, i.e. with a significant interaction effect of treatment and day, including total gas (A), CH_4/SCFA (B), CH_4/CO_2 (C), and NH_3 (D).

Table 1. Total gas production, CH_4 (expressed as daily production and on OM and SCFA basis), CO_2, H_2, and NH_3 values for the different treatments with GA and EA. Data are expressed as the average values for the sampling period (day 6-10).

Parameter	Treatments				SEM	*P*-value
	CTR	GA	EA	EA+GA		
Total gas (ml/day)	4869	4971	4650	4801	140	0.12
CH_4 (ml/day)	175.5^{a}	152.1^{a}	97.0^{b}	71.1^{b}	7.5	<0.001
CH_4/SCFA (ml/g)	3050^{a}	2379^{b}	2070^{b}	1295^{c}	160	<0.001
CH_4/OM (ml/g)	18.2^{a}	14.7^{b}	9.4^{c}	6.4^{d}	0.7	<0.001
CO_2 (ml/day)	1321^{a}	1400^{a}	1063^{b}	1139^{b}	58	0.005
CH_4/CO_2	0.13^{a}	0.11^{b}	0.09^{c}	0.06^{d}	0.003	<0.001
H_2 (ml/day)	6.1	6.9	8.1	7.7	0.8	0.25
NH_3 (mmol/l)	6.1^{a}	4.8^{b}	3.2^{c}	2.6^{d}	0.3	<0.001

Abbreviations: CTR = control; EA = ellagic acid; GA = gallic acid; SEM = standard error of the mean. Means with different superscripts within a row are significantly different ($P < 0.05$).

Regarding the results of protozoal and bacterial count (Figure 1), the number of protozoa (E) was significantly decreased by EA and EA+GA ($P<0.001$), whereas it did not differ between GA and CTR ($P>0.05$). Furthermore, the difference of protozoa counts increased over time among treatments with and without EA ($P<0.05$). From day 8 onwards, the level of protozoa following EA and EA+GA treatments was significantly lower than the one of CTR and GA ($P<0.001$). Conversely, the number of bacteria (F) was higher in all treatments as compared to CTR, and remained stable over days 6 to 10 ($P>0.05$). In particular, GA ($P<0.001$) and EA ($P<0.05$) significantly increased bacterial counts in comparison to CTR, whereas EA+GA only showed an increasing tendency over CTR ($P=0.053$). In addition, the number of bacteria was higher in GA treatments as compared to EA ($P<0.05$) and EA+GA ($P<0.01$).

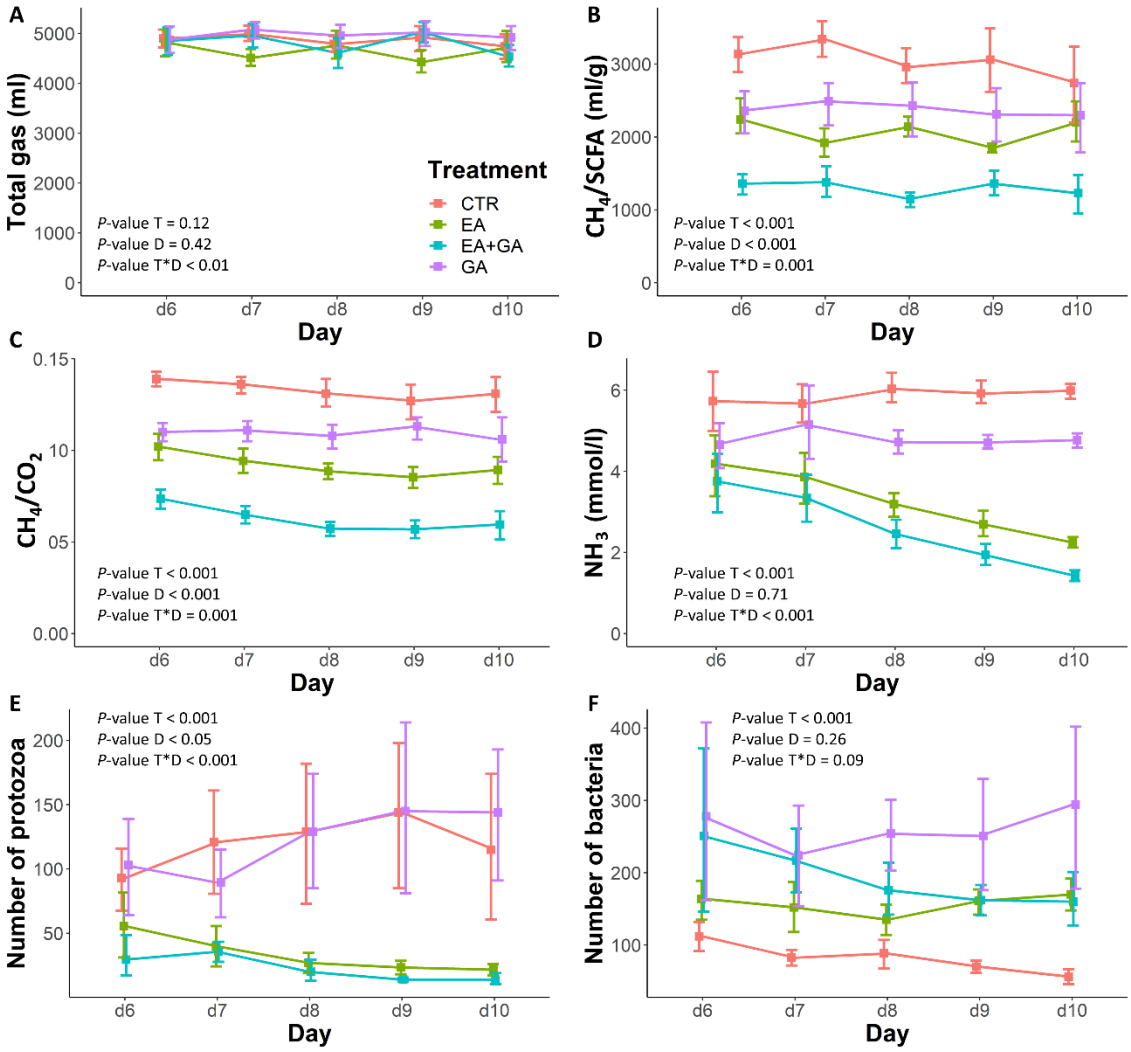

Figure 1. Kinetics of total gas production (1A), CH_4/SCFA (1B), CH_4/CO_2 (1C), and NH_3 (1D) in response to the treatments and during the 5 days of sampling period. Abbreviations: T = treatment; D = day.

SCFA production

Compared to CTR and GA, EA and EA+GA treatments significantly reduced the production of total SCFA (P<0.001). The average reduction of SCFA production was 26% (EA) and 16% (EA+GA) relative to CTR. The reduced total SCFA was also associated with an alteration of the SCFA profile (Table 2). By considering the major SCFA in rumen, which account for 95% of total rumen SCFA (Luo et al., 2015), acetic acid was significantly lowered only by EA (-13%, P<0.001), propionic acid was significantly decreased by all the treatments (P<0.001), but more by EA+GA (-29%), whereas butyric acid was significantly increased by both EA and EA+GA (P<0.05, +30% and +40%, respectively).

Table 2. Total short-chain fatty acids (SCFA), and single SCFA proportion in the incubation fluid of the different treatments with GA and EA. Values are the means for the whole experimental period.

SCFA	Treatments				SEM	*P*-value
	CTR	GA	EA	EA+GA		

Total SCFA (mol/g)	78.4^{a}	83.0^{a}	58.2^{b}	65.7^{b}	3.83	<0.001
Acetic acid (%)	49.1^{a}	52.9^{a}	42.8^{b}	48.2^{a}	1.29	<0.001
Propionic acid (%)	21.2^{a}	18.3^{b}	18.9^{b}	15.0^{c}	0.36	<0.001
Isobutyric acid (%)	0.8^{a}	0.7^{b}	0.6^{c}	0.5^{d}	0.04	<0.001
Butyric acid (%)	19.0^{b}	19.5^{b}	24.8^{a}	26.6^{a}	0.91	0.02
Isovaleric acid (%)	2.7^{a}	2.6^{a}	1.0^{b}	0.8^{c}	0.25	<0.001
Valeric acid (%)	7.3^{bc}	5.9^{c}	11.9^{a}	9.0^{b}	0.51	<0.001

Abbreviations: CTR = control; EA = ellagic acid; GA = gallic acid; SEM = standard error of the mean. Means with different superscripts within a row are significantly different (P < 0.05).

Nutrient degradation

The variation of gas and SCFA production exerted by the treatments was also related to an altered rate of nutrient degradation (Table 3). The EA and EA+GA treatments affected the fermentation of feed by significantly reducing the degradation of all the items reported in Table 3 (P<0.001). By considering the composition of the feed substrate, the fibre degradation is of notable interest, because EA and EA+GA affected CF (-56% and -36%, respectively), NDF (-43% and -47%, respectively), and ADF (-52% and -54%, respectively) more than the other feed components measured. Instead, no significant change was caused by the addition of GA. Since the main perturbation of the rumen fermentation of feed was exerted by EA and EA+GA, but not by GA alone, it is possible that GA affected rumen fermentation to a minor extent.

Table 3. Nutrient degradation of the diet following the 10-day fermentation period with GA and EA.

Nutrient degradation (g/g)	**Treatments**				**SEM**	**P-values**
	CTR	**GA**	**EA**	**EA+GA**		
DM	74.4^{a}	73.8^{a}	66.3^{b}	66.4^{b}	1.20	<0.001
OM	73.0^{a}	70.3^{a}	61.7^{b}	59.1^{b}	1.39	<0.001
CF	43.4^{a}	37.7^{a}	19.0^{b}	27.7^{b}	1.91	<0.001
NDF	51.5^{a}	45.2^{a}	29.3^{b}	27.4^{b}	2.22	<0.001
ADF	45.4^{a}	39.8^{a}	22.0^{b}	20.9^{b}	2.19	<0.001
CP	88.5^{a}	86.4^{ab}	82.1^{bc}	78.7^{c}	1.37	<0.001

Abbreviations: CTR = control; EA = ellagic acid; GA = gallic acid; SEM = standard error of the mean; DM = dry matter; OM = organic matter; CF = crude fibre; NDF = neutral detergent fibre; ADF = acid detergent fibre; CP = crude protein. Means with different superscripts within a row are significantly different (P < 0.05).

Level of urolithin A and urolithin B

The levels of UroA and UroB in rumen fluid were significantly modulated by the treatments EA and EA+GA (P < 0.001), whereas GA did not exert any modulating effect in both cases (P > 0.05). Specifically, the treatments EA and EA+GA showed similar trends by significantly increasing the level of UroA compared to CTR and GA, especially at day 6 (both P < 0.001). For UroB, the only significant difference

was observed at day 10, when EA and EA+GA significantly increased the level of UroB (P<0.05 and P<0.01, respectively) compared to CTR and GA (Figure 2).

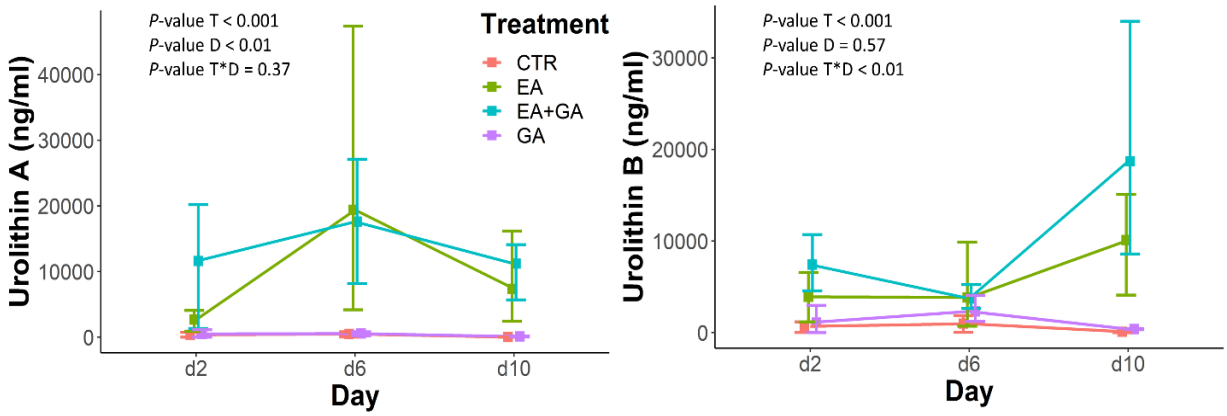

Figure 2. Level of urolithin A and urolithin B in the incubation fluid at day 2, day 6, and day 10. Abbreviations: CTR = control; EA = ellagic acid; GA = gallic acid; T = treatment; D = day.

