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Water buffalo microbiota and immunity during infectious diseases

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Abbreviations

AGP: α_1-acid glycoprotein

AIM2: absent in melanoma 2

Ang4: angiogenin-4

APP: Acute Phase Protein

APR: Acute Phase Reaction

ASV: Amplicon Sequence Variants

AUC: Area Under the Curve

BCVs: Brucella-containing vacuoles

BHBA: β-hydroxybutyrate

BiP: Binding Immunoglobulin Protein

CD14: cluster of differentiation 14

CD16: cluster of differentiation 16

DGP: neutrophils degranulation products

Dicer: Dicer 1, Ribonuclease III

Drosha: Drosha Ribonuclease III

dsRBPs: dsRNA-binding proteins

ER: endoplasmic reticulum

FOXP3: forkhead box P3

GPR: G-protein-coupled receptor

GRAS: Generally Recognised As Safe

Hp: haptoglobin

IFNγ: γ-Interferon IL1β

IKK: IκB kinase

IL1β: Interleukin 1, beta

IL10: Interleukin 10

IL-17A: Interleukin 17A

IL18: Interleukin 18

IL4: Interleukin 4

IL6: Interleukin 6

IL8: Interleukin 8

ILCs: Innate lymphoid cells

IRE1: inositol-requiring enzyme 1

Itln1: Human intelectin-1

LAB: Lactic Acid Bacteria

LPS: Lipopolysaccharides

LTA: Lipoteichoic Acid

MAL: MyD88-adaptor like

miRNA: micro-RNA

MRE: miRNA regulatory element

MVBs: multivesicular bodies

MyD88: Myeloid differentiation primary response 88

NEB: negative energy balance

NEFA: non-esterified fatty acids

NFkB: nuclear factor kappa-light-chain-enhancer of activated B cells

NGS: Next-generation Sequencing

NLRs: nucleotide-binding oligomerization-like receptors

NOD: nucleotide-binding oligomerization

NOS2: nitric oxide synthase 2

OAS: oligoadenylate synthase

OTUs: Operational Taxonomic Units

PACT: protein activator of PKR

PAMPs: pathogen-associated molecular patterns

PBMC: Peripheral Blood Mononuclear Cells

piRNA: Piwi-interacting RNAs

PMN: Polymorphonuclear cells

PPD: Protein Purified Derivates

PRR: pattern recognition receptor

RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted

RegIIIβ: Regenerating islet-derived protein 3 beta

RegIIIγ: Regenerating islet-derived protein 3 gamma

RIGI: retinoic acid-inducible gene

RISC: RNA-induced silencing complex

RNAPII: RNA polymerase II

RORC: RAR Related Orphan Receptor C

ROS: reactive oxygen species

SAA: Serum Amyloid A

SCC: Somatic Cell Count

siRNA: silencing RNAs

STAT3: Signal transducer and activator of transcription 3

STAT4: Signal transducer and activator of transcription 4

STAT5B: Signal Transducer And Activator Of Transcription 5B

TBET: T-box transcription factor

Th1: Type 1 T lymphocytes

Th17: Type 17 T lymphocytes

Th2: Type 2 T lymphocytes

TLRs: Toll-like receptors

TNFα: Tumor Necrosis Factor α

TRBP: transactivation- responsive (TAR) RNA-binding protein

TReg: Regulatory Type T lymphocytes

TSH: Thyroid-stimulating hormone

TIT: single intradermal tuberculin skin test

UPR: unfolded protein response

UTR: Untranslated region

XBP1: X-box binding protein 1

Abstract

The thesis aims to investigate the immune response of water buffaloes (*Bubalus bubalis*) during infectious diseases (e.g. mastitis, brucellosis and tuberculosis) and productions diseases, such as peripartum related metabolic diseases or stress. Given the relationship between the microbial environment and the immune system, the microbiota content of milk has been identified as well.

The immune system of water buffalo has been poorly addressed so far, not to mention the microbiota, which was unknown. Dairy water buffaloes are sensitive to the same diseases as dairy ruminants, such as for example mastitis, tuberculosis, brucellosis, but the impact of these diseases on water buffaloes' immune system are unknown. Water buffalo presents anatomical (e.g. at mammary gland and skin level) and physiological (peripartum and infectious diseases) differences as compared to cow and other dairy ruminants. Therefore, it is evident that both the immune system and microbiota could present several differences.

In my PhD thesis, I tried to address some of these issues. Firstly, the milk microbiota of water buffaloes was investigated in relation to mastitis disease. Secondly, the evaluation of the immune response, in terms of gene expression ad miRNAs, was carried out in animals affected by brucellosis and tuberculosis. Finally, the characterization of the transition period was performed measuring acute phase proteins to assess the inflammation status during the transition period.

The water buffalo milk microbiota was described herein for the first time. The variation in composition and structure was demonstrated depending on the pathological status of the animals. A change in milk microbial community was observed in clinical and subclinical mastitis-affected quarter milk samples as compared with a health condition, as well as a reduction of microbiota diversity in mastitis-affected quarters.

The treatment of subclinical mastitis quarters using inactivated cultures of *Lactobacillus rhamnosus* was performed in order to unravel the effect of alternative strategies to the therapeutic use of antibiotics. A chemotactic response at quarter level was observed, but no change neither in milk microbiota nor in microbiological culture was found after treatment, suggesting that the use of pro- and pre-biotic for mastitis treatment needs to be further investigated.

Given that, so far and in cow, each udder quarter has been regarded as an entity not related to the other three, the hypothesis of the presence of interdependence within milk udder quarters

was demonstrated, studying the intra- and inter-individual variation in milk microbiota. Individual signatures may explain the higher similarity between milk microbiota of quarters within the same udder, suggesting that the independence of milk quarters has been challenged and needs to be taken into account when performing microbiota studies.
Finally, in order to improve the taxonomic resolution of the experimental design, milk microbiota was characterized using a longer amplicon sequencing by using the full-length 16S marker (~ 1500pb) taking advantage of Nanopore technology, that confirmed the higher resolution power as compared with short-read sequencing (~ 250pb) and could provide a supportive role for microbiological culture technique.

The investigation of a possible biomarker for brucellosis in vaginal fluid and serum revealed that levels of 10 miRNAs were modulated in vaginal secretion of seropositive compared to seronegative animals. Furthermore, the diagnostic accuracy of the combination of six miRNAs was good with a sensitivity of 95.45 and a specificity of 85, suggesting that a multi-miRNAs panel would allow an early and fast procedure for brucellosis diagnosis.

A modification in the immune response during tuberculosis disease was observed, aiming to address the main issue of false-positive during routine TB diagnosis. An unbalanced of cytokines expression toward Th1 reaction was found in *M. bovis* positive animals after intra-dermal tuberculin test as compared to those *M. avium* positives. The positive correlation of the expression of IFNγ with two transcriptional factors related to Th1 polarization (TBET and STAT4) demonstrates the pro-inflammatory activity present in animals with tuberculosis.

Acute phase proteins (APPs) were also found to be modulated during peripartum, An increase of Serum Amyloid A (SAA) and haptoglobin (Hp) were observed in the first week after parturition, while the increase of the α_1-acid glycoprotein (AGP) concentrations from prepartum to early lactation was later than for Hp and SAA and was also not limited to a peak value but was continued until 6 weeks post-partum.

Chapter 1: The water buffalo as livestock

The domesticated water buffalo (or Asian buffalo) is scientifically named *Bubalus bubalis*. The two major categories of water buffaloes include river and swamp water buffalo. River buffalo is found in the Middle East, India, Eastern Europe and Italy, while swamp buffalo is distributed in China, Bangladesh and the Southeast Asian countries (Napolitano et al., 2013). Approximately 168 millions of water buffaloes are present worldwide, of which 161 million are located in Asia (95.8%), 3,717 million in Africa (2.24%), 3.3 million in South America (1.96%), 40 000 in Australia (0.02%) and 500 000 in Europe (0.30%) with about 300,000 heads in Italy (www.fao.org).

The Asian water buffalo was domesticated independently in India and China from the wild population of animals similar to *Bubalus arnee* 4000 – 5000 years ago. Water buffalo is a very rustic, adaptable and resilient animal, which is able to survive with coarse feed, straw and crop. It is well-adapted to wet and humid climates, but thermoregulation must be controlled in order to avoid alteration in the animal metabolism. The high susceptibility to thermal stress is due to the low presence of sweat glands and the consequent weak heat dissipation. For this reason, it requires shade and needs to wallow. Similarly to the cow, water buffalo spends 99% of its waking hours ingesting food, ruminating, resting and drinking water; the remaining 1% of the time includes locomotion and other activities (De La Cruz-cruz et al., 2014). This species is used for milk and meat production but also for fork (mainly the swamp type).

Within the scientific classification, water buffaloes and cows belong to the same family (*Bovidae*). However, although the general aspect is similar, water buffaloes have individual signatures, specific of this species. They differ in the dimensions (the water buffaloes is stockier), in the horn form, in the number of chromosomes (48 for swamp and 50 for river buffaloes instead of 60 for cows). The skin is thicker with little hair; the quantity of hair follicles is low (135 – 145 in buffaloes as compared to 2164-2717 in cows) and depends on age, lactation, seasons and other factors (Mason, 1974). The oily skin is due to the presence of more sebaceous glands as compared to cows, while the frequency of sweat glands is very limited. The sexual dimorphism is not so evident: males reach 7-8 quintals, females 6-6.5 quintals. Estrus is not easy to recognize as well (Di Palo et al., 2001). The estrus period is extremely variable (from a few hours to 2-3 days) with a mean of 23 hours. The gestation time is 316 days (from 312 to 321 days), so it is longer than that in the cow (280 days) of about 1 month. Water buffaloes are long-lived animals. They can live up to 20 years or more and the

milk production increases with age. The main characteristics of water buffaloes and comparisons with cow are presented in Table 1.

Table 1 Biological characteristics of water buffaloes as compared to cows

(Hopper, 2014; A. Presicce, 2017; Eastham et al., 2018)

Characteristic	**Water buffalo**	**Cow**
Optimal age for reproduction	24 months	24 months
Lactation period	270 days	360 days
Reproductive cycle	21 days	21 days
Estrus period	1-48 hours	12-18 hours
Gestation period	310 days	280 days
Time between deliveries	14 months	18 months
Milk per day	8-15 L	25-40L
Reproductive career	15 years	3-5 years

The mammary gland of water buffalo strongly differs from that of the cow. The cistern of water buffaloes is smaller and represent 5% of the total volume (~ 10L) as compared to the 25% out of the total volume of~ 30L in cows. Two oxytocin peaks during the milking process are necessary for water buffaloes to fill the cistern, while only one is required for cows. The sphincter is more resistant due to the presence of muscular fibres and is able to recover tonicity in an easier and faster way as compared to bovine, suggesting its role in protection from mastitis. Another mechanism relevant in terms of protection to infection is represented by the presence of high-fat amount that can have a safety role covering the epithelial layer. Furthermore, the teats are longer and the proportion between front/rear quarters is 60/40 (50/50 in cows) (Thomas CS, Svennersten-Sjaunja K, Bhosrekar MR, 2004)

Water buffaloes represent one of the main animal producers in South Asia provide for what concerns milk and meat (www.fao.org). The worldwide production and their profiles are shown in Figure 1.

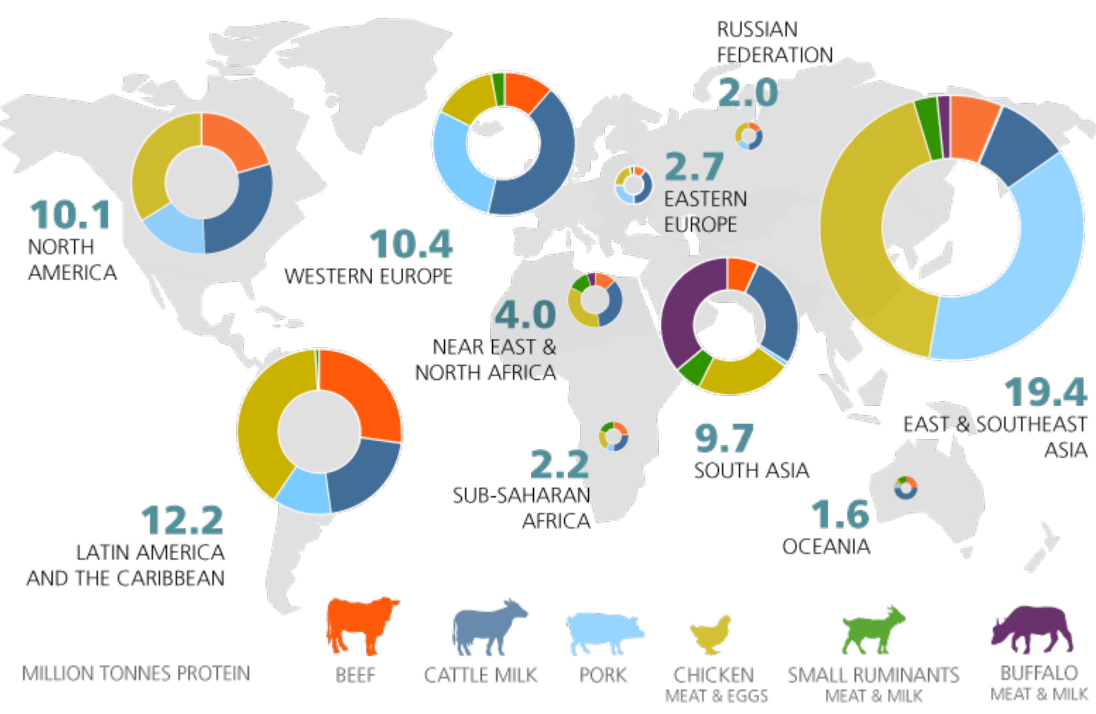

Figure 1 Production percentages of meat and milk worldwide

(from www.fao.org)

World milk production has increased by more than 50% between 1983 and 2013, reaching 769 million tonnes. India is the country with the largest production of milk (18%), followed by United States (12%), China and Brazil (5%) and Russia and Germany (4%). Although 83% of global milk production derives from cattle, water buffalo's dairy production accounts for 14% and the remaining 3% derived from goat, sheep and camel (the last one only 0.1%) (www.fao.org). Water buffalo milk differs from one of other dairy ruminants, including cow, sheep and goat milk (Table 2). It has a higher amount of protein and fat as compared to a cow, with a higher nutritional value. These characteristics are optimal for cheese and other products preparation, among which soft cheeses (e.g. mozzarella), fermented milk (e.g. yogurt), butter, whey (www.fao.org).

Table 2 Composition of human, cow, buffalo, goat and sheep milk

(per 100 g of milk)* (from www.fao.org)

Proximates	Human	Cow		Buffalo		Goat		Sheep	
	Average	Average	Range	Average	Range	Average	Range	Average	Range
Energy (kJ)	291	262	247–274	412	296–495	270	243–289	420	388–451
Energy (kcal)	70	62	59–66	99	71–118	66	58–74	100	93–108
Water (g)	87.5	87.8	87.3–88.1	83.2	82.3–84.0	87.7	86.4–89.0	82.1	80.7–83.0
Total protein (g)	1.0	3.3	3.2–3.4	4.0	2.7–4.6	3.4	2.9–3.8	5.6	5.4–6.0
Total fat (g)	4.4	3.3	3.1–3.3	7.5	5.3–9.0	3.9	3.3–4.5	6.4	5.8–7.0
Lactose (g)	6.9	4.7	4.5–5.1	4.4	3.2–4.9	4.4	4.2–4.5	5.1	4.5–5.4
Ash	0.2	0.7	0.7–0.7	0.8	0.7–0.8	0.8	0.8–0.8	0.9	0.9–1.0

Chapter 2: Water Buffalo mastitis

2.1. Water buffalo mastitis

Mastitis is defined as the inflammation of the mammary gland. Microorganisms are the most frequent cause of mastitis. Contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*, environmental pathogens such as *Streptococcus uberis, disgalactiae*, Coliforms such as *Escherichia coli, Klebsiella* and *Enterobacter, Pseudomonas*, *Prototheca* and yeasts are the most prevalent agents causing infections in water buffalo (Fagiolo and Lai, 2007). Culture-dependent approaches demonstrated that most frequently isolated bacteria during mastitis are coagulase negative, causing 78% of intramammary infections cases of mastitis (Moroni et al., 2006a; Locatelli et al., 2013). *Prototheca* spp. and *Streptococcus pluranimalium* have been found occasionally (Capra et al., 2014; Guccione et al., 2016).

Water buffalo is regarded as less susceptible than a dairy cow to mastitis; however, mastitis in this species still represents a major economic concern. In particular, in developing countries where the animals are farmed in conditions that sometimes do not respect the basic hygiene requirements. The resistance of water buffalo to mastitis is partly due to anatomical factors. The narrow teat canal with a thicker epithelium and keratin layer, the teat skin less sensitive to chapping and sores and the presence of smoother muscular fibres in the teat sphincter of buffalo, as well as the relatively small cistern, have an important role in preventing the microorganism invasion (Thomas CS, Svennersten-Sjaunja K, Bhosrekar MR, 2004). Nevertheless, the longer teats and the pendulous udder of buffalo, with the high quality of milk in term of nutrients, may increase the risk of mastitis, as compared with the cow. The molecular aspects that are at the background of water buffalo relative resistance to mastitis are unknown.

2.2 The water buffalo mammary gland and cellular and immune defences

The bubaline mammary gland starts to grow in prenatal fetuses at 90-109 days of pregnancy, but the full development with the differentiated duct system develops at 251-254 days of gestation (Singh and Roy, 2003). The final functional development occurs before parturition. During early lactation, the cellular junctions in the mammary alveoli are 19.78±0.99 nm wide and 174.16±22.36 nm long and the gap junctions between external and internal nuclear

membranes are 36.39±1.75 nm in fully lactating cells (Laila, 2005; Purohit et al., 2014). Concerning teat dimension and functions, buffalo teats differ from those of cows for some aspects, as already described in chapter 1. Upon milk ejection the teat length and thickness is about 10% more as compared to cows (teat canal length of 3cm vs 0.5-1.5cm, respectively); the cistern of buffaloes is smaller than that in cows (22 cm^2 vs 40-45 cm^2, respectively) and it is larger in early as compared to late lactation (Thomas, 2004). It is demonstrated that buffaloes are able to store a lower milk volume in the cistern after a milking interval of 10 to 11 hours. The milk synthesized between milking in buffalo is stored in the alveolar lumen and the small ducts with low drainage of milk to the cistern. In buffaloes, the milk ejection is based on the same neuro-hormonal mechanisms as in cattle, sheep and goats (Thomas, 2004; Purohit et al., 2014). The process of milk secretion in the dairy cow udder is shown in Figure 2 and is presented as a model for water buffalo milk secretion

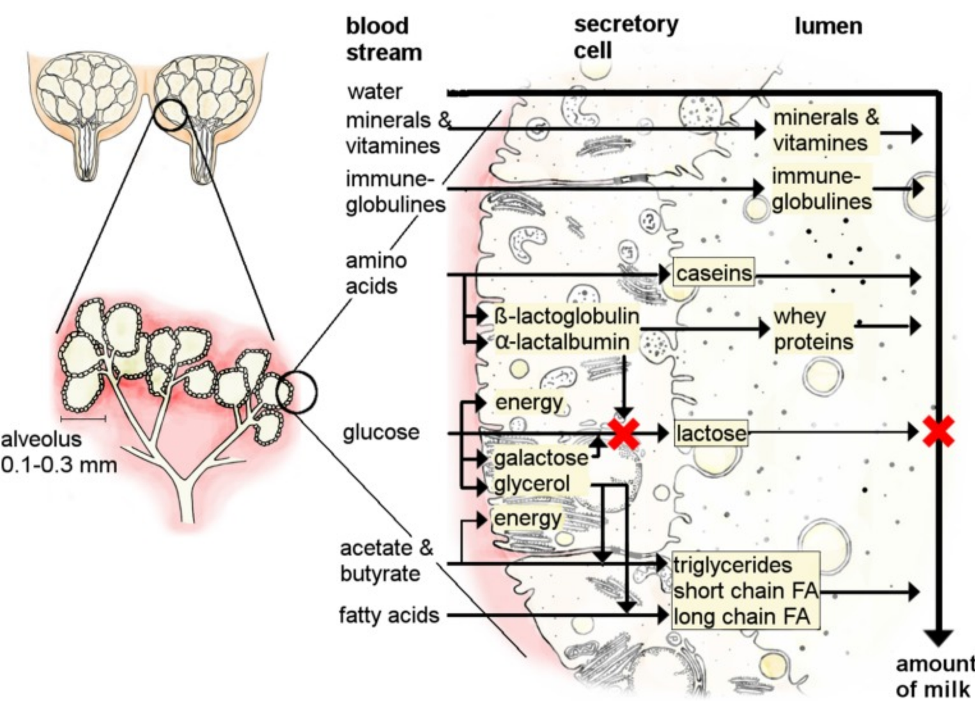

Figure 2 milk secretion process in the udder.

Milk is secreted in the alveoli system of the mammary gland. Several substances can pass the cell membrane from the bloodstream; water, minerals, vitamins, immune-globulins can pass the cell membrane from the blood vessels and others (e.g. proteins, fat, lactose) are produced in the secretory cells (proteins, fat, lactose) (from (Strucken et al., 2015)) .

During infection, polymorphonuclear leukocytes infiltrate the mammary gland as a physiological defensive reaction. Even though the number of Somatic Cell Count (SCC)

increases proportionally to the decrease of milk production, the relationship between infection and SCC in water buffalo is not clear. An increase in SCC can be physiological in response to the activation of the immune system, although, within the milk file chain, is regarded as negative, because it affects the quality of the milk and is believed to be marked of bacterial presence in the mammary gland. In cows, the presence of negative quarters in term of microbiology and SCC < 100.000 cells/ml defined an animal as healthy, while SCC > 200.000 cells/ml are considered as an indicator of subclinical mastitis. On the contrary, in water buffaloes a normal range of SCC, can vary between 50.000 and 375.000 cells/ml (Fagiolo and Lai, 2007), but it can increase progressively during age and parturition (> 400.000 cells/ml) (Ceron-Munoz et al., 2002; Piccinini et al., 2006). Therefore, the relationship between the increase of SCC and the insurgency of intramammary infection in water buffaloes is still under investigation and a matter of discussion.