Real-time qPCR

A real-time qPCR was applied as a first approach to measure the abundance of seven, selected microbial taxa primarily involved in enteric fermentation of feed and subsequent production of H2 and CO2 and subsequent CH4 production (Morgavi et al., 2010). The relative abundance of these selected taxa was measured at days 2, 6, and 10 (Table 4). EA and GA differently modulated the abundances of a panel of five bacterial species, one bacterial group (*Prevotella*) involved in rumen fermentation, and one archaeal genus (*Methanobrevibacter*). Among the six bacteria investigated, the treatments EA and EA+GA decreased the relative abundance of *Butyrivibrio fibrisolvens* (P<0.001), *Fibrobacter succinogenes* (P<0.001), and *Ruminococcus flavefaciens* (P<0.001) compared to CTR considering the same time points (day 6 and day 10), with the only exception of *Ruminococcus flavefaciens* at day 6 for EA, EA+GA, and CTR (P>0.05). The abundance of *Selenomonas ruminantium* was significantly increased only by EA+GA (P<0.001) at day 6 and day 10 if compared to CTR. Regarding the archaea *Methanobrevibacter*, the only significant difference observed was at day 6, when the treatment EA significantly increased the level of *Methanobrevibacter* (P<0.01) compared to CTR and GA (Table 4).

Table 4. Effect of the treatments on a selected panel of bacteria and archaea present in rumen. Data are expressed as average of the relative abundance at the time points day 2, 6, and 10.

Rumen microbes	CTR			GA			EA			EA+GA			SEM	P-value		
	2	6	10	2	6	10	2	6	10	2	6	10		T	D	T*D
B. fibr	0.39^{a}	0.30^{a}	0.35^{a}	0.39^{a}	0.15^{abc}	0.20^{abc}	0.3^{ab}	0.08^{bc}	0.01^{c}	0.32^{ab}	0.09^{bc}	0.01^{c}	0.07	<0.001	<0.001	<0.01
F. succ	0.23^{a}	0.52^{a}	0.20^{a}	0.26^{a}	0.36^{a}	0.13^{a}	0.22^{a}	0.009^{b}	$< 0.001^{c}$	0.21^{a}	0.003^{b}	$< 0.001^{c}$	0.03	<0.001	0.052	<0.001
R. alb	0.22	2.13	1.18	0.24	3.69	1.44	0.26	0.65	1.81	0.20	0.87	2.90	0.77	0.08	<0.01	0.25
R. flav	0.27^{ab}	0.17^{bcde}	0.20^{bcd}	0.31^{a}	0.15^{cde}	0.13^{de}	0.31^{a}	0.11^{def}	0.08^{ef}	0.25^{abc}	0.09^{def}	0.005^{f}	0.02	<0.001	<0.01	<0.01
S. rum	8.93^{d}	20.10^{cd}	21.10^{cd}	8.92^{d}	20.40^{cd}	24.60^{bcd}	11.30^{d}	42.80^{abc}	40.60^{abc}	8.45^{d}	57.20^{a}	46.40^{ab}	3.37	<0.001	0.13	<0.001

Methanobrev	2.51^{abc}	1.16^{bc}	1.69^{abc}	3.26^{a}	1.12^{c}	1.53^{abc}	3.10^{ab}	3.33^{a}	2.84^{ab}	2.76^{abc}	1.17^{abc}	1.74^{abc}	0.72	<0.01	<0.01	<0.05
Prevotella	1.09	0.67	0.44	1.05	0.78	0.67	1.08	0.95	0.52	1.09	0.92	0.55	0.09	0.32	<0.001	0.39

Abbreviations: *B. fibr = Butyrivibrio fibrisolvens; F. succ = Fibrobacter succinogenes; R. alb = Ruminococcus albus; 2R. flav = Ruminococcus flavefaciens; S. rum = Selenomonas ruminantium; Methanobrev* = *Methanobrevibacter*; CTR = control; EA = ellagic acid; GA = gallic acid; SEM = standard error of the mean; T = treatment; D = day. Means with different superscripts within a row are significantly different (P < 0.05).

Bacterial and archaeal communities

Following the results of qPCR, a further investigation applying high-throughput sequencing was carried out to go more into detail in the modulation of rumen microbiota. A total of 17'836 bacterial and 274 archaeal ASVs were detected, of which 6584 and 110 were classified to the species level. The number of detected species or candidate species was 669 for bacteria and 18 for archaea. Bacterial ASV richness, evenness, and diversity significantly decreased over time in all treatments (P<0.05, Figure 3A-C), but the decrease was largest in samples treated with EA and EA+GA. These two treatments revealed significantly lower ASV richness after 10 days of treatment compared to CTR and GA (-59.2%, P<0.05), whereas bacterial ASV evenness and diversity were significantly decreased at day 6 (P<0.05) by the treatments EA and EA+GA compared to CTR and GA. In contrast to bacteria, archaeal ASV richness, evenness, and diversity did not significantly change over the duration of the experiment and among most treatments (Figure 3D-F). Only the archaeal ASV richness revealed a small, but significant difference among treatments at day 2 (GA vs. CTR, P<0.001), and a slightly bigger at day 10 (CTR vs. EA+GA and GA vs. EA, P<0.05 for both).

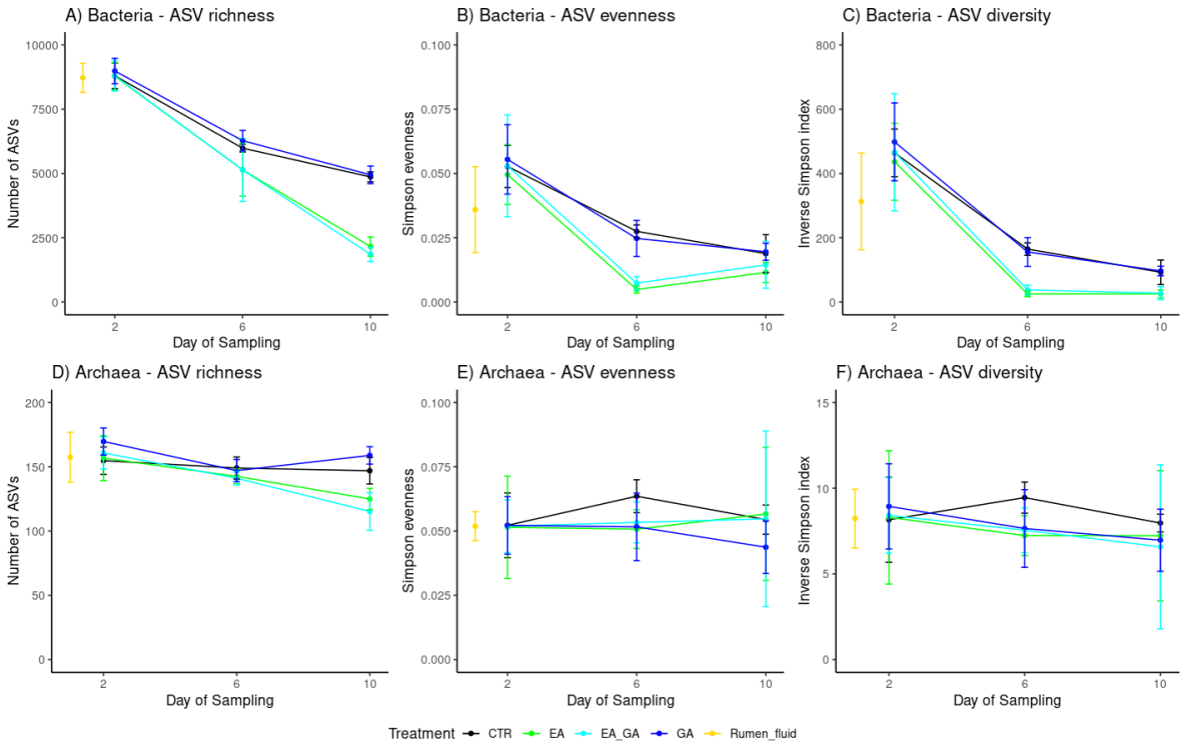

Figure 3. Bacterial (A-C) and archaeal (D-F) ASV richness (A, D), ASV evenness (B, E), and ASV diversity (C, F). Values are means of 1000 iterative subsamples of raw communities to the lowest read number of a sample. Abbreviations: CTR = control; EA = ellagic acid; GA = gallic acid.

Bacterial and archaeal community compositions differed significantly among the three experimental runs (Figure 4), revealing differences among the initially sampled rumen fluids. On average, 46% of bacterial and 75% of archaeal ASVs were shared among samples from different rumen fluids. The proportion of shared ASVs among experimental runs II and III (55% bacterial and 82% archaeal ASVs) was higher, compared to the proportion of shared ASVs among the first and the other two runs (41%, 72%). Despite these differences in the rumen fluids, the treatments EA and EA+GA caused a consistent community shift along the same ordination axis (NMDS1, Figure 4A). A similar effect of EA addition was also detected on archaeal communities, that also shifted along the first ordination axis (Figure 4B). Overall, treatment, days, and run had a significant effect on bacterial and archaeal communities (PERMANOVA, $p < 0.05$).

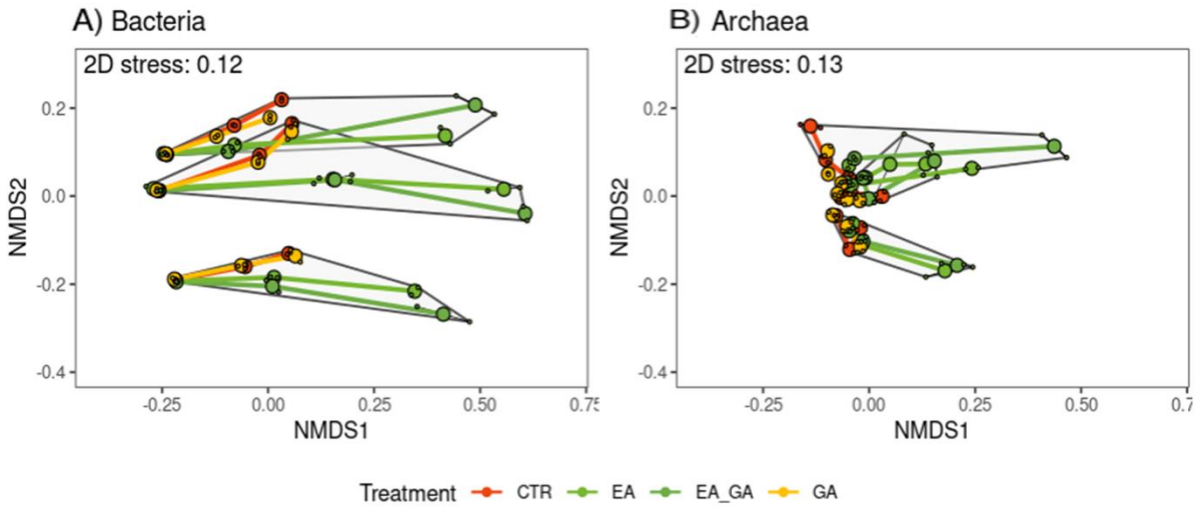

Figure 4. Bacterial (A) and archaeal (B) communities visualized by nonmetric multidimensional scaling. Grey polygons represent the three experimental runs. Small black dots linked to the means by thin lines indicate the measured samples.

To better understand the community shifts caused by the addition of EA, we compared treatments EA and EA+GA (EA+) to CTR and GA (EA-) more in detail. The number of shared bacterial ASVs among EA+ and EA- decreased from 96.3% at day 2 to 53.4% at day 10 (Figure 5A), while a smaller decrease from 98.0% to 90.8% was found for archaea (Figure 5B). In parallel to the decrease of shared bacterial ASVs among EA+ and EA-, we could observe an increase of bacterial ASVs that were detected in EA- only. The percentage of bacterial ASVs detected only in EA- increased from 1.7% at day 2 to 12.4% at day 6 and 37.9% at day 10. This suggested that many bacterial species were sensitive to the addition of EA, which lead to their disappearance in EA+. The most important change in the taxonomic composition of bacterial communities was the increase of Megasphaeraceae in EA+ treatments (Figure 5C). Other minor alterations of the bacterial communities were the decrease of the candidate genus UBA932 (family Bacteroidaceae) and the increase of the family Lachnosphiraceae in EA+. The taxonomic composition of archaeal communities was characterized by a decrease of the most abundant candidate genus UBA71 (family Methanomethylophilaceae) in all treatments over time, as well as an increase of the candidate genus JAKSHX01 (family Methanomethylophilaceae) in EA- and the genus *Methanomethylophilus* in EA+. Furthermore, *Methanobrevibacter* became with a mean relative abundance of 23.0% much more abundant in EA+ at day 10, as compared to days 2 (1.0%) and 6 (1.2%).

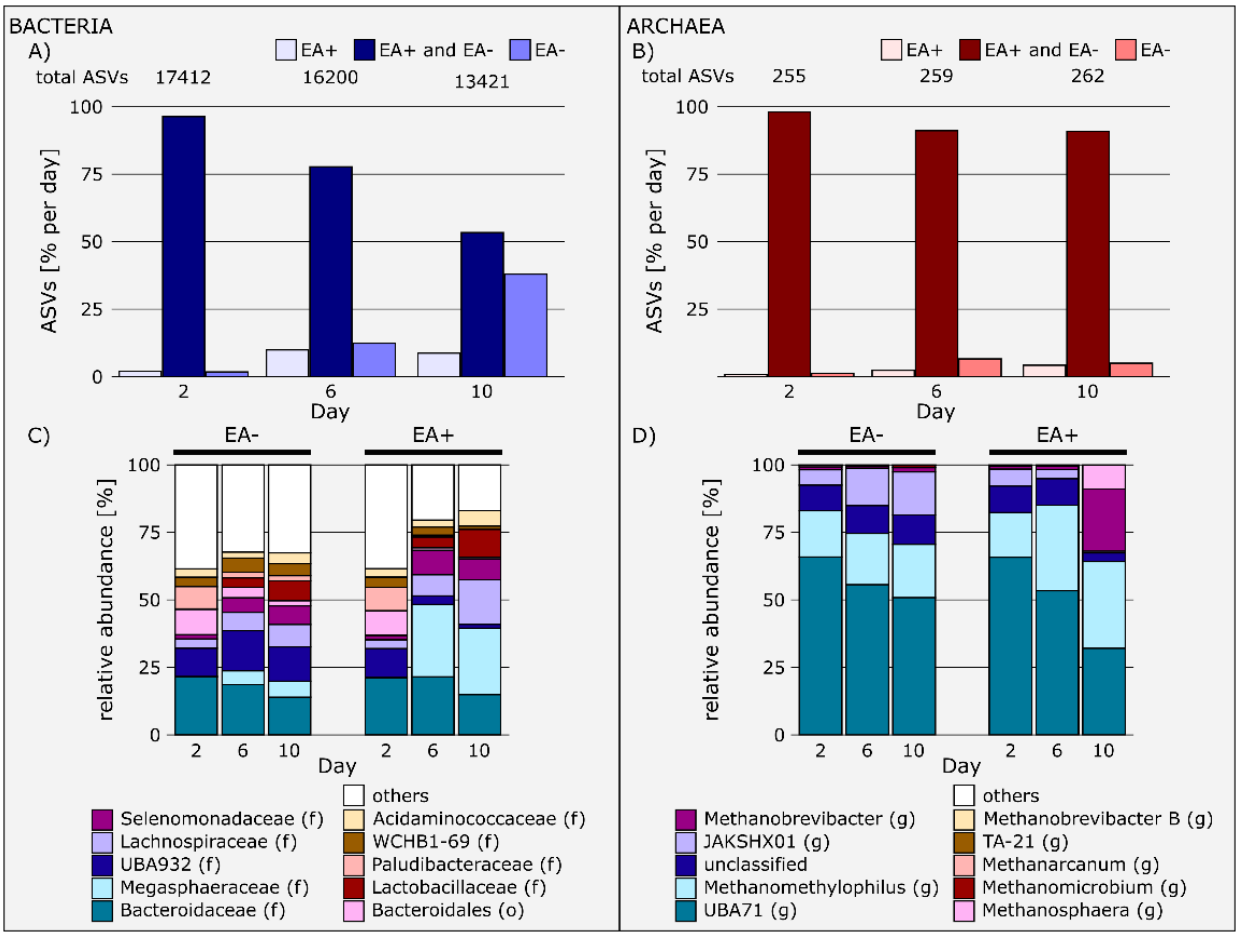

Figure 5. Differences in microbial community compositions of treatments with EA (EA+) or without (EA-). Percentages of bacterial (A) and archaeal (B) ASVs detected only in samples with EA (light blue/red), detected in samples with and without EA (dark blue/red), and detected only in samples without EA (blue/red). Lower panels show relative abundances of the ten most abundant bacterial families (C) and archaeal genera (D). Mean relative abundances of samples with (EA+) or without (EA-) are shown per day (2, 6, 10). Less abundant families and genera were summed in the category 'others' (white).

Comparison of qPCR and high-throughput sequencing

With the exception of *B. fibrisolvens*, all taxa quantified by qPCR were represented by multiple ASVs. The number of ASVs within a taxon targeted by qPCR ranged from 2 (*R. albus*) to 1362 (*Prevotella*) ASVs, which corresponded to 2 to 37 species. Correlations of qPCR values with summed relative abundances obtained by high-throughput sequencing varied widely ranging from -0.33 to 0.98. Insignificant, negative or weak correlations below 0.6 were obtained for *Butyrivibrio fibrisolvens* (-0.33, p=0.004), *Selenomonas ruminantium* (0.17, p=0.146), *and Methanobrevibacter* (0.55, p<0.0001), strong correlations were obtained for *Ruminococcus flavefaciens* (0.73, p<0.0001), *Prevotella* (0.80, p<0.0001), *Fibrobacter succinogenes* (0.93, p<0.0001), *and Ruminococcus albus* (0.98, p<0.0001). Two species of *Ruminococcus albus*, *Ruminococcus* D *albus* and *Ruminococcus* D *albus* C were genetically

identified, which responded differently to the treatments, with the first being more abundant in EA- and the second being most abundant in EA+ (Figure 6A-C).

Correlations of bacterial and archaeal ASVs to CH_4 production

To find possible links between microbial communities and CH_4 production, we correlated relative abundances of bacterial and archaeal ASVs detected at day 10 with CH_4 emissions. Among the 13421 detected bacterial and 262 detected archaeal ASVs at day 10, 35 bacterial and 8 archaeal ASVs were positively correlated and 3 bacterial and no archaeal ASVs were negatively correlated to CH_4 emissions ($p.adj < 0.05$, $|rho| > 0.7$). The three bacterial ASVs, which were strongly and negatively correlated to CH_4 emissions, were classified to *Megasphaera elsdenii* ($r = -0.73$, $p < 0.0001$), the genus *Megasphaera* ($r = -0.73$, $p < 0.0001$), and the family Lachnospiraceae ($r = -0.71$, $p < 0.0001$). Relative abundances summed at the species level also showed *Megasphaera elsdenii* with the strongest negative correlation with CH_4 emissions ($r = 0.78$, $P < 0.0001$, Figure 6D). *M. elsdenii* was the dominant bacterial species in both EA- and EA+, but its relative abundance at day 10 was with 19.3% much higher in EA+ as compared to EA- (4.6%). The strongest positive correlations of bacterial and archaeal species with CH_4 emissions at day 10 were detected for candidate species of the bacterial family Anaerovoracaceae ($r = 0.90$, $P < 0.0001$, Figure 6E) and of the archaeal family Methanomethylophilaceae ($r = 0.86$, $P < 0.0001$, Figure 6F).

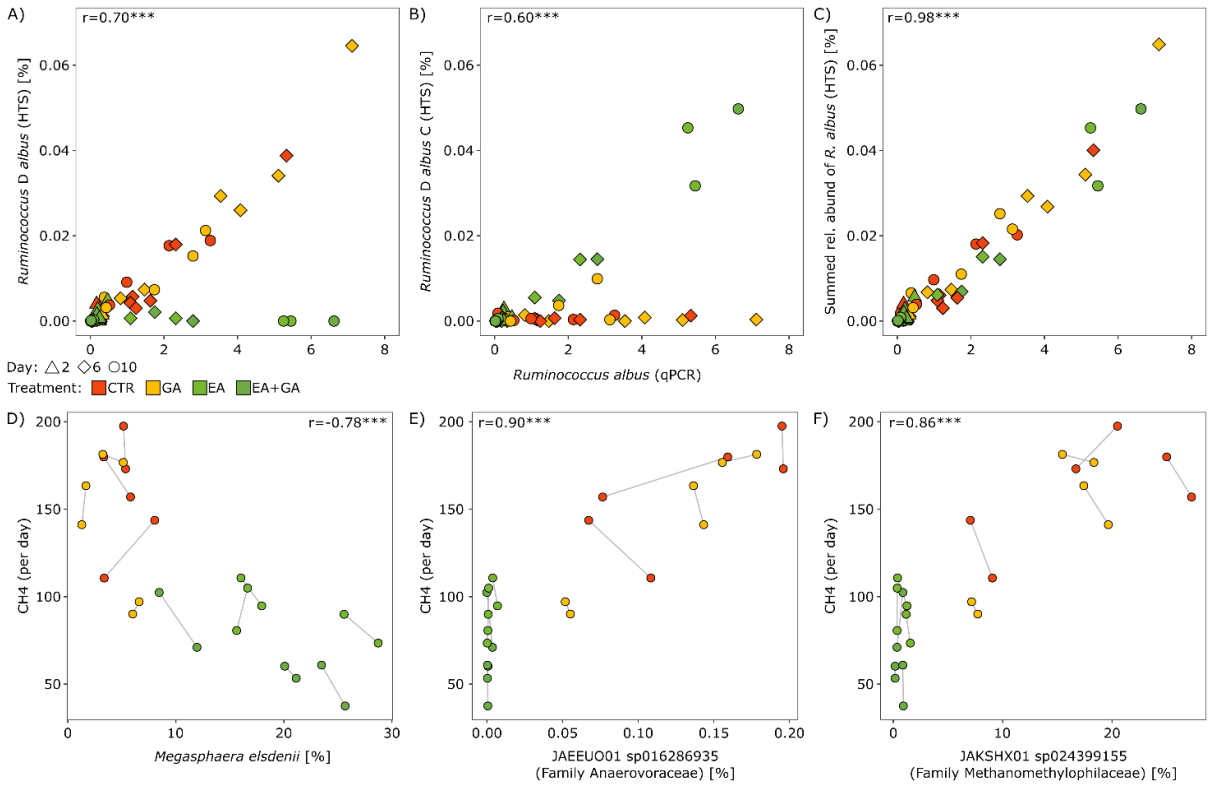

Figure 6. Correlations of qPCR quantification with relative abundances generated by high-throughput sequencing (A-C) of two Ruminococcus species, i.e., Ruminococcus D albus (A) and Ruminococcus D albus C (B), as well as their sum (C). Lower panels show correlations of relative abundances of species

with daily CH_4 emissions at day 10. The bacterial species with the strongest negative (D) and positive (E) correlations, and the archaeal species with the strongest positive (F) correlation are shown. No archaeal species was strongly negatively correlated to CH_4 ($r < -0.7$). Colors indicate treatments, i.e., control (CTR), gallic acid (GA), ellagic acid (EA) and their combination (EA+GA), and shapes show sampling time points.

Discussion

The results of this study showed that the total gas production was not significantly altered by the treatments, if compared to CTR (Table 1), in accordance with the results obtained in the previous trial (Manoni et al., 2023). A meta-analysis of Jayanegara et al. (2018) discussed six Rusitec studies with different tannin plant extracts, ranging 0-135 mg/g DM of inclusion in the incubation fluid. The authors observed that increasing level of dietary tannins did not affect total gas production. Furthermore, Wei et al. (2019) applied Rusitec with increasing concentrations of GA and observed that up to 20 mg/g GA did not affect total gas production. In our experiment, even 75 mg/g GA did not alter total gas production. The gas production rate could be related to the bacterial count in the rumen fluid (Figure 1). Indeed, the number of bacteria was higher following all tannin treatments compared to CTR. The higher bacterial level might have maintained gas production over time, although the reduced bacterial ASV richness (Figure 4A) and the decreased abundance of fibre-and cellulose-degrading bacteria such as *B. fibrisolvens, F. succinogenes, and R. flavefaciens* (Table 4) might have reduced the nutrient degradation (Table 3). The higher bacterial count following all tannins treatments could be also related to the kinetics of UroA and UroB (Figure 2). The UroA is one of the end-products of the bacterial metabolism of HT in rumen, especially ellagitannins such as EA (Lotfi, 2020; Quatrin et al., 2020). It has been reported that tannin-degrading bacteria in rumen can convert UroA to UroB (González-Barrio et al., 2012; Espìn et al., 2013). In addition, González-Barrio et al. (2012) identified UroA, Iso-UroA, and UroB in rumen samples collected from beef cattle fed oak leaves. These literature findings could be related to our results, because part of the significantly higher level of UroA detected at day 6 might have been converted to UroB, for which we observed a significant higher level at day 10, by tannin-degrading bacteria, thus suggesting that some specific bacterial families able to degrade HT were still metabolically active at late stages of the trial. So far, there are no studies dealing with the measurement of urolithins in rumen fluid after Rusitec. This is why further studies are needed to unravel the metabolism of tannin-degrading and urolithin-producing bacteria, as well as the knowledge about the fate of urolithins in rumen after HT biotransformation.