Late innate immune response during mastitis has been largely studied in cattle, but not in buffaloes. Microbiome environment and immunity response are regarded as relevant factors in determining and controlling infections. The low sensibility of water buffalos to intramammary infection still has to be understood and probably the mechanisms involved in mastitis onset and developing are different from the cow. If anatomical and physical barrier is not intact, bacteria can establish in the mammary gland tissue. It happens mainly around the parturition and in early lactation, and a reduced SCC can increase the risk of mastitis. The late innate immune response begins its function with the bond between a pattern recognition receptor (PRR) located on or within the host cell and the pathogen-associated molecular patterns (PAMPS), which is unspecific and derives from the microorganism. In particular, PRR expressed on two types of cells in the mammary gland: leucocytes and epithelial cells. In cattle, ten Toll-like receptors - a type of PRR - are known. The innate response is central in mastitis process: bacteria are phagocytosed and killed through the action of superoxide ions, hypochlorite, and hydrolytic enzymes produced by macrophages and neutrophils, and lactoferrin and defensins synthesized by epithelial cells. The activation of TLR leads to the synthesis of transcription factors, (e.g. NFkB), which are important for the immune modulation. CD14 is another receptor found on neutrophils and macrophages in the mammary gland: it binds LPS-protein complexes, inducing the transcription and the translation of TNFα and its release. In other words, after the first recognition, leucocytes and neutrophils are attracted from blood due to the chemokine's environment, such as IL-8 and RANTES, and then activated in the mammary gland through cytokines. The response of the immune system firstly depends on the type of bacteria causing the infection. In gram-negative *E. coli* infection,

the LPS-TLR-4 binding between bacteria and epithelial mammary cells, respectively, induces a rapid and strong increase of SCC, antimicrobial defense protein such as lactoferrin and lysozyme, lipid mediators, and an intense release of TNFα and cytokines, being responsible for severe clinical signs and fever, including sometimes the death of the animal due to septicemia. Conversely, the Gram-positive *S. aureus* causes a more moderate and delayed increase of SCC, leading to subclinical and chronic infections. In addition, TNFα is synthesized but in a reduced amount, which explains the afebrile forms of *S. aureus* mastitis. LPS induces the activation of the immune response for what concerns Gram-negative bacteria, while peptidoglycans and lipoteichoic acid (LTA), which are recognized by TLR-2, are the major molecules involved during Gram-positive-derived infections. This binding with peptidoglycans and LTA induces the expression of pro-inflammatory cytokines such as TNFα and IL-6 and chemokines like IL-8, but in lower concentration than in mastitis caused by Gram-negative bacteria (Rainard and Riollet, 2006; Wellnitz and Bruckmaier, 2012).

The humoral factors that determine which kind of innate and adaptive immunity defensive strategy is activated in response to intramammary infection in water buffaloes are mostly unknown. Lymphocytes play a strategic role. There is a precise order of cytokine upregulation and the cytokine expression pattern is related to lymphocyte polarization. During inflammation or infection, mononuclear cells can acquire distinct functional phenotypes, orchestrating the immune response and aiding in activating other cell types of both innate and adaptive immunity. Macrophage and T helper (Th) lymphocytes polarization is driven by cues in the tissue microenvironment, which can include cytokines, growth factors and microorganism-associated molecular patterns, reshaping the function of mononuclear cells on the basis of the physiological or pathophysiological context. Two well-established polarized phenotypes are often referred to as classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages). The M1/M2 phenotypes are associated with specific cytokines environment, namely IFNγ or IL-4 and IL-13 and are linked to T helper 1 (Th1)- and Th2-type immune responses, respectively (Nfejdjof et al., 2012).

A further regulatory level is provided by microRNA (miRNA), a recently discovered class of small, noncoding RNA regulating post-transcriptionally protein expression, which emerges as important regulators of host-pathogen interaction processes (Harris et al., 2013). Bovine miRNAs that are actively secreted into milk (Lawless et al., 2013), probably contributing to cell-cell communication and ranking among the most promising clinical biomarkers for the diagnosis, prognosis and therapeutic options of a variety of diseases.

There is very few information about both the cytokine and microRNA network in water buffalo mammary gland. To the best of my knowledge, the impact of both cytokines and microRNA and microbiota on water buffalo mastitis are unknown.

2.3. The microbiota and innate immunity

The microbiota is the collection microorganisms in a specific environment. The most used approach to investigate the microbiota composition is by targeted sequencing of the 16S rRNA gene. The metagenome represents all the genes and the genomes of the microbiota. The microbiome includes the genes and the genomes of the microbiota, together with the products from the microbiota and the host (Marchesi and Ravel, 2015) (Figure 3).

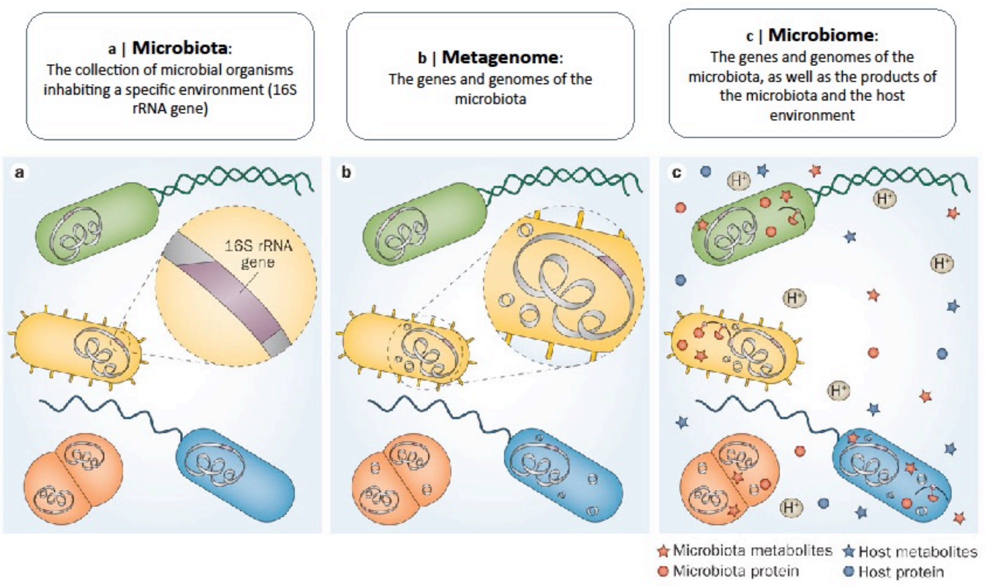

Figure 3 Microbiota (a), metagenome (b) and microbiome (c).

From (Whiteside et al., 2015)

The development of culture-independent techniques by means of high-throughput DNA sequencing has just begun to unravel the impact of the large community of microorganisms, on human and animal health (Stubbendieck et al., 2016). Microbiota establishes a mutual relationship with its hosts and the resulting cross-talk extends beyond the balance between tolerance to commensal micro-organisms and developing protection against pathogens

(Thaiss et al., 2016b). Metagenomic techniques have also revealed how the "healthy" microbiota - the microbial community belonging to healthy individuals - includes potential pathogens. Recent studies on gut microbiota have provided the evidence that the onset of a disease can be the result of a change in the interaction with other microorganisms (Rogers, GB; Hoffman, LR; Carroll, MP; Bruce, 2013). A new concept of pathobiome, which can be defined as the microbiota environment integrating also pathogenic agents, is taking shape and has been recently discussed and thoughtfully reviewed (Vayssier-Taussat et al., 2014).

Within the innate immune system, the PRR including the Toll-like receptors (TLRs), the nucleotide-binding oligomerization (NOD)-like receptors (NLRs), the RIG-I-like receptors, the C-type lectin receptors, the absent in melanoma 2 (AIM2)-like receptors and the OAS-like receptors, plays a relevant role in sense and control microorganisms (Thaiss et al., 2016a). Considering that the number of microorganisms in the human body is about 3.8×10^{13} (the human cells are 3×10^{13}) (Sender et al., 2016) and that the amount of bacterial protein is around 8 million (against the only 22,000 human protein-coding genes) (Rup, 2012), the innate immunity recognition should continuously be active against them with the consequent damaging effects. However, the microbiota sensing at the body surface is controlled, and the innate immune system becomes active only in response to microorganisms' penetration (Thaiss et al., 2016b). As it is demonstrated that the host-microbiota mutualism is lost in the absence of innate immune recognition (Rakoff-Nahoum et al., 2004; Slack et al., 2009), the innate system is able to guarantee regulated crosstalk between host and its microbiota. The relationship between innate immunity and microbiota is characterized by a network of interaction that includes epithelial cells, myeloid cells and innate lymphoid cells (Thaiss et al., 2014). Epithelial cells, in particular in the intestine, respond to PRR with the production of antimicrobial peptides (including RegIIIγ, RegIIIβ, Ang4 and Itln1) and mucus. The epithelial IL-18 guarantees positive feedback for the production of the antimicrobial peptide and requires transcriptional activation through TLRs or the G-protein-coupled receptor GPR109a and posttranscriptional cleavage through the NLRP6 inflammasome. IL-18, together with IL-22 is also produced by other immune cells and has a supporting role for epithelial cells response (Thaiss et al., 2016b). The development of myeloid cells is also modulated by the microbiota, which can influence their migration and gene expression, regulating the production of local metabolites and mediators (Thaiss et al., 2016b). Moreover, the absence of commensal bacteria and metabolites can have a negative impact on myelopoiesis in the bone marrow (Khosravi et al., 2014). Innate lymphoid cells (ILCs) normally develop without the presence of microbiota (Sawa et al., 2010). However, their

function is strictly depending on microbiota, influencing their maturation and activity (Sawa et al., 2011). ILCs include cytotoxic cells (natural killer cells) and non-cytotoxic subsets (ILC1, ILC2 and ILC3). The main relationship between ILCs and microbiota occurs within the non-cytotoxic subset cells and this crosstalk is mediated by epithelial cells or myeloid cells. ILCs can act by using cytokines, PRR ligands and antimicrobial peptides. ILC1 cells are activated by IL-12 from myeloid cells, ILC2 cells are activated by epithelial-derived cytokines and interact with mast cells, eosinophils, basophils and macrophages, ILC3 cells interact with cells of both the innate and adaptive immune systems and are able to support epithelial cells against microorganisms and produce antimicrobial molecules (Sonnenberg et al., 2012; Thaiss et al., 2016b). The basis of the crosstalk in intestinal mucosa are shown in Figure 4.

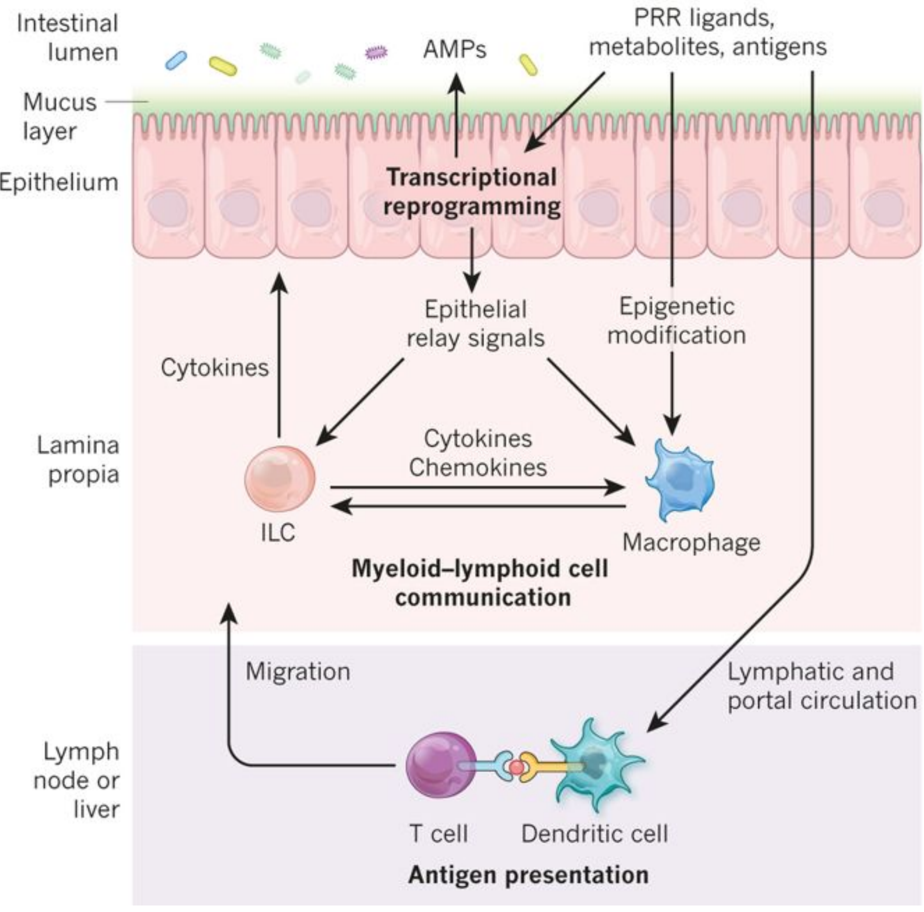

Figure 4 Cross-talk between innate immunity and microbiota in the intestinal mucosa.