The daily production of CH_4 per ml of total gas was significantly reduced by EA and EA+GA if compared to CTR, but not by GA. A similar trend was observed for the daily CO_2 production per ml of total gas, which was significantly reduced by EA and EA+GA as well. Instead, the daily H_2 production per ml of total gas was not significantly altered by the treatments (Table 1). Similarly to Manoni et al. (2023), the

treatments EA and EA+GA were the most effective in reducing daily CH_4 production. Wei et al. (2019) observed no effects of GA up to 20 mg/g DM in a Rusitec fermentation model on total gas, daily CH_4 and H_2 production. Here, GA did not reduce total gas, CH_4 and H_2 production even at 75 mg/g DM. As reported above, the effect of tannins on CH_4 production is a consequence of the direct modulation of archaea methanogens or the indirect modulation of other microbial components and H_2 suppliers, mostly bacteria (Hassan et al., 2020), but the specific effect of individual HT components has not yet been addressed. Overall, a treatment effect was more evident on bacteria rather than archaea. We observed that EA+ treatments reduced bacterial richness over time. Considering the taxonomic composition of the bacterial community, the EA+ treatments reduced the relative abundance of *B. fibrisolvens*, *F. succinogenes*, and *R. flavefaciens* whose metabolic processes of feed fermentation are known to be a source of H_2 (Tapio et al., 2017). Instead, the same treatments increased the relative abundance of *S. ruminantium*, a nitrate-reducing bacterial species capable of growing on tannic acid or condensed tannins (Skene and Brooker, 1995) and associated with reduced methanogenesis (Iwamoto et al., 2002). Then, high throughput sequencing showed that EA+ treatments increased the bacterial families Lachnospiraceae and Megasphaeraceae, both negatively correlated with CH_4 production. Lachnospiraceae are known to be positively correlated with butyrate production (Kaminsky et al., 2023), in accordance with our results. Then, the *Megaspheraceae* bacterial family, in particular *M. elsdenii*, was the bacterial family mostly increased by EA+ treatments at day 10 (compared to EA- treatments) and showing the highest negative correlation with CH_4 production. *M. elsdenii* is a lactate-consuming bacterial species, that has already been correlated with lower rumen CH_4 production (Seradj et al., 2014; Li et al., 2021; Susanto et al., 2023). The action of *M. elsdenii* on CH_4 mitigation could be explained by the conversion of lactate to propionate, which works as an alternative H_2 sink that subtracts H_2 from CH_4 production (Pereira et al., 2022). In this study, propionate was not increased by the treatments. It was reported that in long-term fermentation simulation techniques such as Rusitec, *M. elsdenii* uses H_2 to convert lactate to pyruvate (instead of propionate) by an NAD-independent lactate dehydrogenase enzyme (Prabhu et al., 2012; Li et al., 2021). Therefore, the observed CH_4 reduction could be ascribed more to a redirection of H_2 to an alternative H_2 sink, which is not propionate but pyruvate.

Moving to archaea, it is known that rumen archaea are more stable and more conserved than bacteria across ruminant species (Henderson et al., 2015). Indeed, our results showed that the archaeal community was less affected by the treatments in terms of community shifts. Overall, the archaeal community was dominated by the Methanomethylophilaceae family. The Methanomethylophilaceae are methylotrophic archaea, the minor components of archaeal methanogens (Morgavi et al., 2010; Henderson et al., 2015). The methylotrophic methanogens produce CH_4 using H_2 to reduce methylated compounds, such as methanol, instead of hydrogenotrophic methanogens that use H_2 to reduce CO_2

(Li et al., 2021). By observing the rumen archaeal taxonomy, the candidate genus JAKSHX01 of the family Methanomethylophilaceae had the highest positive correlation with CH_4 production and was reduced in EA+ treatments and increased in EA- treatments. Instead, the hydrogenotrophic archaea *Methanobrevibacter* was increased by EA+ treatments, although no positive correlation with CH_4 production and no *Methanobrevibacter*-mediated H_2 depletion were observed. Other studies evaluating the effect of various sources of HT- and CT-plant extracts on the modulation of rumen microbiota showed that *Methanobrevibacter* was not reduced by any tannin source or dose of supplementation (Salami et al., 2018; Min et al., 2022). It is thus possible that the metabolic activity but not the abundance of *Methanobrevibacter* was affected by the EA+ treatments. In any case, the cause-effect relationship between reduced ruminal CH_4 emission and relative abundance of methanogens still needs to be demonstrated, because CH_4 emissions can be reduced even without reducing the presence of methanogens (Danielsson et al., 2017). This can also depend on the low vs high CH_4-yield emission phenotype of the animals considered, given by the microbial composition and the number of gene transcripts related to methanogenesis (Stepanchenko et al., 2023). In addition, we observed that EA alone decreased both acetate and propionate and increased butyrate. Instead, the treatment EA+GA behaved similarly but without affecting acetate (Table 2). Acetate and butyrate are positively associated with net production of H_2, while propionate is positively associated with net consumption of H_2, as reported above (Lyons et al., 2018; Ungerfeld, 2020). Here, the modulation of the rumen microbial community might have influenced the SCFA profile and in turn H_2 production, and an example is the positive correlation between Lachnospiraceae and butyrate (Kaminsky et al., 2023). However, a clear pattern between SCFA profile and CH_4 production should be further addressed.

Furthermore, protozoa are known to indirectly sustain CH_4 emission through the production of H_2 (Aboagye and Beauchemin, 2019). The EA+ treatments reduced the level of protozoa. Wei et al. (2019) observed that up to 20 mg/g GA did not alter protozoal count. In our study the use of 75 mg/g GA did not affect protozoa either. In addition to the reduction of CH_4, the strong reduction of protozoa caused by EA+ was also associated with reduced NH_3 formation (Table 1). It is known that protozoa are key contributors of protein degradation and deamination (Males and Purser, 1990; Soliva et al., 2011), and their depletion is linearly correlated with reduced CH_4 emissions and NH_3 formation (Spanghero et al., 2022). The NH_3 formation is also related to CP degradation, which is affected by the presence of tannins due to the formation of tannin-protein complexes, subtracting proteins from microbial degradation in rumen (Aboagye and Beauchemin, 2019). It is likely that the combination of decreased protozoa, reduced nutrient degradation and microbial protein catabolism could have caused reduced NH_3 formation. The lower OM, NDF, and CP degradation is the main concern on the use of tannins to modulate rumen fermentation, because the binding capacity of tannins with proteins and fibres can subtract them from microbial degradation in rumen (Aboagye and Beauchemin, 2019). Another

explanation of the reduced feed degradation can be the reduced relative abundance of bacteria able to ferment fibres and cellulose at rumen level, such as *B. fibrisolvens*, *F. succinogenes,* and *R. flavefaciens*. Last, the amount of substrate per unit of fermentation medium volume, which is of course smaller than the *in vivo* condition, could have affected the results too (Hristov et al., 2012). The impact of the treatments on the degradation of nutrients is in line with other studies dealing with other sources of tannin compounds such as chestnut extract (Wischer et al., 2013), fibrous feed sources (brachiaria, beet and apple pomace) (Soliva et al., 2015), feed concentrates (barley and soybean meal) (Smith et al., 2020), and choline (Li et al., 2021).

To summarize, EA+ and EA- treatments differently modulated rumen microbiota by altering the taxonomy of the rumen archaea and bacteria communities and lowering the number of protozoa. The major effect of EA+ was observed on the increased abundance of lactic acid consuming bacteria M. elsdenii which likely functioned as H_2 sink and showed the most negative correlation with CH_4 production. The EA+ treatments did not directly reduce the population of hydrogenotrophic methanogenic archaea, but reduced the bacterial richness. Consequently, EA+ treatments reduced total gas and CH_4 production, NH_3 formation, SCFA production and nutrient degradation. We observed that lower concentrations of EA (7.5% DM) alone or in combination with GA showed both beneficial and detrimental effects on rumen fermentation similarly to what obtained after using double the concentration of EA (15% DM) during HGT (Manoni et al., 2023). It is possible that the longer retention time in rumen fluid might have enhanced the effect of EA (and EA+GA) on the modulation of microbiota and related enteric fermentation. All these outcomes were not observed in the case of GA, which was less effective and in turn less detrimental on rumen fermentation than EA.

Conclusion

This research allowed us to further investigate the role of EA and GA after 10 days of continuous and standardized rumen fermentation. The outcomes of this study were similar to what was observed after the 24-h HGT trial, but with Rusitec we could observe the kinetics of gas production over 5 days of measurement. We observed a stronger impact of EA+ treatments on modulating rumen fermentation and in turn reducing CH_4 production rather than GA, and this affected all the related outcomes of rumen fermentation, such as SCFA, NH_3, and nutrient degradation. The GA treatment showed minor detrimental effects on SCFA production and nutrient degradation than EA. Some parameters, such as total gas and bacteria, were stable along the 5 days of measurement, whereas some others, such as CH_4 and protozoa, showed a decreasing trend starting from day 6 onwards. In addition, the coupling with high-throughput sequencing enabled us to observe the impact of EA and GA on the modulation of rumen microbiota. If on one hand both treatments and time did not affect archaea in terms of

stability of the community in the rumen environment, the increased relative abundance of *M. elsdenii*, the reduced presence of Methanomethylophilaceae, the depletion of protozoa, and the reduced bacterial richness over time caused by EA+ treatments might partially explain the observed CH_4 decrease and the related effects. Further investigations regarding the composition of the rumen bacterial community facing its reduced richness after the EA+ treatments, the effect of these treatments on specific rumen microbes (e.g. *M. elsdenii*) and more details on the action of urolithins in the rumen environment could improve the outcomes of this study.

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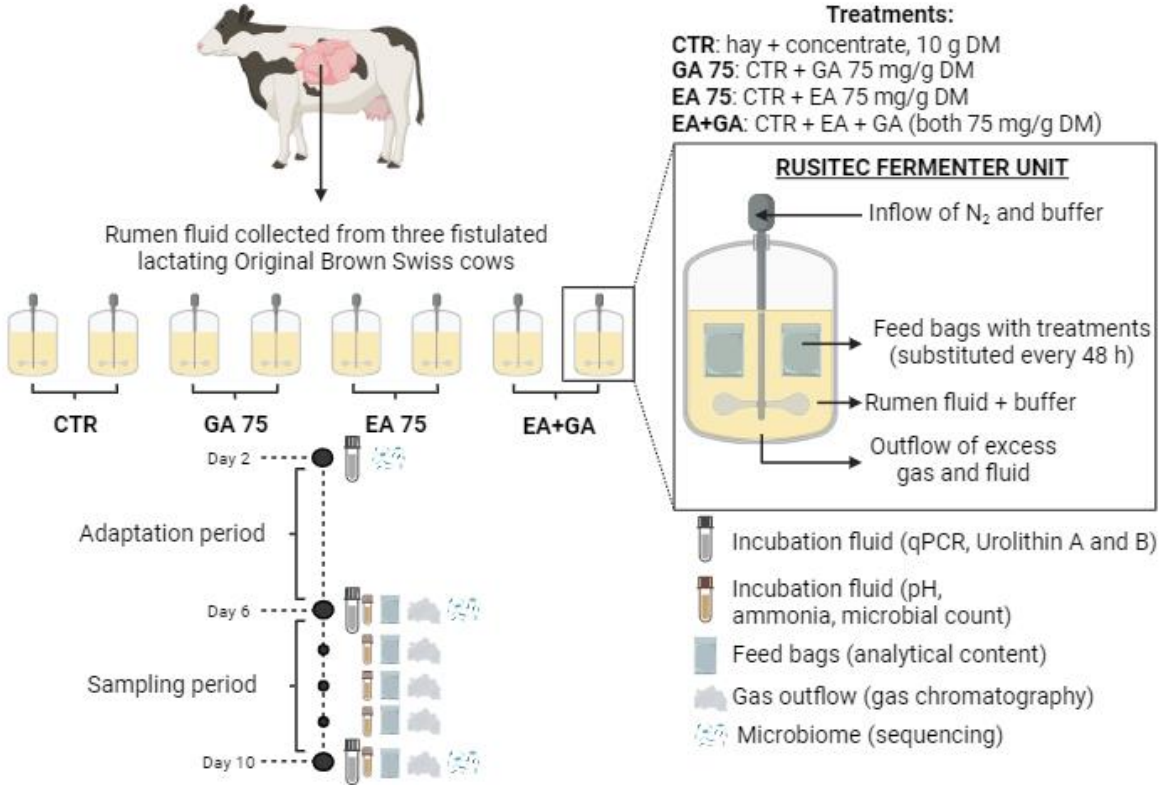
Supplementary Figures

Supplementary Figure S1

Schematic representation of the experimental set-up including treatments and sampling scheme.



Discussion

To mitigate the environmental footprint of animal farming, the production of livestock must be directed to sustainable choices at multiple levels of the production chain. The whole sector of feedstuffs production represents the largest cost for livestock production. Many strategies were discussed and applied by involving dietary treatments and innovative feeding approaches. For example, the use of less food-competing feedstuffs for animal production, such as crop and food by-products, is currently regarded as a valid option to redirect a third of global cereal production to humans, since it is currently used for livestock feeding. Furthermore, the inclusion of dietary additives and/or supplements to counteract the emissions of GHG from livestock are currently studied, such as the use of rumen-affecting compounds, either natural or synthetic, or the reduced protein supply to decrease the N-ammonia emissions. Animal feeding is therefore seen as a reasonable step of the livestock sector that can be targeted with innovative strategies in the frame of sustainable livestock farming. In this thesis, two main projects were carried out, both involving the application of methodological in vitro approaches to evaluate the effect of two dietary strategies aimed at raising the sustainability of livestock production.

Metabolic impact of former food products in pig diet (Section A)

The first experimental project (Section A) was oriented to the research of alternative feedstuffs that could alleviate the feed-food competition and increase the sustainability of livestock production. We used FFPs because they are food by-products already processed to obtain feedstuffs. The dietary inclusion of FFPs showed no detrimental alteration of the growth and nutritional parameters in post-weaning piglets (Luciano et al., 2021) and pigs (Mazzoleni et al., 2023). What we addressed here was a further step in the validation of FFPs as applicable ingredients in pig feeding. This step involved the investigation of the impact of FFPs on the metabolism of the pigs and particularly on the lipid metabolism, considering the peculiar fat composition of FFPs, i.e. the higher content of MUFA than the control diet (+12%). It is known that the fatty acid profile of the lipid dietary source is reflected in the fatty acid profile of the adipose tissue (Schumacher et al., 2022), and here we had a clear example of this. In fact, the lipid profiles of the diets and of the adipose tissue of post-weaning piglets were similar, but the adipose tissue of FFPs-fed piglets showed minor differences between the level of SFA, MUFA, and PUFA than the control-fed piglets. The piglets' abdominal fat resembled the characteristics of the dietary fat but the piglets were able to rebalance the fatty acid profile of the diets in their adipose tissue, probably because the mechanism of fat deposition was not altered enough to lead to an unbalanced fatty acid profile in the adipose tissue. In this sense, the KEGG pathway analysis of the

metabolites detected with the metabolomics investigation did not highlight any evidence of altered pathways belonging to fat deposition in piglets. It is possible that, since the diets containing FFPs were formulated to be isoenergetic and isonitrogenous, they did not lead to any observable alteration of the metabolism. Also, the effect of FFPs on adipose tissue and lipid metabolism was observable but limited due to the fact that young animals such as post-weaning piglets were involved in this study and the duration of the feeding trial lasted for only 42 days. Unlike our findings, Sun et al. (2015) performed an untargeted metabolomics assessment to observe the impact of an unbalanced diet with high-fat and high-cholesterol levels (4 times higher fat than control diet) on the metabolism of 4-week old pigs. The authors observed an altered level of bile acids, lipid-secondary metabolites, fatty acids and amino acids in plasma, urine and fecal samples that significantly increased over time (Sun et al., 2015). Similarly, another study highlighted a high plasma concentration of total cholesterol and triglycerides in 8-week old pigs fed with a high-fat diet (around 3-fold times higher fat than control diet) for three months (Yang et al., 2018). These latter studies showed that the unbalanced diets tested altered the lipid metabolism in response to a higher intake of fat, whereas in our case an altered lipid metabolism was not really observed. Instead, the investigation of the liver proteome of tissues collected at the slaughtering age showed that the SA and SU FFPs affected the level of several proteins related to the mechanisms of lipogenesis and lipid oxidation (e.g. CA3, NNMT, and HMGCS2), and in turn fat oxidation. Duran-Montgé et al. (2009) proved that the use of seven lipid dietary sources with a peculiar fatty acids profile exerted a differential gene expression of lipogenic and lipolytic genes in liver, with PUFA-rich fish oil being associated with a downregulation in lipogenesis, whereas MUFA-rich sunflower oil with increased expression of lipogenesis-related genes (Duran-Montgé et al., 2009). As seen above, the SA and SU FFPs diets had a higher level of MUFA than the control diet, but we did not observe any increase of lipogenesis-related proteins in liver. We hypothesized that on one hand the longer experimental time interval involving FFPs and the higher age of pigs involved in the proteomics-peptidomics study have influenced the lipid metabolism in liver, but on the other hand the functional evaluation of the modulated proteins did not show any correlation with modulated pathways, thus giving the idea of the minimal impact of FFPs on the liver metabolism involving lipid accumulation and/or oxidation. Sejersen et al. (2013) observed that growing pigs fed with a high-fat diet showed an increased fat deposition (+50% on average than control group) and a higher level of total cholesterol and triglycerides in plasma, as like as what reported by Sun et al. (2015) and Yang et al. (2018). However, Sejersen and co-authors observed that the high-fat diet did not cause lipid accumulation in liver, but instead increased the expression of three proteins related to fatty acid oxidation (Sejersen et al., 2013). This finding is similar to what we observed, because both the SA and SU FFPs diets increased the lipid oxidation-related proteins (e.g. HMGCS2) and decreased lipogenesis-related proteins (e.g. CA3). Apart from the lipid metabolism, a higher level of caffeine and theobromine was observed in the

plasma of pigs fed with FFPs, probably because they were already present in the ingredients used to prepare the FFPs. Although caffeine was associated with the regulation of lipid metabolism (Harpaz et al., 2017), we did not observe any evident correlation between these two items. It is possible that the higher level of caffeine in the FFPs diets was not enough to affect lipid metabolism. In addition, the peptidomics investigation did not detect significant differences in terms of differentially expressed peptides among the three dietary groups considered, despite four serum proteins identified only in the SA FFPs-fed group. These proteins triggered the activation of an adaptive physiological mechanism related to the higher level of anti-hypertensive peptides, probably aimed at counteracting the higher presence of sodium (around 2-fold) in the SA FFPs diet, compared to the SU FFPs and the control diets. In the literature, it was reported that an increasing concentration of dietary sodium chloride was positively correlated with the upregulation of lipolysis-related genes, together with the downregulation of lipogenesis-related genes, and in turn contributing to a reduced fat deposition in the abdominal adipose tissue in mice (Cui et al., 2017). It is possible that the modulation of liver proteins in the SA FFPs group was also given by the higher presence of dietary sodium, even though this correlation was not investigated. By considering all these findings together, the FFPs diets exerted a minimal impact on the metabolism of the pigs involved in the study, and this impact was not detrimental. Indeed, the two FFPs diets lowered the lipid accumulation in liver and the SA FFPs diet exerted an anti-hypertensive effect on the pigs. The fact that the particular nutrient composition of FFPs did not deeply alter the metabolism of piglets and adult pigs suggested us that FFPs could be recycled as feed ingredients instead of human-edible cereals, to alleviate the feed-food competition. Further work could be directed to assess the safety of FFPs in terms of packaging remnants and to compare the outcomes of the use of multiple sources of FFPs from different geographical areas.

Tannins and rumen fermentation

The second experimental project (Section B) was based on the research of dietary strategies to reduce the CH_4 production from enteric fermentation and consequently reduce the impact of ruminant production. We used GA and EA, two single tannin molecules, to observe their impact on rumen fermentation and on the modulation of the rumen microbiota. We applied two in vitro methodologies, first to obtain preliminary results and then to further validate the treatments. The HGT was useful to choose the best candidates, whereas the Rusitec was useful to standardize and stabilize the fermentation and thus to observe the alteration on the system caused only by the presence or absence of tannins in the reaction medium and not by other factors (e.g. effect of the basal diet on fermentation). We observed that the EA-containing treatments, EA and EA+GA, were more efficient than GA in terms of reduced CH_4 and ammonia production. The higher modulating effect of EA rather

than GA on rumen fermentation was observed in another HGT-adapted trial, in which the inclusion of EA up to 5% DM reduced total gas, CH_4, and ammonia production more than the same concentration of GA (Hundal et al., 2016). At the same doses of supplementation (7.5% DM), the efficiency of EA on CH_4 and ammonia production increased from the HGT to the Rusitec trial to a higher extent than GA. The effect of the EA-containing treatments on CH_4 and ammonia was the spotlight of an overall altered enteric fermentation. In fact, the effect of EA and EA+GA reverberated also on the other parameters investigated, causing the reduction of total gas production, IVOMD, and SCFA production. A negative correlation between the concentration of tannins and total gas, ammonia, and SCFA production and nutrient degradation was observed in various HGT trials (Jayanegara et al., 2012). Regarding SCFA, the effect of GA and EA on the single SCFA species was different in the two trials, but the only similarities were the decreased percentage of propionic acid and isobutyric acid following both GA and EA treatments. Propionic acid is an alternative H_2 sink that can compete with methanogens for the use of H2, and a higher level of propionic acid is usually correlated with a lower CH_4 production (Tavendale et al., 2005; Bhatta et al., 2009). It was reported that the use of quercetin up to 6% DM in an Ankom in vitro 24-h trial in association with two different feed substrates (maize silage and grass silage) reduced CH_4 production and did not affect SCFA production in both cases, but propionic acid was increased only in the case of maize silage, whereas it was decreased in the case of grass silage (Battelli et al., 2023). This result was reported to show that an increased level of propionic acid is usually an indication of CH_4 mitigation, but in some cases (including our trial) this is not observed. In our trial, the treatment GA 150 was in most cases more similar to the treatment EA 75 than EA 150, meaning that EA affected rumen fermentation at lower doses than GA. Because of a lower impact on rumen fermentation, GA was less detrimental than EA on SCFA production and nutrient degradation in rumen. The lower impairing effect of GA on rumen fermentation was also observed by Getachew et al. (2008), who compared the effect of quebracho tannins to the one of GA and tannic acid up to 150 g/kg DM added to alfalfa as standard HGT substrate. All the tested compounds reduced the rate of total gas production at increasing doses, but only GA increased the total SCFA production and did not affect the rumen degradation of dietary protein, thus being less detrimental for rumen fermentation (Getachew et al., 2008). Considering that our aim was to evaluate the potential of GA and EA on CH_4 and ammonia mitigation, we obtained the expected results more with EA, but along with a depression of rumen fermentation. The stronger effect of EA and EA+GA rather than GA alone was also observed on the bacterial and archaeal communities, for which the EA-containing treatments (EA+ treatments) clustered together and separately from CTR and GA (EA- treatments). No variation in the level of bacteria and protozoa for the first 24 h (HGT) was observed. Instead, we observed an overall reduction of bacterial ASV richness, evenness, and diversity in the face of an increased bacterial and a reduced protozoal level caused by EA and EA+GA after the 10-d Rusitec trial. As discussed in the introduction,

the role of protozoa in nutrient digestion and in rumen fermentation is known (Jayanegara et al., 2012; Hristov et al., 2012), but a correlation between the tannin-induced reduction of the level of protozoa and the CH_4 mitigation has yet to be addressed. In our trial, the depletion of protozoa might have negatively affected nutrient degradation and in turn CH_4 production, but it is more likely that the major effect of EA (and GA to a lesser extent) was the modulation of the rumen bacterial community, along with an overall reduction of the bacterial abundance. No major variation of the abundance of archaea hydrogenotrophic methanogens in the EA-treated samples was observed after the two trials, as also depicted by the fact that *Methanobrevibacter* was not reduced by EA and EA+ga, as observed with rt-qPCR. Instead, a reduced relative abundance of some of the main fibrolytic and cellulolytic bacteria (i.e. *B. fibrisolvens, F. succinogenes, R. flavefaciens, Prevotella*) was observed after HGT and after Rusitec. The reduced abundance of protozoa and bacteria was also probably related to the decreased nutrient degradation observed after the Rusitec trial, because in the literature a strong correlation between the microbial abundance and the degradation of nutrients at rumen level was reported (Bach et al., 2005; Dai and Faciola, 2019). Considering all the modulation of the rumen microbiota, we hypothesized that the lower presence of protozoa and the strong negative correlation between *M. eldsenii* (whose abundance was increased by EA+ treatments) and CH_4 production could in part explain the observed results on CH_4 production, because of the conversion of propionate to pyruvate and the resultant consumption of the H_2 reservoir (Prabhu et al., 2012; Li et al., 2021; Pereira et al., 2022). This latter could be an explanation for the lower level of propionate discussed above. To conclude, EA was more effective than GA (at the same concentration) in reducing CH_4 emissions and ammonia formation, i.e. our major objectives of the study. Since these are in vitro results, caution must be paid when moving to the *in vivo* condition. In these *in vitro* studies, we allowed ourselves to explore the effect on tannins in rumen by "promoting" the experimental conditions and applying concentrations that should be adapted to the in vivo condition, but this enabled us to observe how tannins can be beneficial and detrimental at the same time on rumen microbiota and enteric fermentation. Despite this, HGT and Rusitec were useful to obtain preliminary results and to test them in a more robust context, to get an idea of the "molecular behaviour" of GA and EA in rumen. The final outcome of these trials is that EA could be explored as a valid phytogenic supplement for CH_4 mitigation, but the dose of supplementation of 7.5% should be tailored and further investigated and other combinations with GA could be explored.