From (Thaiss et al., 2016b)

2.4. The microbiota and next-generation sequencing (NGS)

The 16S small ribosomal subunit gene (16S rRNA) is the most widely used gene for microbiota experiments (Kuczynski et al., 2011). In fact, the 16S rRNA gene is ubiquitously present in Bacteria and Archea and contains 9 hypervariable regions that can differentiate taxa (Clarridge and III, 2004) and conserved regions used for designing "universal" amplification primers (Figure 5). Furthermore, several databases are available for the 16S rRNA gene (Balvočiute and Huson, 2017). The most used method for microbiota studies is sequencing specific hypervariable regions of the 16S rRNA gene. Next-Generation Sequencing (NGS) machine are conventionally divided into second and third-generation sequencing platforms. The second-generation machines (e.g. 454 pyrosequencing, Ion Torrent Personal Genome Machine, Illumina platforms) have sequencing length limitation (35-700bp) but lower error rate (from 0.1 to 1%) as compared to the third generation ones (Goodwin et al., 2016), that can reach a 15% of error rate, but are able to sequence long reads (up to 60,000 pb for Pacific Biosciences and 1Mb for oxford Nanopore technology) (Quick and Loman, 2017). The second-generation sequencing approach is also called clonal template generation sequencing strategy because the DNA is clonally amplified on a solid surface (bead-based or emulsion PCR or solid state or bridge PCR), instead the third generation sequencing method does not rely on PCR and consists of the single molecule real-time sequencing.

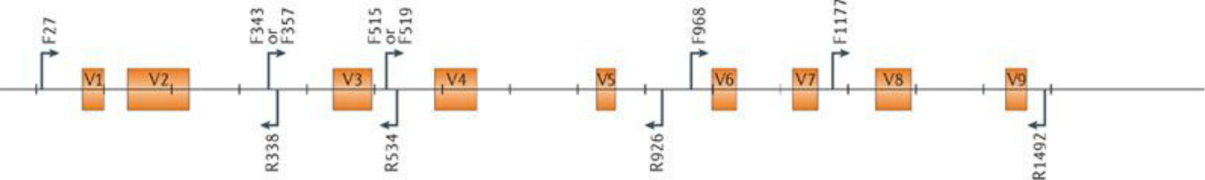

Figure 5 16S rRNA gene and the most used primers for microbiota studies.

The hypervariable regions (from V1 to V9) are shown. From (Kuczynski et al., 2011).

The two commonly high-throughput sequencing methods used to characterize microbial communities are i) targeted (amplicon) sequencing that arose in the early 2000s. ii) metagenomics (shotgun) sequencing, initially used in the late 2000s and gaining popularity in the late early 2010s (Figure 6).

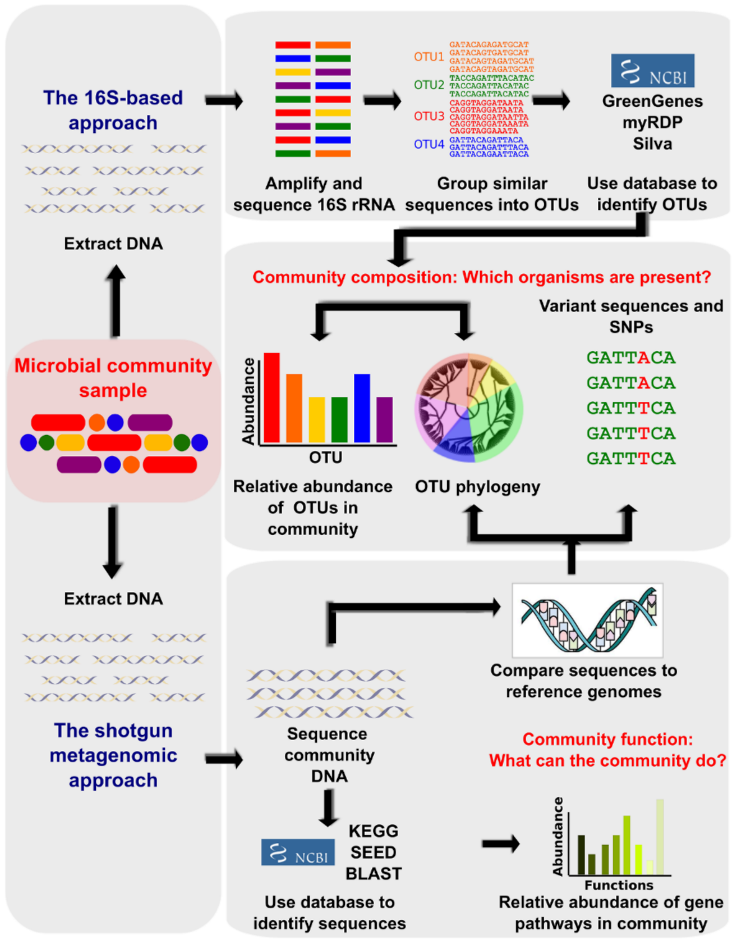

Figure 6 Comparison between 16S-based and shotgun metagenomics approaches.

From (Morgan and Huttenhower, 2012)

The targeted amplicon sequencing bioinformatics workflow is shown in Figure 7.

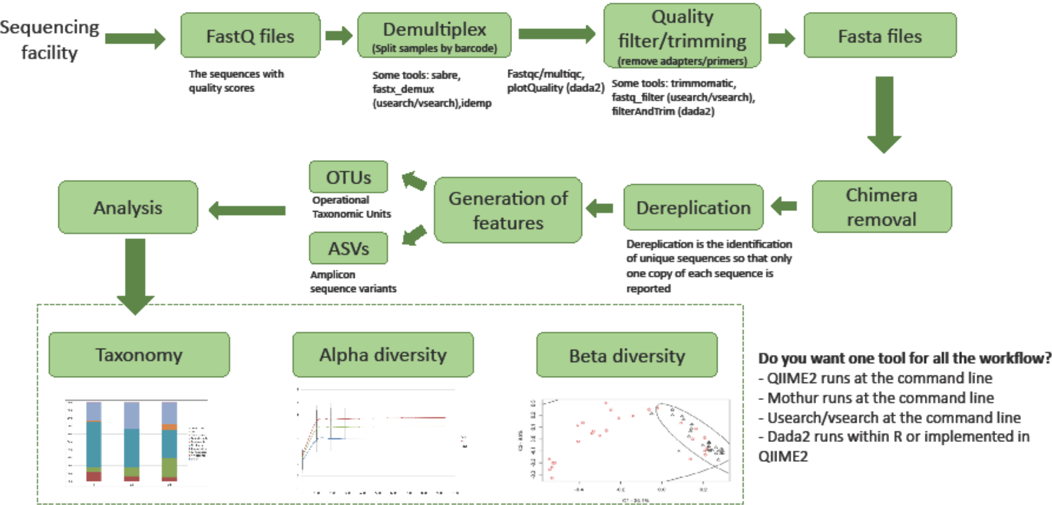

Figure 7 Bioinformatic workflow for 16S targeted sequencing data

(From Sabah Ui-Hasam, ANGUS – analysis sequencing data course - July 12, 2018)

FastQ files got from the sequencing facility, are composed of sequences and quality scores for each base. Sequences need to be split by samples depending on the barcode. Then, reads are filtered based on their quality and primers/adapter are removed. The obtained fasta files (without the quality scores) are processed. After chimera removal, dereplication is applied by the identification of unique sequences in order to report only one copy of each sequence (features) together with its frequency. Operational taxonomic units (OTUs) are unique sequences derived from clusters of sequences with a specific identity between each other (generally 97%): if 1000 sequences have almost 97% of identity, they are clustered together and only one sequence is picked up to assign taxonomy. Amplicon sequence variants (ASVs) are unique sequences that enable single-nucleotide resolution, so each ASV is picked up to assign taxonomy. After taxonomy assignment, data are normalized by means of the relative abundance for what concerns taxonomy. To normalize alpha and beta diversity analysis the same sequence depth is selected for all samples. Taxonomy analysis describes which taxa are found in the samples together with their abundance. The alpha diversity represents how many taxa are present in the samples and can be assessed using a qualitative or a quantitative approach. The richness (e.g. observed species method) is calculated for the qualitative approach, while the evenness (e.g. Shannon index) takes into account the relative abundance

in addition to the taxon type. The beta diversity analysis describes how many taxa are shared between samples, by means of qualitative and a quantitative (e.g. UniFrac method) (Figure 8).

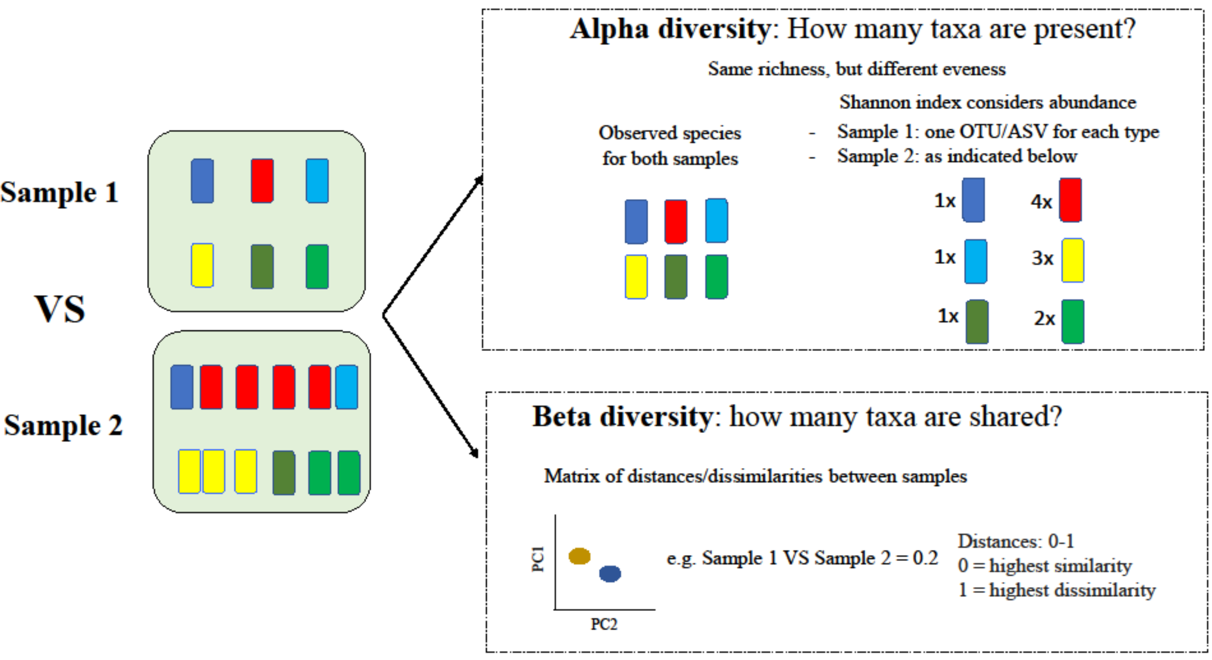

Figure 8 Alpha and beta diversity

The metagenomic bioinformatic workflow is shown in Figure 9.