Conclusions

The sustainable livestock farming comes through applying alternative approaches to the production chain of feed and food. Here, the metabolic investigation of the impact of FFPs as dietary ingredients for pigs followed two in vivo trials in which the value of FFPs had been already observed. Instead, the investigation of GA and EA as rumen supplements was performed as a preliminary test to validate the potential of these molecules in the frame of the CH_4 mitigation strategies. We concluded with promising results from the two areas of research and with the indication that the in vitro methodologies that we applied were useful to increase the knowledge towards the topics investigated. To conclude, the application of FFPs and tannins as dietary supplements for pigs and dairy cows, respectively, are innovative and sustainable strategies, but they must be further investigated and sustained all over the livestock production chain.

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Further research activity – List of articles (with abstract)

1. Pinotti, L., **Manoni, M.**, Fumagalli, F., Rovere, N., Tretola, M., Baldi, A. **The role of micronutrients in high-yielding dairy ruminants: Choline and vitamin E**. Ankara Üniv. Vet. Fak. Derg. 2020, 67, 209- 214. DOI: 10.33988/auvfd.695432

Abstract: This review addresses the potential role of antioxidants and methyl-group sources in optimising the metabolic health of dairy ruminants. The productivity of high-yielding dairy cows has increased over the past 40 years and the milk yield has doubled. Such increases in milk production have been observed not only in dairy cows but also to some extent in other dairy ruminants such as ewes, goats and buffaloes (Bubalus bubalis). As a consequence, in all specialized dairy ruminants it is essential to optimize the macro and micro-nutrient supply, especially during the most critical period in the animals' production cycle i.e. from parturition until the peak of lactation. In this critical phase, an array of factors can enhance the balance between the intake and demand for nutrients, although the availability and supply of the selected micronutrients is also important. The supplementation of dietary antioxidants or boosting the endogenous methyl group status, via vitamin E, selenium and choline are proposed as possible strategies in maintaining stable metabolic health and optimising milk production.

2. Pinotti, L., **Manoni, M.**, Fumagalli, F., Rovere, N., Luciano, A., Ottoboni, M., et al. **Reduce, Reuse, Recycle for Food Waste: A Second Life for Fresh-Cut Leafy Salad Crops in Animal Diets.** Animals 2020, 10, 1082. DOI: 10.3390/ani10061082

Abstract: The world’s population is growing rapidly, which means that the environmental impact of food production needs to be reduced and that food should be considered as something precious and not wasted. Moreover, an urgent challenge facing the planet is the competition between the food produced for humans and the feed for animals. There are various solutions such as the use of plant/vegetable by-products (PBPs) and former foodstuffs, which are the co/by-products of processing industries, or the food losses generated by the food production chain for human consumption. This paper reviews the by-co-products derived from the transformation of fresh-cut leafy salad crops. A preliminary nutritional evaluation of these materials is thus proposed. Based on their composition and nutritional features, in some cases similar to fresh forage and grasses, this biomass seems to be a suitable feedstuff for selected farm animals, such as ruminants. In conclusion, although the present data are not exhaustive and further studies are needed to weigh up the possible advantages and disadvantages of these materials, fresh-cut leafy salad crops represent a potential unconventional feed ingredient that could help in exploiting the circular economy in livestock production, thereby improving sustainability.

3. **Manoni, M.**, Di Lorenzo, C., Ottoboni, M., Tretola, M., Pinotti, L. **Comparative Proteomics of Milk Fat Globule Membrane (MFGM) Proteome across Species and Lactation Stages and the Potentials of MFGM Fractions in Infant Formula Preparation.** Foods 2020, 9, 1251. DOI: 10.3390/foods9091251

Abstract: Milk is a lipid-in-water emulsion with a primary role in the nutrition of newborns. Milk fat globules (MFGs) are a mixture of proteins and lipids with nutraceutical properties related to the milk fat globule membrane (MFGM), which protects them, thus preventing their coalescence. Human and bovine MFGM proteomes have been extensively characterized in terms of their formation, maturation, and composition. Here, we review the most recent comparative proteomic analyses of MFGM proteome, above all from humans and bovines, but also from other species. The major MFGM proteins are found in all the MFGM proteomes of the different species, although there are variations in protein expression levels and molecular functions across species and lactation stages. Given the similarities between the human and bovine MFGM and the bioactive properties of MFGM components, several attempts have been made to supplement infant formulas (IFs), mainly with polar lipid fractions of bovine MFGM and to a lesser extent with protein fractions. The aim is thus to narrow the gap between human breast milk and cow-based IFs. Despite the few attempts made to date, supplementation with MFGM proteins seems promising as MFGM lipid supplementation. A deeper understanding of MFGM proteomes should lead to better results.

4. Cazzola, R., Della Porta, M., **Manoni, M.**, Iotti, S., Pinotti, L., Maier, J.A. **Going to the roots of reduced Magnesium dietary intake: a tradeoff between climate changes and sources.** Helyion 2020, 6, e05390. DOI: 10.1016/j.heliyon.2020.e05390

Abstract: Magnesium is essential in plants where it is associated with chlorophyll pigments and serves as a cofactor of enzymes implicated in photosynthesis and metabolism. It is an essential nutrient for animals, involved in hundreds metabolic reaction and crucial for the biological activity of ATP. Not surprisingly, magnesium deficiency is detrimental for the health of plants and animals. In humans, subclinical magnesium deficiency is common and generates chronic inflammation, which is the common denominator of a wide range of mental and physical health problems from metabolic diseases to cognitive impairment, from osteopenia and sarcopenia to depression. It is ascertained that magnesium content in fruits and vegetables dropped in the last fifty years, and about 80% of this metal is lost during food processing. As a consequence, a large percentage of people all over the world does not meet the minimum daily magnesium requirement. In this scoping review, we summarize how agronomic and environmental factors, including global warming, affect magnesium content and availability in the soil and, consequently, in the food chain, with the aim of attracting the interest of

botanists, agronomists, animal and human nutritionists and physicians to work on a strategy that grants adequate magnesium intake for everybody.

5. Giromini, C., Tretola, M., Baldi, A., Ottoboni, M., Rebucci, R., **Manoni, M.**, Pinotti, L. **Total phenolic content and antioxidant capacity of former food products intended as alternative feed ingredients.** Ital. J. Anim. Sci. 2020, 19, 1387-1392. DOI: 10.1080/1828051X.2020.1844086

Abstract: The application of Former Food Products (FFPs) as feed ingredients is already documented in swine, as well as their beneficial nutritional value. To date, FFPs extra-nutritional bioactive effect in feed has not been elucidated. Therefore, the aim of the present study was to investigate and compare the total phenolic content and antioxidant capacity (AOC) in six samples of FFPs extracted by different solvent systems. After methanol and acetone extraction, total phenolic content and AOC were determined in FFPs and wheat sample (CTR) using Folin–Ciocalteu and 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)-ABTS assay, respectively. Results demonstrated that FFPs samples were characterised by an average amount of total phenolic content of 129.3 ± 15.1 mg tannic acid equivalents (TAE)/100g in methanol extracts and 156.4 ± 25.8 mg TAE/100g in acetone extracts. Whereas, the ABTS assay revealed that FFPs showed also anAOC of 138.0 ± 14.3 mg Trolox Equivalent (TE)/100 g in methanol extracts and 173.3 ± 18.8 mg TE/ 100 g in acetone extracts. Former Food Products represent relevant sources of phenols and antioxidant compounds, which can be beneficial for animal health.

6. Piuri, G., Zocchi, M., Della Porta, M., Ficara, V., **Manoni, M.**, Zuccotti, G.V., et al. **Magnesium in Obesity, Metabolic Syndrome, and Type 2 Diabetes.** Nutrients 2021, 13, 320. DOI: 10.3390/nu13020320

Abstract: Magnesium (Mg^{2+}) deficiency is probably the most underestimated electrolyte imbalance in Western countries. It is frequent in obese patients, subjects with type-2 diabetes and metabolic syndrome, both in adulthood and in childhood. This narrative review aims to offer insights into the pathophysiological mechanisms linking Mg^{2+} deficiency with obesity and the risk of developing metabolic syndrome and type 2 diabetes. Literature highlights critical issues about the treatment of Mg^{2+} deficiency, such as the lack of a clear definition of Mg^{2+} nutritional status, the use of different Mg^{2+} salts and dosage and the different duration of the Mg^{2+} supplementation. Despite the lack of agreement, an appropriate dietary pattern, including the right intake of Mg^{2+}, improves metabolic syndrome by reducing blood pressure, hyperglycemia, and hypertriglyceridemia. This occurs through the modulation of gene expression and proteomic profile as well as through a positive influence on the composition of the intestinal microbiota and the metabolism of vitamins B1 and D.

7. **Manoni, M.**, Cattaneo, D., Mazzoleni, S., Giromini, C., Baldi, A., Pinotti, L. **Milk Fat Globule Membrane Proteome and Micronutrients in the Milk Lipid Fraction: Insights into Milk Bioactive Compounds.** Dairy 2021, 2, 202–217. DOI: 10.3390/dairy2020018

Abstract: Milk lipids are composed of milk fat globules (MFGs) surrounded by the milk fat globule membrane (MFGM). MFGM protects MFGs from coalescence and enzymatic degradation. The milk lipid fraction is a "natural solvent" for macronutrients such as phospholipids, proteins and cholesterol, and micronutrients such as minerals and vitamins. The research focused largely on the polar lipids of MFGM, given their wide bioactive properties. In this review we discussed (i) the composition of MFGM proteome and its variations among species and phases of lactation and (ii) the micronutrient content of human and cow's milk lipid fraction. The major MFGM proteins are shared among species, but the molecular function and protein expression of MFGM proteins vary among species and phases of lactation. The main minerals in the milk lipid fraction are iron, zinc, copper and calcium, whereas the major vitamins are vitamin A, β-carotene, riboflavin and α-tocopherol. The update and the combination of this knowledge could lead to the exploitation of the MFGM proteome and the milk lipid fraction at nutritional, biological or technological levels. An example is the design of innovative and value-added products, such as MFGM-supplemented infant formulas.

8. Pinotti, L., **Manoni, M.**, Ferrari, L., Tretola, M., Cazzola, R., Givens, I. **The Contribution of Dietary Magnesium in Farm Animals and Human Nutrition.** Nutrients 2021, 13, 509. DOI: 10.3390/nu13020509

Abstract: Magnesium (Mg) is a mineral that plays an essential role as cofactor of more than 300 enzymes. Mg in farm animals' and human nutrition is recommended to avoid Mg deficiency, ensure adequate growth and health maintenance. Mg supplementation above the estimated minimum requirements is the best practice to improve farm animals' performances (fertility and yield) and food products' quality, since the performance of farm animals has grown in recent decades. Mg supplementation in pigs increases meat quality and sows' fertility; in poultry, it helps to avoid deficiencyrelated health conditions and to improve meat quality and egg production by laying hens; in dairy cows, it serves to avoid grass tetany and milk fever, two conditions related to hypomagnesaemia, and to support their growth. Thus, Mg supplementation increases food products' quality and prevents Mg deficiency in farm animals, ensuring an adequate Mg content in animal-source food. These latter are excellent Mg sources in human diets. Sub-optimal Mg intake by humans has several implications in bone development, muscle function, and health maintenance. This review summarizes the main knowledge about Mg in farm animals and in human nutrition.

9. Pinotti, L., Luciano, A., Ottoboni, M., **Manoni, M.**, Ferrari, L., Marchis, D., Tretola, M. **Recycling food leftovers in feed as opportunity to increase the sustainability of livestock production.** J. Clear Prod. 2021, 294, 126290. DOI: 10.1016/j.jclepro.2021.126290

Abstract: With the diminishing availability of farmland, climate change and the threat of declining water resources, livestock needs to meet the growing demand for food and feed by using fewer resources. The reuse of food losses as sustainable ingredients for feed formulations could represent a promising alternative to cereal grains for both monogastrics and ruminants, increasing livestock sustainability and reducing the competition between animal and human nutrition. The acceptance of food leftover for feeding animals it is still far to be completely welcomed in several countries, where the outdated stereotypical image of the garbage used as feed is still existing. To implement this practice, a renewed image of food leftover as feed is needed, mainly disseminating the most recent findings about their properties, the new technologies applied for their production and their impact on the environment. This paper aims to disseminate a wide understanding of food losses and explores the potential benefits of using two main categories of food leftovers, namely former food products (FFPs) and bakery by-products (BBPs), as alternative feed ingredients in pig and ruminant nutrition. Several characteristics of those two categories of food losses are examined and compared to a standard diet, such as nutritional-related properties, safety, efficiency and environmental implications. The literature shows that both categories of food leftovers hold a significant nutritional value and are a sustainable alternative to traditional feed ingredients. They resulted as a low risk category for animal health. In addition, when used in complete feed to replace traditional feed ingredients, neither FFPs nor BBPs do not decrease animal's growth performances. These findings valorize food losses into animal feed as a well-suited strategy to contribute to a reduced environmental and climate footprint of animal products and food waste prevention. However, a greater participation by feed/food processors and stakeholders is crucial to allow the sector to increase its contribution in the entire EU food and feed chain.

10. Luciano, A., Mazzoleni, S., Ottoboni, M., Tretola, M., Calvini, R., Ulrici, A., et al. **Former Foodstuff Products (FFPs) as Circular Feed: Types of Packaging Remnants and Methods for Their Detection.** Sustainability 2022, 14, 13911. DOI: 10.3390/su142113911

Abstract: Alternative feed ingredients in farm animal diets are a sustainable option from several perspectives. Former food products (FFPs) provide an interesting case study, as they represent a way of converting food industry losses into ingredients for the feed industry. A key concern regarding FFPs is the possible packaging residues that can become part of the product, leading to potential contamination of the feed. Although the level of contamination has been reported as negligible, to ensure a good risk evaluation and assessment of the presence of packaging remnants in FFPs, several

techniques have been proposed or are currently being studied, of which the main ones are summarized in this review. Accordingly visual inspections, computer vision (CV), multivariate image analysis (MIA), and electric nose (e-nose) are discussed. All the proposed methods work mainly by providing qualitative results, while further research is needed to quantify FFP-derived packaging remnants in feed and to evaluate feed safety as required by the food industries.

11. Ferrari, L., Fumagalli, F., Rizzi, N., Grandi, E., Vailati, S., **Manoni, M.**, et al. **An Eight-Year Survey on Aflatoxin B1 Indicates High Feed Safety in Animal Feed and Forages in Northern Italy.** Toxins 2022, 14, 763. DOI: 10.3390/toxins14110763

Abstract: Aflatoxins (AFs) remain the main concern for the agricultural and dairy industries due to their effects on the performances and quality of livestock production. Aflatoxins are always unavoidable and should be monitored. The objective of this paper is to bring to light a significant volume of data on AF contamination in several animal feed ingredients in Northern Italy. The Regional Breeders Association of Lombardy has been conducting a survey program to monitor mycotoxin contamination in animal feeds, and in this paper, we present data relating to AFB1 contamination. In most cases (95%), the concentrations were low enough to ensure compliance with the European Union's (EU's) maximum admitted levels for animal feed ingredients. However, the data show a high variability in AF contamination between different matrices and, within the same matrix, a high variability year over year. High levels of AFs were detected in maize and cotton, especially in the central part of the second decade of this century, i.e., 2015–2018, which has shown a higher risk of AF contamination in feed materials in Northern Italy. Variability due to climate change and the international commodity market affect future prospects to predict the presence of AFs. Supplier monitoring and control and reduced buying of contaminated raw materials, as well as performing analyses of each batch, help reduce AF spread.

12. Rakita, S., Kokić, B., **Manoni, M.**, Mazzoleni, S., Lin, P., Luciano, A., et al. **Cold-Pressed Oilseed Cakes as Alternative and Sustainable Feed Ingredients: A Review.** Foods 2023, 12, 432. DOI: 10.3390/foods12030432

Abstract: Due to the increasing demand for alternative protein feed ingredients, the utilization of oilseed by-products in animal nutrition has been sought as a promising solution to ensure cheap and environmentally sustainable feedstuffs. This review aimed to summarize the nutritional value of six cold-pressed cakes (rapeseed, hempseed, linseed, sunflower seed, camelina seed, and pumpkin seed) and the effects of their inclusion in diet for ruminant, pig, and poultry on nutrient digestibility, growth and productive performance, and quality of the products. The presented results indicated that these unconventional feed ingredients are a good protein and lipid source and have a balanced amino acid and fatty acid profile. However, contradictory results of animal production performances can be found

in the literature depending on the cake type and chemical composition, dietary inclusion level, animal category, and trial duration. Due to the substantial amount of essential fatty acid, these cakes can be efficiently used in the production of animal products rich in n-3 and n-6 polyunsaturated fatty acids. However, the utilization of cakes in pig and poultry nutrition is limited because of the presence of antinutritive factors that can deteriorate feed intake and nutrient utilization.

13. Pinotti, L., Ferrari, L., Fumagalli, F., Luciano, A., **Manoni, M.**, Mazzoleni, S., et al. **Review: Pig-based bioconversion: the use of former food products to keep nutrients in the food chain.** Animal 2023, 17, 100918. DOI: 10.1016/j.animal.2023.100918

Abstract: The primary challenge of agriculture and livestock production is to face the growing competition between food, feed, fibre, and fuel, converting them from resource-intensive to resource-efficient. A circular economy approach, using agricultural by-products/co-products, in the livestock production system would allow to reduce, reuse, and redistribute the resources. Former food products (FFPs), also named ex-foods, could represent a valid option in strengthening resilience in animal nutrition. FFPs have a promising potential to be included regularly in animal diets due to their nutritive value, although their potential in animal nutrition remains understudied. A thorough investigation of the compositional and dietary features, thus, is essential to provide new and fundamental insights to effectively reuse FFPs as upgraded products for swine nutrition. Safety aspects, such as the microbial load or the presence of packaging remnants, should be considered with caution. Here, with a holistic approach, we review several aspects of FFPs and their use as feed ingredients: the nutritional and functional evaluation, the impact of the inclusion of FFPs in pigs' diet on growth performance and welfare, and further aspects related to safety and sustainability of FFPs.

Further research activity – List of conference abstracts and posters

1. **Manoni, M.**, Rovere, N., Luciano, A., Fumagalli, F., Ottoboni, M., Bernardi, C.E.M., Pinotti, L. **Nutrients content and microbiological quality of salad crops as feed ingredient for ruminant diets.** European Federation of Animal Science (EAAP) Congress 2020 (poster)

Abstract: This study evaluated the nutritional composition and microbiological quality coupled with electronic nose odor profile of selected salad crops (SC). Eighteen sample SC deriving from a plant producing salad crops have been collected during the summer season in two sites: at the production site (factory plant) and after transport and one-day storage (to mimic farm site). The samples were then analyzed for pH, dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin. Eight selected samples were analysed for the enumeration of Total Viable Count (TVC) (ISO 4833), Enterobacteriaceae (ISO 21528-1), Escherichia coli (ISO 16649-1), coagulase-positive Staphylococci (CPS) (ISO 6888), presumptive B. cereus and its spores (ISO 7932), sulphite reducing Clostridia (ISO 7937), Yeasts and Moulds (ISO 21527-1) and the presence in 25 g of Salmonella spp. (ISO 6579). Data obtained were analysed using IBM SPSS Statistics version 21 software (SPSS Inc.). Salad crops at the production site (factory plant) and after transport and one-day storage (to mimic farm site), have shown pH value of 6.0 and 6.4, respectively. For the same material nutrient composition was comparable to that of common fresh grass: the overall mean of CP content was 210 g/kg DM, while average NDF, ADF and ADL contents were 360, 238, and 74 g/kg DM respectively. The main difference between fresh grass and SC was for DM content, that was lower (5.8±0.8 g/kg) in SC samples compared to literature grass values. Total Viable Count was limited and Salmonella was always absent. Salad crops has some potential as feed ingredient for ruminant's diets, even though a full verification of their potential need further deep investigation. The present work has been done in the frame of Progetto di Grande Rilavenza IT-RS "Sustainable animal nutrition" (SUN) funded the Ministero degli Affari Esteri e della Cooperazione Internazionale.

2. **Manoni, M.**, Luciano, A., Ottoboni, M., Ferrari, L., Tretola, M., Pinotti, L. **Effect of salty or sweet food leftover-based diets on serum metabolites in piglets.** "Associazione per la Scienza e le Produzioni Animali" (ASPA) Congress 2021 (poster)

Abstract: Nowadays, agriculture and livestock production face several challenges for what concerns sustainability. In particular, animal feed production competes with human food production for the use of lands and natural resources. The use of alternative feed ingredients could thus increase the sustainability of livestock production. In this study, conventional cereal grains have been partially replaced by confectionery or bakery former food products (FFPs) in pigs' diets to evaluate their effect on pig serum metabolites. FFPs are safe and healthy ingredients produced for human consumption,

but no longer intended for this purpose. Specifically, 36 post-weaning female piglets were fed with three different diets: (1) control diet (CTR), 0% FFPs; (2) FFP-C diet, (30% cereals replaced by 30% confectionery FFPs); (3) FFP-B diet, (30% cereals replaced by 30% bakery FFPs). The diets were formulated to meet the NRC requirements and to be iso-energetic (15.3MJ/kg DM) and iso-nitrogenous (19% CP DM). During the 42 days of trial, blood samples were collected on days 0 (t0), 21 (t1) and 42 (t2). The serum metabolites, measured using a standard enzymatic colorimetric analysis, were: total proteins, albumin, globulin, albumin/globulin (A/G), urea, alanine aminotransferase (ALT-GPT), aspartate aminotransferase (AST-GOT), alkaline phosphatase (ALP), total bilirubin, glucose, triglycerides (TG), non-esterified fatty acids (NEFA), α-amylase, total cholesterol, calcium, phosphorus, magnesium. Data were analyzed using one-way analysis of variance (one-way ANOVA) and further explored by principal component analysis (PCA). The results showed that the levels of serum metabolites were similar across the three experimental groups in the three different time points. The only exceptions were observed for α-amylase and TG. In particular, α-amylase tended to be lower in both experimental groups (FFP-C and FFP-B) in comparison with CRT group throughout the experiment, whereas TG were lower in the FFP-C experimental group only at t1. In conclusion, the inclusion of FFPs (up to a level of 30%) in post-weaning piglets' diet does not lead to a significant perturbation of the level of several serum metabolites, thereby enhancing the hypothesis of their reuse in animal nutrition. However, further studies are needed to strengthen these results and increase the knowledge.

3. **Manoni, M.**, Terranova, M., Amelchanka, S., Pinotti, L., Silacci, P., Tretola, M. **Ellagic acid and gallic acid can mitigate methane production and ammonia formation in an in vitro model of short-term rumen fermentation.** "Società Italiana delle Scienze Veterinarie" (SISVET) Congress 2022 (oral presentation)

Abstract: Livestock production is of particular importance for human nutrition, but it has a high environmental impact. In particular, ruminants are the major contributors to the total livestock greenhouse gas emissions. Several feeding strategies aimed to decrease the environmental footprint of ruminants were and are currently investigated. One of the most promising strategies is based on the dietary supplementation with tannins, that can mitigate methane production and ammonia formation, but also affect feed digestibility in ruminants. The aim of this study was to assess the effect of dietary supplementation with two metabolites of hydrolysable tannins, ellagic acid (EA) and gallic acid (GA), on gas production using an in vitro rumen fermentation system. Rumen fluid was collected from one of four fistulated, lactating cows in each of the four runs. Fermenters were fed with a standard diet (200 mg DM hay). Five different supplementation conditions were investigated (% of DM): i) EA 7.5, ii) EA 15, iii) GA 7.5, iv) GA 15, v) EA 7.5 + GA 7.5. After an incubation of 24 h at 39 °C, several parameters were measured with the rumen samples: pH, ammonia, total SCFA production,

microbial count (bacteria and protozoa) and in vitro dry matter digestibility (IVDMD). After gas phase collection, total gas production, methane and CO_2 were evaluated using gas chromatography. The treatments did not affect microbial count and pH (P>0.05). Total SCFA production decreased by 10% after all treatments (P<0.001). Total gas production decreased by 10% after all treatments (P<0.05). Methane production was significantly decreased by EA 15 (-20%, P< 0.001) and EA+GA (-25%, P< 0.001) treatments. CO_2 production was also decreased (P<0.001), but to a lesser extent as compared to methane. Ammonia production was significantly decreased by EA 15 (-13%, P<0.001) and EA+GA (-20%, P<0.001). All the treatments except GA 7.5 caused a slight but significant 10% reduction of IVDMD (P<0.001). These results showed that EA and GA can decrease gas production but affect digestibility of dry matter. The high doses of EA and GA were used to mimic a diet including natural extracts rich in hydrolysable tannins. Indeed, further work will concentrate on the dosage of tannin-secondary metabolites from rumen samples and on long-term in vitro incubation screening also with lower doses of metabolites, in order to see whether the decrease in gas production and decrease digestibility can be dissociated.