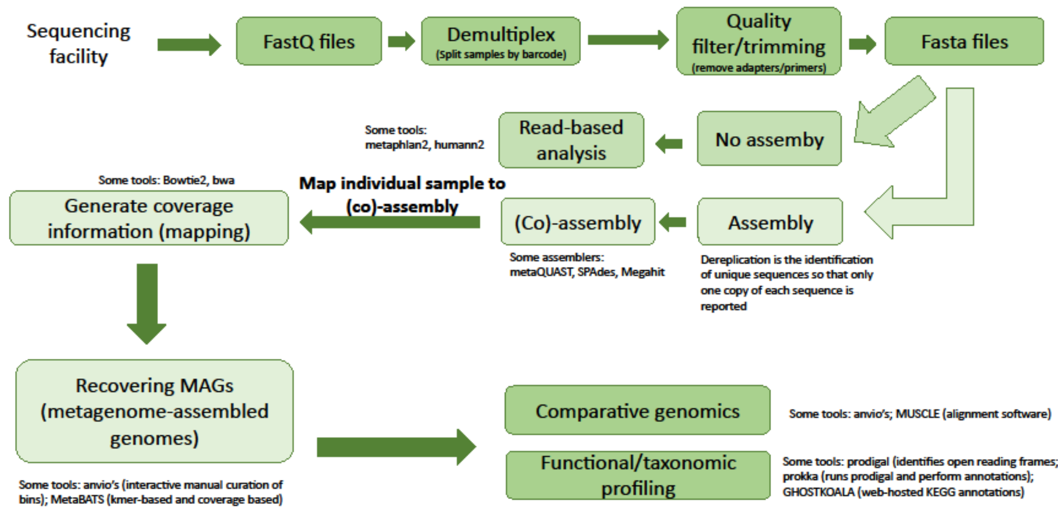

Figure 9 Bioinformatic workflow for metagenomic sequencing data

(From Sabah Ui-Hasam, ANGUS – analysis sequencing data course - July 12, 2018)

The first steps are the same as seen for the target amplicon sequencing workflow. Then, it is possible to perform the assembly or not depending on the study. It is possible to do a read based analysis without making the assembly, aligning the sequences reads against the database of interest (Quince et al., 2017). The analysis allows to do it in less time as compared to the assembly approach and enables profiling also low-abundant microorganisms (it could be difficult using the assembly approach). Some disadvantages include the fact that the database is needed and that undefined microbes are difficult to profile. The metagenome assembly is similar to the whole genome assembly, but different communities are present in the metagenome. The strategy is to assemble the reads into a contig, then the contigs into scaffolds and then the scaffold into chromosomes. It is necessary to perform a co-assembly, putting together the reads of all samples and perform the assembly using all these sequences. The co-assembly will be used later for the single samples and allows to have a higher read depth. Furthermore, it increases the ability to recover the genomes from each independent sample. Once completed the co-assembly, the taxonomy can be assigned and the microbial composition can be assessed, together with its functional profile (Quince et al., 2017). Most applications require sequencing each base multiple time to achieve high confidence base calls. Sequencing coverage (or sequencing depth), at the nucleotide level, is the number of times a reference base is represented within a set of sequencing reads. When describing sequencing coverage at the whole genome level, this value is expressed as an average or median of all the per base coverage values. For example, a genome with 30× coverage will have an average of 30 reads spanning any given position within the genome—some regions will have higher coverage and some will have lower coverage. Sequencing at increased levels of coverage enables the generation of a high quality, highly accurate consensus sequence, providing confidence in the results and allowing accurate detection of variants or assembly of complex genomes. Human WGS studies are typically performed at 30×–40× coverage or higher (From Illumina website). Depending on the study design and final application, more or less coverage may be required. For example, certain applications, such as tumour-normal analysis or rare variant detection, may require higher coverage levels to identify low-frequency variants.

A comparison between targeted amplicon sequencing and metagenomics approaches is shown in Table 3.

Table 3 Targeted amplicon sequencing and metagenomics approaches and their pros and cons.

Targeted amplicon sequencing	Metagenomic sequencing
Pros	
Widely used for microbiota studies with curated database for 16S rRNA gene	Functional potential
Evaluate changes in community structure	Insight into the 'unculturables'
"Cheap" and fast	Better for relative abundance (but cell/lysis efficiencies can be different – also in targeted amplicon sequencing)
Cons	
PCR bias: under/over representation	Expensive
Universal primer (only for what we know)	Complex analysis
Sequencing of only 16S rRNA regions (on the fragment of one gene). Be aware of Multiple copies of 16S rRNA gene	Need for high quality starting material
No functional analysis	'Contamination' from host DNA
No difference between live and dead bacteria	

Critical steps, including source material, DNA extraction, primer sequences and sequencing platform may affect microbiota results. No standard methodologies are present for metagenomics studies. Costea and colleagues (Costea et al., 2017) compared i) DNA extraction methods from fecal samples through seven commonly used extraction kits (Invitek's PSPStool, Mobio's PowerSoil, Omega Bio Tek's EZNAstool, Promega Maxwell, Qiagen's QIAampStoolMinikit, Bio101's G'Nome, MP-Biomedicals's FastDNAspinSoil and Roche's MagNA PureIII) and non-kit-based protocols; ii) two different library preparation.

Authors found that DNA extraction had the largest effect on microbiota composition, even if, regarding the mock community analysis, results were approximately in accordance with each others. On the contrary, Vesty and collaborators (Vesty et al., 2017) investigated the effect of DNA extraction methods on microbiota results, focusing on bacterial and fungal diversity; they demonstrated no differences in bacterial genera composition across DNA extraction methods – using three commercial kits, namely MoBio PowerSoil DNA Isolation Kit, QIAamp DNA Mini Kit and Zymo Bacterial/Fungal DNA Mini Prep, and phenol/ chloroform-based DNA isolation protocol – from plaque and saliva. In another work, Fouhy et al. (Fouhy et al., 2016) used two different DNA extraction methods from fecal samples (QIAamp DNA Stool Mini kit and repeat bead beating method with elements of the Qiagen faecal extraction kit), different primer sequences (V4-V5, V1-V2 and V1-V2 degenerate primers) and two different sequencing platforms (Illumina MiSeq and Life Technologies Ion Torrent PGM); authors found that primers set and sequencing machine may influence microbiota composition results; in this study, all of the bacterial components from mock community were detected only from V4-V5 and PGM combination.

To the best of my knowledge, also for food microbial ecology, including milk microbiota, no standardized protocols are available. High-throughput sequencing techniques already considered the issues about the variable region amplified, the sequencing platform, the taxonomic resolution and the database used (Ercolini, 2013). Regarding milk microbiota, V1-V2 with 454 FLX (Oikonomou et al., 2012; Kuehn et al., 2013) or Ion Torrent PGM (Catozzi et al., 2017a), V1-V3 with 454 FLX (Ercolini et al., 2012) and V4 with Illumina MiSeq (Ganda et al., 2016; Bonsaglia et al., 2017) are the preferred 16S rRNA region amplified and sequencing platform.

Another critical point is represented by reagent and laboratory contamination: some genera belonging to *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Deinococcus-Thermus* and *Acidobacteria* were already found as contaminants in negative controls, and contamination could be more evident in the sample containing a low microbial biomass (Salter et al., 2014), as milk. Therefore, some recommendations in order to reduce the impact of contaminants include: i) to maximize or enrich the sample starting material ii) to minimize the risk of contamination from sample collection to sequencing iii) to process samples using random order and replicates/triplicates iv) quantify negative controls from extraction and PCR (Salter et al., 2014).

2.5. The ruminant's milk microbiota

Milk microbiota composition is complex (Quigley et al., 2013). Different microorganisms are present in different species and their presence and abundance are driven by many factors, including genetics, disease, environmental conditions. The majority of the studies regarding milk microbiota has been assessed in human and cow. *Staphylococcus* and *Streptococcus* are considered to be the main genera dominating the human milk microbiota, regardless of geographic location or analytic methods (Fitzstevens et al., 2017). Other taxa found in human milk are *Corynebacterium, Lactococcus, Leuconostoc, Streptococcus, Staphylococcus, Weissella, Propionibacterium, Pseudomonas, Sphingomonas, Ralstonia* and *Serratia* (Hunt et al., 2011; Quigley et al., 2013). Some of them are shared with cow milk, where *Acinetobacter, Aerococcus, Brevibacterium linens, Corynebacterium casei, Enterococcus faecalis, Lactococcus lactis, Leuconostoc, Staphylococcus saprophyticus/succinus, Streptococcus thermophiles, Pseudomonas* and *Weissella hellenica* are the most prevalent genera (Quigley et al., 2012). The core cow milk microbiota is composed of four bacterial genera, namely *Faecalibacterium, Lachnospiraceae, Propionibacterium, Aerobacillus* (Bicalho, 2014). *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Streptococcus* are the more frequent genera also in sheep milk microbiota (Quigley et al., 2013).The impact of the microbiota in the development of mastitis has been only described (Oikonomou et al., 2012; Bicalho, 2014) and recently reviewed (Addis et al., 2016a). Milk harbours a wide range of bacteria, many of which cannot be identified by microbiological culture technique. In fact, not all mastitis-causing pathogens can be detected because negative microbiological culture results may occur, even during acute mastitis with clinical symptoms (Taponen et al., 2009). Furthermore, cow subclinical mastitis samples generally show a high somatic cell count higher than 200,000 cells/ml without necessarily presenting the causative agent by culture-dependent methods, as well as it happens also in water buffalo (Catozzi et al., 2017a). Therefore, molecular techniques have developed to improve mastitis diagnosis and support microbiological culture methods (Oultram et al., 2017). The microbiota of raw and pasteurized milk showed the presence of a rich and diverse bacterial community (Quigley et al., 2012). Milk from healthy and mastitis-affected animals revealed differences at microbiota level (Oikonomou et al., 2012; Kuehn et al., 2013), although the use of culture-independent technique and the concept of milk microbiota has been recently challenged (Rainard, 2017). However, *Streptococcus uberis* and *Staphylococcus aureus,* known as a common pathogen of the mammary gland, have been detected in milk from animals without any inflammatory reaction, suggesting the hypothesis that the development of mastitis can be regarded more as

a dysbacteriosis than a primary infection (Bicalho, 2014). Fifteen genera represent the water buffalo milk core microbiota, namely *Acinetobacter*, *Aerococcus*, *Facklamia*, *Clostridium*, *5-7N15*, *Micrococcus*, *Propionibacterium*, *02d06*, *SMB53*, *Solibacillus*, *Staphylococcus*, *Trichococcus*, *Turicibacter*, *Pseudo*monas and *Psychrobacter* and differences between healthy, clinical and subclinical mastitis affected quarter milk samples have been reported (Catozzi et al., 2017a).