4. **Manoni, M.**, Terranova, M., Amelchanka, S., Pinotti, L., Silacci, P., Tretola, M. **Ellagic acid and gallic acid mitigate methane production in an in vitro model of rumen fermentation.** European Federation of Animal Science (EAAP) Congress 2022– **Winner of Scholarship EAAP 2022** (oral presentation)

Abstract: Ruminant production accounts for 81% of greenhouse gas emissions in the livestock sector. Of this quantity, enteric fermentation methane accounts for 90%. Dietary supplementation with tannins is known to mitigate methane production, but also affect feed digestibility. The aim of this study was to investigate the effect of ellagic acid (EA) and gallic acid (GA), alone or in combination, on rumen fermentation in a short-term in vitro experiment using Hohenheim Gas Test. EA and GA were supplemented to a control diet (hay, 200 mg DM). Five different conditions were applied to this study (% of DM): i) EA 7.5, ii) EA 15, iii) GA 7.5, iv) GA 15 and v) EA 7.5 + GA 7.5. After an incubation of 24 h at 39°C, pH, ammonia formation, gas production, short-chain fatty acids (SCFA), in vitro organic matter digestibility (IVOMD) and the microbial count were determined. The treatments did not alter microbial count and pH. Total SCFA production decreased by approximately 10% after all treatments (P<0.001). In the ruminal SCFA profile, the differences were still significant (except for valeric acid) but less evident than total SCFA. Total gas production slightly decreased (-10%) after all treatments, except for GA 7.5. EA 15 and EA+GA treatments decreased methane production per g DM by 20% and 25%, respectively. These two treatments decreased by 10% the production of CO_2 per g DM and the production of ammonia on DM basis, respectively by 13% and 20%. All the treatments caused a slight but significant 10% reduction of IVOMD. In conclusion, diet supplementation with EA and GA, alone or in combination, may be a promising dietary strategy to mitigate methane production in ruminants.

Further long-term in vitro ruminal fermentation studies are needed to validate these results, before assessing these treatments in vivo.

5. **Manoni, M.**, Luciano, A., Mazzoleni, S., Ottoboni, M., Tretola, M., Pinotti, L. **Inclusion of former food products in post-weaning piglets' diet: impact on the fatty acid profile of subcutaneous adipose tissue and on serum metabolites.** American Society of Animal Science (ASAS) Congress 2023 (oral presentation)

Abstract: Currently, the global demand for food is expected to raise significantly by 2050, when the global population will reach around 10 billion. In particular, animal-source products are in high demand. However, one third of the global food production is wasted every year, thus causing a significant environmental footprint in terms of greenhouse gas (GHG) emissions. Also, livestock production is facing the feed-food competition, use of natural resources and environmental sustainability. To address these issues, the use of food by-products in animal nutrition could satisfy the principles of circular economy. Among the different food by-products, former food products (FFPs) consist mainly of unsold/defective food from industrial production (i.e. bread, croissants, biscuits, chips). FFPs are classified in sugary confectionary FFPs (FFPs-C) and salty bakery FFPs (FFPs-B). The aim of this study was to assess the impact of the inclusion of FFPs into post-weaning piglets' diet on the fatty acid profile of subcutaneous adipose tissue and on serum metabolites. Thirty-six post-weaning female piglets (Large White × Landrace, BW 8.52 ± 1.73 kg) were assigned to a standard diet (CTR), or with a 30% replacement (w/w) of conventional cereals with FFPs-C or FFPs-B for 42 days. Growth performance and feeding behaviour were measured. Subcutaneous abdominal fat samples were collected from 12 selected piglets after slaughtering. Gas chromatography was performed on feed samples from control diet and experimental diets and on adipose tissue samples. Untargeted metabolomics was performed on blood samples (d 0 and d 42) using UHPLC/MS-MS. Data were analyzed with the software MetaboAnalyst (version 5.0). The inclusion of FFPs did not alter growth performance and feeding behaviour. The CTR diet had higher PUFA level than FFPs diets. In FFPs diets, the fatty acid profile was equally distributed among SFA, MUFA, and PUFA. The ω-6/ω-3 ratio was higher in FFPs-C diets than CTR and FFPs-B diets. The subcutaneous adipose tissue showed no differences between groups for SFA level. The MUFA level was reduced in CTR compared to FFPs groups, whereas PUFA level was reduced in FFPs-C group compared to FFPs-B and CTR groups. The ω-6/ω-3 ratio was lower in FFPs-C group compared to CTR and FFPs-B groups. The untargeted metabolomics identified a total of 104 metabolites, with 2 significantly different between groups. Specifically, the serum concentration of theobromine and caffeine was higher in FFPs groups compared to CTR. To conclude, piglets were able to rebalance the fatty acids profile despite the differences observed in the diets. The metabolic status was not strongly altered by the inclusion of 30% FFPs in the diet. Thus, FFPs could be safely applied in post-weaning pig diets. Further studies are needed to

assess the effect of FFPs on pig physiology and metabolic status in growing phase pigs and for a longer feeding time.

6. **Manoni, M.**, Amelchanka, S., Terranova, M., Pinotti, L., Silacci, P., Tretola, M. **Effect of hydrolysable tannins' metabolites on feed fermentation, gas production and ruminal ecosystem using a long-term in vitro rumen fermentation approach (Rusitec).** "Società Italiana delle Scienze Veterinarie" (SISVET) Congress 2023 (oral presentation)

Abstract: Livestock production is currently facing the growing demand for animal-source food and the need to reduce the impact on the environment. Ruminants are among the largest contributors to greenhouse gas (GHG) emissions, in particular methane (CH_4). Different strategies to lower greenhouse gas (GHG) emissions in ruminants are being investigated, and one of them relies on the dietary tannin supplementation. The dietary supplementation with tannins in ruminants can affect the rumen microbial community and thus the enteric fermentation, leading to reduced methane emissions. Following a previous in vitro screening, we investigated the effect of two hydrolysable tannin metabolites, named ellagic acid (EA) and gallic acid (GA), in a long-term in vitro rumen fermentation approach. EA and GA were supplemented to a control diet (CTR: ryegrass hay and barley concentrate (7.5:2.5), 10 g DM/day) in an 8-fermenter rumen simulation technique (Rusitec), running for 10 days. Three experimental conditions were investigated: i) EA 75 mg/g DM, ii) GA 75 mg/g DM, iii) EA 75 mg/g DM + GA 75 mg/g DM. The measurements were performed in the last 5 days of the experimental period. Total gas production was not significantly altered over the last 5 days, whereas daily CH_4 production was significantly decreased by EA (-45%) and EA+GA (-60%), compared to control. CH4 production per unit of dietary organic matter (OM) and short-chain fatty acids (SCFA) was also reduced by EA (-48% and -32%) and EA+GA (-65% and -58%), and less by GA (-19% and -22%). Ammonia formation was significantly reduced by EA (-46%), GA (-19%) and EA+GA (-86%). Total SCFA production was decreased by EA and EA+GA (-26%, -16%), but not by GA. Similarly, EA and EA+GA, but not GA, reduced rumen degradability of OM, crude fibre (CF) and crude protein (CP). All the treatments increased the bacterial count and decreased the protozoal count (except for GA). Furthermore, EA and EA+GA modulated the relative abundance of selected fibrolytic and cellulolytic rumen bacterial taxa. The results showed that both EA and GA decreased average daily CH4 production and ammonia formation, with EA being most effective than GA for almost all the parameters observed. Nevertheless, GA showed a lower impairing effect on the degradability of nutrients in rumen and on SCFA production. Further research on the dynamics of rumen microbiota and the metabolism of hydrolysable tannins in rumen will strengthen the outcome of this study.

7. **Manoni, M.**, Luciano, A., Mazzoleni, S., Ottoboni, M., Tretola, M., Pinotti, L. **Effect of the**

dietary inclusion of former food products on subcutaneous adipose tissue and on serum metabolites in post-weaning piglets. "Società Italiana delle Scienze Veterinarie" (SISVET) Congress 2023 – **Winner Poster Award 2023 (ARNA Society)** (poster)

Abstract: The 30% of food production is lost after harvest and wasted in retail and households. This amount accounts for 10% of global greenhouse gas (GHG) emissions. In parallel, animal feed production is the largest cost of livestock production, accounting for around 40% of GHG emissions of livestock production. The use of food by-products as feed ingredients could reduce food waste and increase environmental sustainability. Among food by-products, former food products (FFPs) generate high amounts of waste. FFPs are divided into sugary confectionary FFPs (FFPs-C) and salty bakery FFPs (FFPs-B). The aim of this study was to assess the impact of the inclusion of FFPs into post-weaning piglets' diet on fatty acid profile of subcutaneous adipose tissue and on selected metabolites. Thirty-six post-weaning female piglets (Large White × Landrace) were assigned to a standard diet (CTR), or with a 30% replacement of conventional cereals with FFPs-C or FFPs-B for 42 days. Growth performance and feeding behaviour were measured. Subcutaneous abdominal fat samples were collected from 12 selected piglets after slaughtering. Gas chromatography was performed on feed samples and adipose tissue samples. Untargeted metabolomics was performed on blood samples with UHPLC/MS-MS. Data were analyzed with MetaboAnalyst (v5.0). The growth performance and feeding behaviour were not altered by FFPs. The PUFA level was significantly higher ($P<0.05$) in CTR (47%) than FFPs-C (34%) and FFPs-B (27%) diets. In FFPs diets, the fatty acid profile was equally distributed ($P>0.05$) among SFA, MUFA, and PUFA. The subcutaneous adipose tissue showed no differences ($P>0.05$) between groups for SFA level. The MUFA level was reduced ($P<0.001$) in CTR (34%) compared to FFPs-C (41%) and FFPs-B (40%), whereas PUFA level was reduced ($P<0.01$) in FFPs-C (12%) compared to FFPs-B (19%) and CTR (20%). The untargeted metabolomics identified a total of 104 metabolites, with only two being significantly different ($P<0.001$) between groups. Specifically, the serum concentration of theobromine and caffeine was higher in FFPs groups compared to CTR. To conclude, piglets rebalanced the fatty acids profile despite the differences in the diets. The metabolic status was not deeply altered by the dietary inclusion of FFPs. Thus, FFPs could be safely applied in post-weaning pig diets. Further studies are needed to assess the effect of FFPs on pig physiology and metabolic status in growing phase and for a longer feeding time.

8. **Manoni, M.**, Amelchanka, S., Terranova, M., Pinotti, L., Silacci, P., Tretola, M. **Ellagic acid and gallic acid reduced methane and ammonia in an in vitro rumen fermentation model.** European Federation of Animal Science (EAAP) Congress 2023 (poster)

Abstract: Livestock production must meet the growing demand for animal-source food and reduce the impact on the environment. Different strategies are studied to lower greenhouse gas (GHG)

emissions in ruminants, and one of them is tannin supplementation. The dietary supplementation with tannins can affect the enteric fermentation, reducing methane emissions. Following a previous screening, we investigated the effect of two hydrolysable tannins, ellagic acid (EA) and gallic acid (GA), in a long-term *in vitro* rumen fermentation. EA and GA were supplemented to a control diet (CTR: ryegrass hay and barley concentrate, 10 g DM/day) in an 8-fermenter rumen simulation technique (Rusitec), for 10 days. Three experimental conditions were investigated: i) EA 75 mg/g DM, ii) GA 75 mg/g DM, iii) EA 75 mg/g DM + GA 75 mg/g DM. The data were collected in the last 5 days of the incubation time. Total gas production was not altered over the last 5 days, whereas daily methane (CH_4) production was significantly decreased by EA (-45%) and EA+GA (-60%), compared to control. CH_4 production per unit of dietary organic matter (OM) and short-chain fatty acids (SCFA) was also reduced by EA (-48% and -32%) and EA+GA (-65% and -58%), and less by GA (-19% and -22%). Ammonia formation was significantly reduced by EA (-46%), GA (-19%) and EA+GA (-86%). Total SCFA production was decreased by EA and EA+GA (-26%, -16%), but not by GA. Similarly, EA and EA+GA, but not GA, reduced rumen degradability of OM, crude fibre (CF) and crude protein (CP). All the treatments increased the bacterial count and decreased the protozoal count (except for GA). EA and EA+GA modulated the relative abundance of selected rumen bacterial taxa. These data proved that both EA and GA decreased GHG emissions and ammonia formation, with EA being most effective than GA. Nevertheless, GA showed a lower interfering effect on nutrient rumen degradability. Further details on rumen microbiota dynamics and hydrolysable tannins metabolism will complete the outcome of the study.