2.6. Use of antibiotics and probiotics during mastitis

Antibiotic is the most widely used drug to treat mastitis in dairy ruminants; however, it is also used to prevent mastitis in healthy animals during the dry period. Antibiotic may negatively impact on animal health over time and antibiotic residues may remain in animal products, including milk. Furthermore, antibiotic use promotes bacteria to become resistant to antimicrobial drugs. Many mastitis-causing pathogens, among which *Streptococcus agalactiae* (Berghash et al., 1983) and *Staphylococcus aureus* (Yang et al., 2016), became antibiotic-resistant (Ceniti et al., 2017). Although antibiotic treatment is necessary to treat mastitis disease (Oliver and Murinda, 2012), the anti-microbial resistance persists in the bacterial community (Andersson and Hughes, 2011). In water buffalo, subclinical mastitis is more frequent than clinical mastitis and the antibiotic treatment is the currently used treatment; however, during the dry period and in healthy animals, the preventive use of antibiotic is discouraged (Moroni et al., 2006b). Moreover, the antibiotic use has been demonstrated to alter the microbiota, reducing the microbial diversity, which is restored after about 5 days. So, one of the hypothesis is to use an alternative strategy to control the infection minimizing the antibiotic use, but without affecting the microbiome; new therapeutic approaches, such as bacteriophages, vaccines, nanoparticles, cytokines and plant-, animal- or bacteria-derived antimicrobials have been studied and recently reviewed (Gomes and Henriques, 2016). Generally Recognised As Safe (GRAS) bacteria include Lactic Acid Bacteria (LAB) and beneficial effects are already demonstrated on human gut microbiota (Pessione, 2012). The innovative research field is represented by the use of probiotic on mammary gland microbiota; in-vitro studies have shown promising results on bovine mammary epithelial cells: *Lactococcus lactis* showed antibacterial and immunomodulatory properties by producing nisin A (Malvisi et al., 2016); *Lactobacillus casei* was able to prevent *Staphylococcus aureus* invasion (Bouchard et al., 2013); *Lactobacillus perolens* inhibited the 85.7% of the mastitis-causing pathogens (Frola et al., 2012); *Lactobacllus rhamnosus* improved the inflammation caused by *Escherichia coli* (Wu et al., 2016); *Lactobacillus brevis*

and *Lactobacillus plantarum* showed high colonization properties and anti-inflammatory activity (Bouchard et al., 2015). Results from in-vivo experiments remain critical and the inoculation in the mammary gland is necessary as the oral administration of probiotics is not effective in ruminant species (Uyeno et al., 2015). Even though *Lactococcus lactis* is known to stimulate the bovine intramammary immune system, by polymorphonucleocytes (PMN) and leucocytes recruitment and increasing haptoglobin and milk amyloid A concentration (Crispie et al., 2008), only in some instances the live cultures of *Lactococcus lactis* were effective in cow mastitis treatment (Klostermann et al., 2008). In another study (Camperio et al., 2017), mammary glands of mastitis mice models, infected by *Staphylococcus chromogenes* and treated with live cultures of *Lactococcus lactis*, showed high levels of IL-1β and TNFα, in addition to tissues damages, suggesting that this GRAS bacteria cannot be used for mastitis treatment. As confirmed also in the experiment by Mignacca and colleagues (Mignacca et al., 2017) on ewes affected by subclinical mastitis, *Lactococcus lactis* did not show good probiotic properties: briefly, despite the fast activation of the mammary gland immune response, animals treated with live cultures of *Lactococcus lactis* showed mild or moderate clinical mastitis symptoms, without improving *Staphylococcus aureus* and coagulase-negative Staphylococci infections. Similarly, *Lactobacillus acidophilus* and *Lactobacillus casei* were not able to treat cow mastitis and caused a local inflammatory response (Greene et al., 1991), as well as it was demonstrated using *Lactobacillus perolens* (Frola et al., 2012). Clinical studies on human mastitis are focused on *Lactobacillus fermentum*, *Lactobacillus Salivarius* and *Lactobacillus gasseri* (Di Cerbo et al., 2016); the oral administration of *Lactobacillus salivarius* and *Lactobacillus gasseri* was effective against human infectious mastitis during lactation (Jiménez et al., 2008). *Lactobacillus fermentum* (Arroyo et al., 2010) and *Lactobacillus salivarius* (Arroyo et al., 2010; Fernández et al., 2016) showed better results than those from the antibiotic group in terms of improvement and recurrence of mastitis in women.

Chapter 3: Brucellosis

3.1. Water buffalo brucellosis

In ruminants, brucellosis is one of the most important zoonotic diseases. *Brucella* genus is the causative agent. It is a gram-negative and facultative intracellular pathogen (Corbel, 1997; Martirosyan et al., 2011). Macrophages, dendritic cells and placental trophoblast are the cells target of *Brucella* (Carvalho Neta et al., 2008). More than 500,000 new cases of brucellosis in human are reported annually. In animal production field, it causes economic losses (Mcdermott et al., 2013; Singh et al., 2015). In western regions, brucellosis progressively disappeared by using strategies of animal screening and vaccination; however, in the Middle East, Asia, Africa and South America, as well as in some areas of Italy, it remains endemic (Garofolo et al., 2017). The transmission of brucellosis to human is due to the consumption of raw milk or after direct contact with infected animals. The pathogenesis of *Brucella* consists on: a) Onset of infection; b) Acute phase with the first clinical, hematological and pathological symptoms; c) Chronic phase with clinical symptoms and evident pathological sign (Martirosyan et al., 2011).

The Brucella life cycle is illustrated in Figure 10 Within the host cells (1), *Brucella* from Brucella-containing vacuoles (BCVs) (2); then, after the fusion with lysosomes (3), about the 90% of the bacterium is degraded, while the 10% survive (4). The BCVs reach the endoplasmic reticulim (ER) (5) and replicate at this level (6). The BCVs traffic towards the autophagy like vacuoles system (7-8) until the cell membrane where they can leave the target cells and spread to the others (9) (Ke et al., 2015).

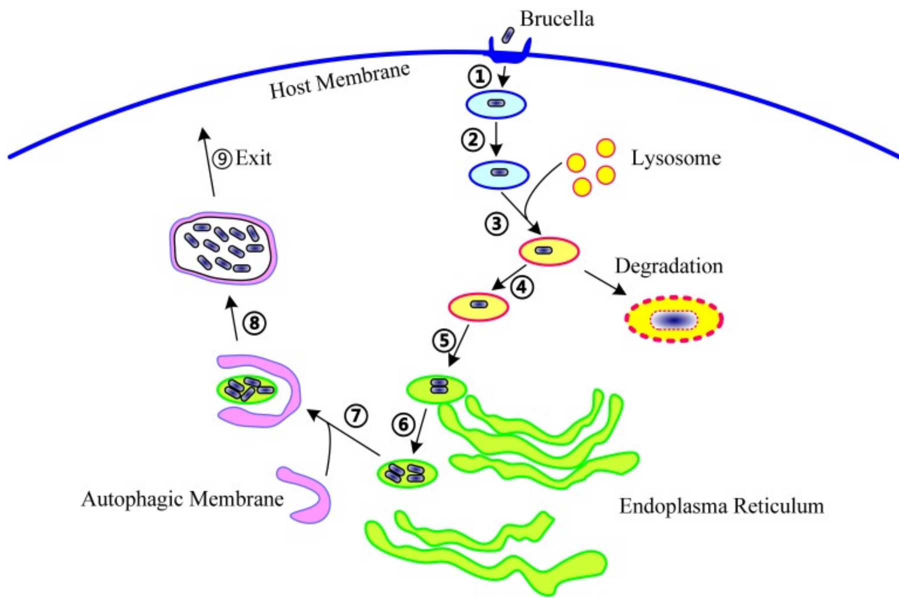

Figure 10 The life cycle of Brucella within host cells.

From (Ke et al., 2015)

Brucella can both down- and upregulate the inflammatory immune response (Figure 11). On one side, the outcome of signalling pathway by TLR4 during the infection is reduced by the presence of elongated fatty acid chains able to reduce the LPS toxicity and by blocking downstream IKK phosphorylation via MyD88 binding with *Brucella* TIR-containing proteins, BtpA and BtpB, inducing an increased polyubiquitination and degradation of MyD88-adaptor like (MAL). The inhibition of MyD88 pathways at the early stage is crucial for the bacterial replication. On the other side, the interaction with the endoplasmic reticulum (ER) molecular chaperone called BiP leads to the release and phosphorylation of inositol-requiring enzyme 1 (IRE1), inducing the mRNA splicing of X-box binding protein 1 (XBP1) and the activation of unfolded protein response (UPR); it also promotes a pro-inflammatory effect through NF-kB pathway at the late stage, suggesting the right moment to leave the cells and spread to the others (De Figueiredo et al., 2015).

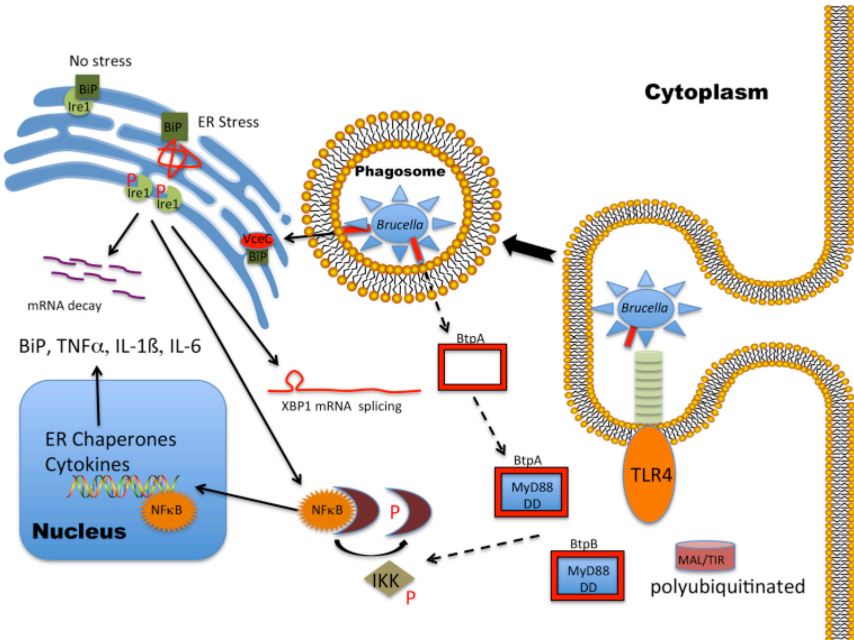

Figure 11 Immune response induced by Brucella.

From (De Figueiredo et al., 2015)

B. melitensis and *B. abortus* typically infect small and large ruminants, respectively. *B. abortus* biovar 1 is the main causative agent in water buffalo in South America (Fosgate et al., 2002; Megid et al., 2005), Pakistan (Ali et al., 2017), Italy (Borriello et al., 2013) and Africa (Wareth et al., 2014). In pregnant animals, *Brucella*'s targets are placenta and then fetus, inducing abortion especially in the last third of the pregnancy (Anderson et al., 1986b; a). In non-pregnant animals, the bacterium is released through secretion and clinical sign are not always present (Capparelli et al., 2009). It is mandatory to have animals free of brucellosis, following the eradication and surveillance programs (Ducrotoy et al., 2016, 2018); for this reason, the diagnosis must be early in order to monitor disease spreading. The diagnosis is based on bacteriological and immunological tests; however, negative results can occur in serological tests, even when the bacterium is present, especially in the first disease phases. As miRNAs are involved in many biological processes, including infections, they can also be involved in brucellosis pathogenesis and could be used as a biomarker to early diagnose the disease.

3.2. microRNAs, their potential functions and their potential as biomarkers

The non-coding RNAs are divided in small- and long- non-coding RNA depending on their length, less or more than 200 nucleotides; both categories are described in Figure 12 (Esteller, 2011).

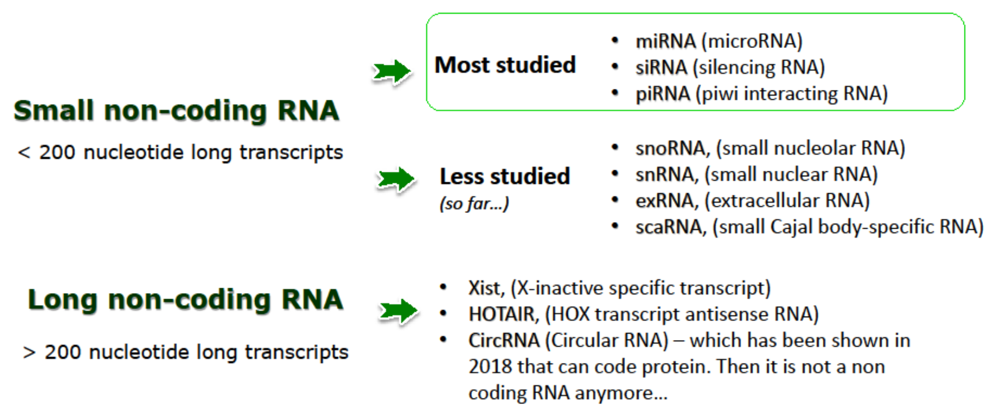

Figure 12 Non-coding RNA

Among the most studied small non-coding RNAs, silencing RNAs (siRNA) are double-stranded small non-coding RNA molecule (about 22 nucleotides) derived from viral origin or can be products of repeated transcription. They bind to mRNA only in the case of complete complementarity, leading to the mRNA break. Piwi-interacting RNAs (piRNA) are longer single-stranded RNA (26-31 nucleotides), which inhibit the transcription of defined loci by histone methylation and maintain genomic integrity by stopping transposable elements. MicroRNAs (miRNAs) are double-stranded small non-coding RNA, about 22 nucleotides in length, with a role as post-transcriptional regulators and as controller of many cellular processes in eukaryotic organisms. They are encoded in genes of the genome and do not require the full complementarity to bind the target mRNA; in fact, one type of miRNA may regulate many genes, as well as one gene, can be regulated by several miRNAs. miRNA acts together with Argonaute proteins as RISC (RNA-induced silencing complex), inducing the mRNA degradation by binding to 3'UTR coding sequences or 5'UTR, recruiting deadenylation factors that remove the poly(A) from the mRNA or impeding the protein translation. So, the target mRNA is either destroyed or stored (Esteller, 2011). miRNAs are

named using a code with three letters from the organism (has: Homo sapiens; bta: Bos Taurus) and a number (the numbering of miRNAs is sequential). Lettered suffixes denote closely related mature sequences (has-miR-121a and has-miR-121b); when two miRNA sequences originate from the same predicted precursor, they are identified using names such as miR-142-5p (from the 5' arm) and miR-142-3p (from the 3' arm). The first miRNA was identified in 1993 from the Caenorhabditis elegans nematode and called *lin*-4 (Lee et al., 1993); instead of producing a mRNA encoding proteins, it produces short non-coding RNAs, one of which was a 22-nucleotides RNA that contained sequences partially to multiple sequences in the 3' UTR of the *lin-14* mRNA, inhibiting its translation into LIN-14 protein (Esteller, 2011). In 2000, a second small RNA was discovered and called let-7 miRNA, which was conserved in many species and was demonstrated to suppress lin-41 (Reinhart et al., 2000). The related-miRNA discoveries are shown in Figure 13 (Weiss and Ito, 2017).

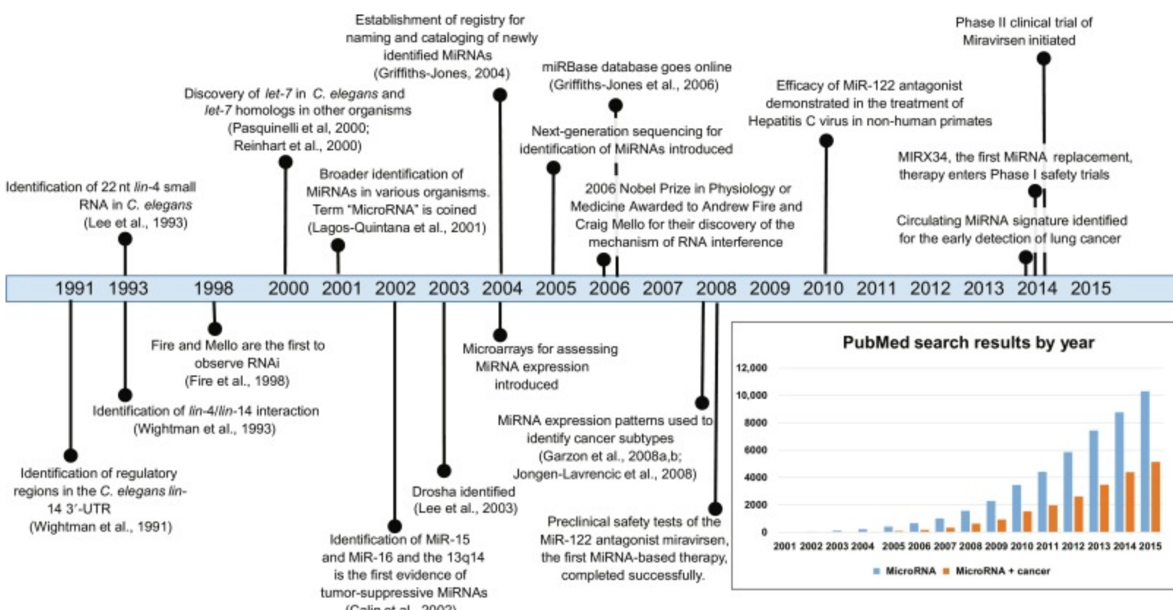

Figure 13 miRNA discovery timeline from 1991 to 2015.

From (Weiss and Ito, 2017)

The presence of miRNA has been reported in a wide range of organisms, from algae to humans (Bartel and Chen, 2004; Zhao et al., 2007). More than 60% of mammalian mRNA is targeted by at least one miRNA (Friedman et al., 2008). The genomic location of miRNAs can be inside exons, inside introns, intergenic (no splicing), in clusters (miRNA clusters either intronic and exonic that produce a long primary transcript), excited from introns after microprocessing (the so-called mirtrons) (Olena and Patton, 2010). miRNA biogenesis starts

with the transcription of precursor molecules (pri-miRNAs) by RNA polymerase II (RNAPII). The pri-miRNA has hairpins used as substrates for two members of the RNase III family of enzymes – Drosha and Dicer, that act together with dsRNA-binding proteins (dsRBPs) like DGCR8, transactivation- responsive (TAR) RNA-binding protein (TRBP) and protein activator of PKR (PACT) (Krol et al., 2010). The detailed process of the miRNA biogenesis is shown in Figure 14.

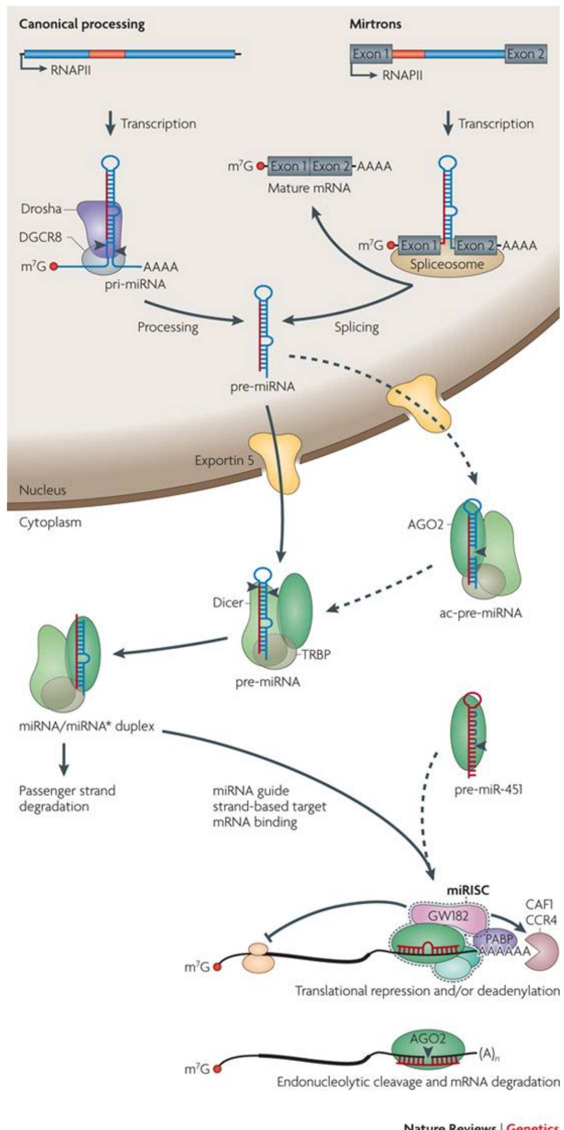

Figure 14 miRNAs biogenesis and roles.

From (Krol et al., 2010)

The miRNAs bind the 3'UTR of a target gene by base pairing. The binding between the miRNA seed sequence (nucleotides 2-8 at the 5' end of the miRNA sequence) and the miRNA regulatory element (MRE) at the 3'UTR of a target gene determines the specific type of

regulation. MiRNAs act as inhibitors of translation if the binding at the 3'UTR of target genes is partially complementary. Instead, when the binding complementary is perfect, miRNAs induce mRNA degradation. MiRNAs can also function in an unconventional manner, activating the translation by binding non-canonical sites in the 5'UTR of target genes (Oliveto et al., 2017). MiRNAs are collected into multivesicular bodies (MVBs) that are specialized endosomal vesicles that can be delivered to lysosomes to be degraded or released in fluids as exosomes (Stoorvogel, 2012).

miRNAs are involved in many physiological processes, from molecular pathways to cell-cell communication and pathogen-host interactions (Eulalio et al., 2012; Ghai and Wang, 2016). miRNAs are also stable in body fluids and can be easily purified (Turchinovich et al., 2011). Exogenous miRNAs were detected by RNA-seq in human plasma and breast milk and in the cow as well. A study on mice revealed that feeding a diet depleted of bovine milk exosomes and miRNAs caused more than a 60% decrease in plasma miRNAs as compared with controls. The RNAs encapsulated in bovine milk exosomes survive harsh conditions such as low pH and digestion under simulated gastrointestinal tract conditions (Zempleni, 2017). MiRNA can be considered promising biomarker for diagnosis of many diseases, including infectious diseases (Correia et al., 2017), in both humans (Hawley et al., 2017; Markopoulos et al., 2017) and animals (Dong et al., 2017; Lecchi et al., 2018).

Two strategies can be applied to study miRNAs: i) target a priori miRNAs that could be differentially expressed (following literature) and validate them using quantitative PCR. ii) Sequence miRNAs by OMIC approach and validate them by PCR. The targeted approach (i) results to be cheaper (target probes) but the number of selected targets could be small and the risk that those miRNAs are not regulated is high. On the contrary, OMICS (ii) allows detecting a wide number of targets (and possibly the right ones) but this approach is very expensive as compared to the other and de-regulated miRNAs must be validated by PCR.

Chapter 4: Tuberculosis and monocyte polarisation

4.1. Water buffalo tuberculosis

Bovine tuberculosis (TB) is a zoonotic disease caused by *Mycobacterium tuberculosis* complex (MTBC), which includes genetically related species belonging to the genus *Mycobacterium*. MTB has been well adapted to wild and domestic animals and to humans as well (Malone and Gordon, 2017). In 2016, 147,000 new cases of zoonotic TB were estimated in people globally, with more than 12,000 deaths associated to the disease; Africa followed by South-East Asian and Western Pacific countries is the most affected region (www.fao.org). The pathogenesis of TB in humans is shown in Figure 15.

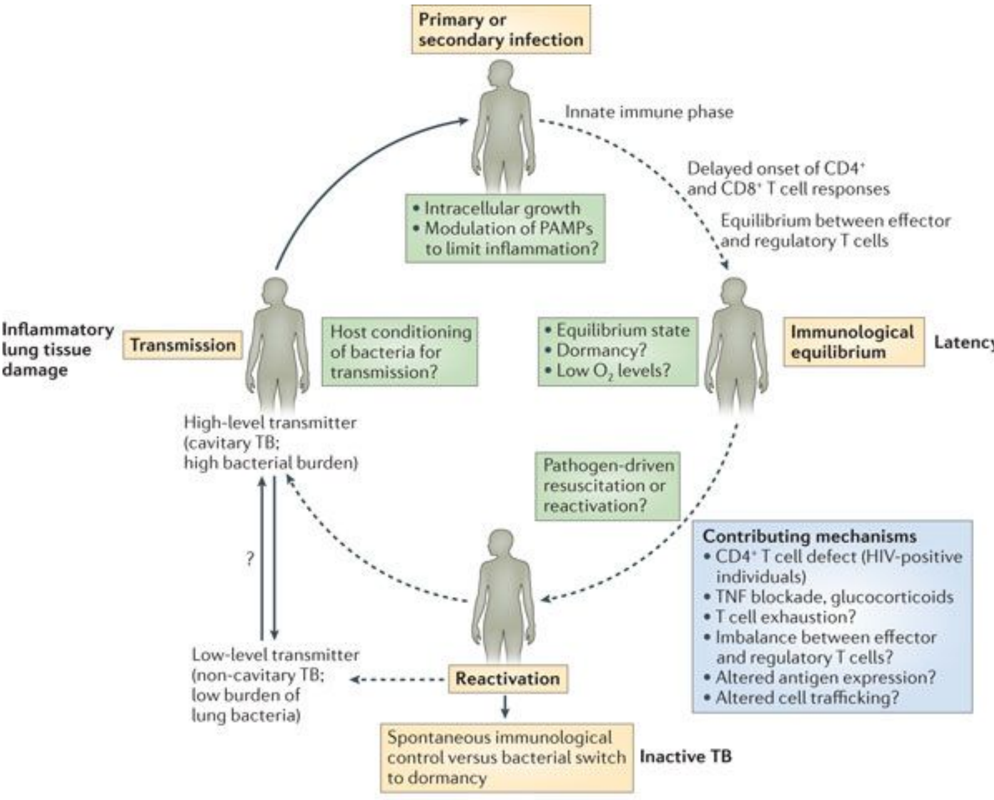

Figure 15 Immunological life cycle during tuberculosis disease.

From (Ernst, 2012)

Mycobacterium bovis represents the major infectious agent in ruminants (Pesciaroli et al., 2014), including water buffalos (Cousins, 2001; Michel et al., 2007; de Garine-Wichatitsky et al., 2010). *M. bovis* is an obligate intracellular pathogen and the transmission between animals primarily occurs by inhalation (Menzies and Neill, 2000; Smith, 2003); afterwards, the microorganism adheres to the alveolar surface of the lungs, where it is phagocyted by macrophages which in turn activate T cells (Neill et al., 2001). During the first TB phase, the disease is localized, and the bacterium is generally eliminated by macrophages together with cytotoxic T lymphocytes. The phase of equilibrium between effector and regulatory cells is characterized by the formation of the granuloma, which occurs in order to isolate the pathogen: *M. bovis* is surrounded by immune cells, including macrophages, T and B cells, T reg and dendritic cells (Figure 16) (Salgame, 2011a) .

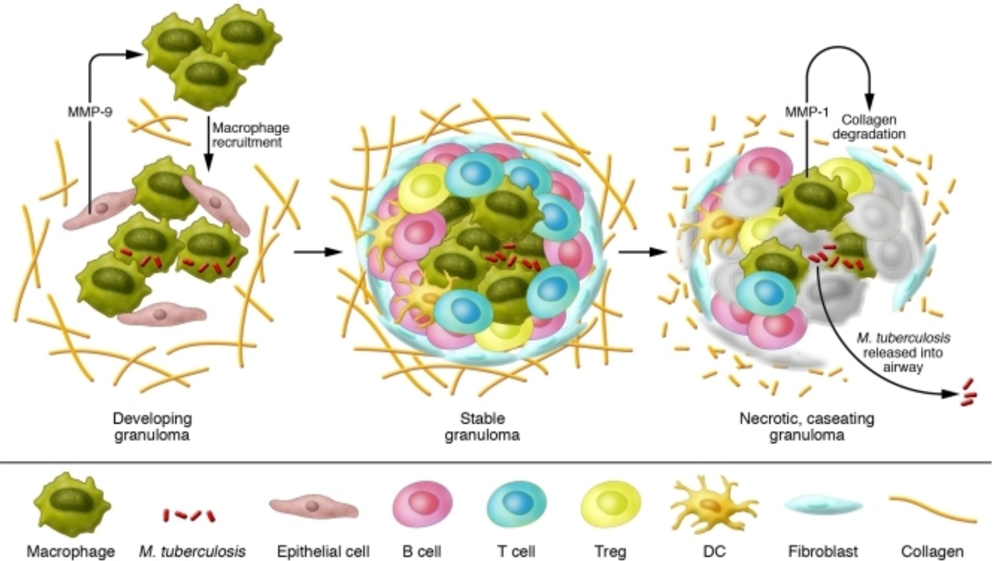

Figure 16 Granuloma composition during tuberculosis.

MMP: Matrix metallopeptidase 9. From (Salgame, 2011a)

However, the disease resistance depends on innate and adaptive immunity; in fact, when the immune system drops down, the reactivation and the transmission of the disease occur again.

The programs of control and eradication of tuberculosis in cattle are present in many countries with the aim of reducing infections and animal culling (Caminiti et al., 2016). The disease is monitored by using the single intradermal tuberculin skin test (TIT) to determine the presence of tuberculosis in live animals. Protein purified derivates (PPD) from *M.bovis* are injected in the animal dermis and the reaction in terms of skin thickness is evaluated after 72 hours (Good et al., 2018). In addition to SIT, supplemental tests (e.g. IFNγ and antibodies quantifications)

are also included to confirm TB diagnosis. If the animal is positive to SIT, it is sent to the slaughterhouse, where the diagnosis is confirmed by the presence of TB lesion. The microbiological culture is also integrated to support the diagnosis. The tests for TB control available for cows are also used for water buffalo. However, the efficacy of the SIT using *M. bovis* PPD in water buffaloes is lower than in cows, with reduced sensitivity and specificity. In fact, the great cutis thickness of water buffalo leads to a difficult interpretation of the result. Moreover, as water buffaloes are used to wallow in the mud, *Mycobacterium avium* - a non-tuberculous *Mycobacterium* generally present in the environment - may interfere with the *M. bovis* PPD test.

4.2 Monocyte polarisation during infection

It is known that in humans the circulating monocytes belong to three different class: i) the CD14+ CD16− (classical) monocytes (85%); ii) CD14+ CD16+ (intermediate) monocytes (15%); iii) non-classical monocytes (Passlick et al., 1989; Wong et al., 2011; Patel et al., 2017). As well as for human monocytes, bovine monocytes are characterized by three main subgroups based on the surface expression of CD14 and CD16 (Hussen et al., 2013), as shown in Figure 17.

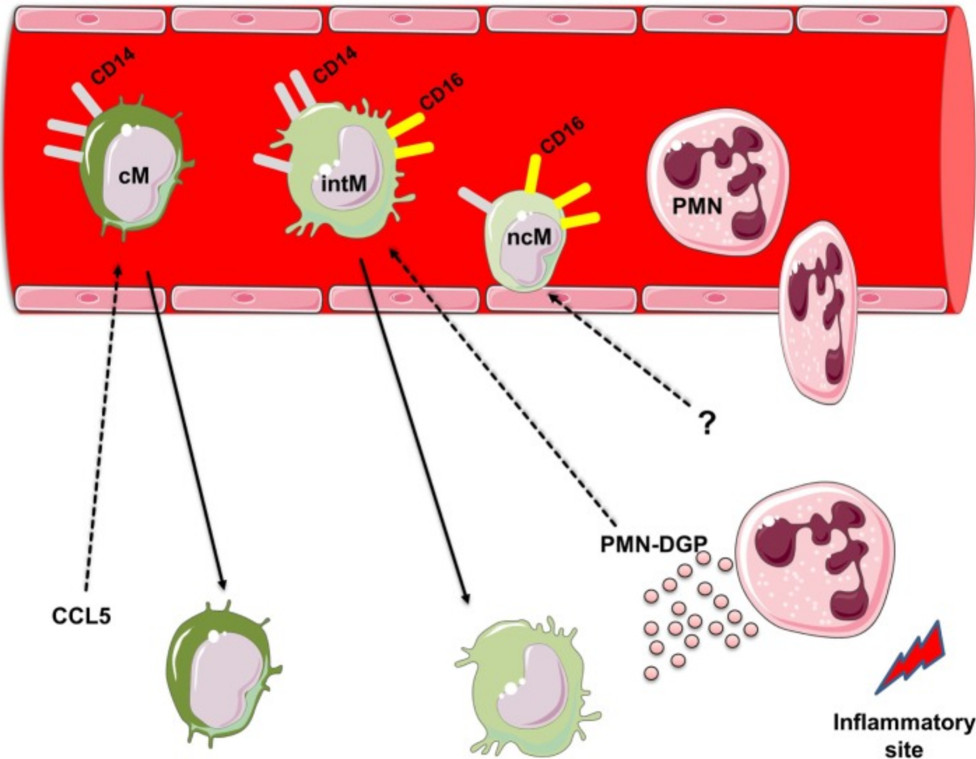

Figure 17 Bovine monocyte subsets.

Classical monocytes (cM) with high CD14 but no CD16 expression (CD14++ CD16−); intermediate monocytes (intM) with high CD14 and low CD16 expression (CD14++ CD16+);non-classical monocytes (ncM) with high CD16 but no CD14 expression (CD14− CD16++) from (Hussen and Schuberth, 2017).

The surface protein CD172a, also called signal-regulatory protein alpha, was identified as monocyte marker in cows, similar to the pig (Moreno et al., 2010) and rat (Melgert et al., 2012) and it is higher in intermediate and non-classical monocytes. CD163 is mostly expressed on classical monocytes. Monocytes are regarded as effector immune cells belonging to the innate immune system; they actively participate during infections through phagocytosis and microorganism killing but also secreting cytokines and chemokines (Auffray et al., 2009). Among the bovine monocytes' subsets, especially the classical ones are able to phagocyte microorganisms. The intermediate ones show a middle capacity to make phagocytosis in favour of reactive oxygen species (ROS) and inflammatory cytokines (TNF-α, IL-1β) production. Non-classical monocytes show a reduced activity as compared to the other two classes (Hussen and Schuberth, 2017). In general, the bovine CD14 positive monocytes are those participating in the response against microorganisms. Conversely, the CD16 positive monocytes are considered the pro-inflammatory subset in humans (Ziegler-Heitbrock, 2007). Classical and intermediate monocytes in cows are also able to respond to

the neutrophils degranulation products (DGP) produced by PMNs; in fact, PMN-DPG can activate monocytes by inducing Ca^{2+}-mobilization promoting their migration (Hussen et al., 2016). The differentiation of monocytes to monocyte-derived macrophages or dendritic cells occurs after leaving the bloodstream (Baquero and Plattner, 2016; Holzmuller et al., 2016). The polarization of monocytes, depending on their activity, is mostly based on the differentiation into pro- and anti-inflammatory macrophages, called M1 and M2, respectively. The pro-inflammatory pattern, with a high expression of *TNF, IL1,* nitric oxide synthase 2 (*NOS2)*, and *CXCL8,* was identified in LPS-stimulated macrophages derived from bovine classical and intermediate monocytes; conversely, the anti-inflammatory phenotype of non-classical monocytes-derived macrophages was demonstrated by the greater expression of type-I arginase (*ARG1)* (Hussen and Schuberth, 2017). NOS and Arg-I are the enzymes that are involved in L-arginine/nitric oxide (NO) metabolism and are typically associated with pro- and anti-inflammatory patterns, respectively (Yang and Ming, 2014). The modulation of bovine monocytes differentiation can also be regulated by PMN-DPG, which promote features associated with M2 macrophages, with a lower expression of MHC-II (79). However, the activity of these cells resulted to be increased in cytokines production, both anti- (e.g. IL10) and pro-inflammatory (IL12) (Ambarus et al., 2012; Hussen and Schuberth, 2017).

4.3. T-cell polarization and microRNA related to tuberculosis

The polarization of T lymphocytes population is fundamental in controlling tuberculosis. This differentiation is promoted by many factors, including the response of monocyte-derived macrophages, whose maturation have already been discussed in the previous paragraph. Th1 polarization guarantees the production of pro-inflammatory cytokines (e.g. IFNγ, IL1β, IL12, TNFα) and plays a major role in the cell-mediated anti-mycobacterial immunity in humans (Winslow et al., 2008; Lin and Flynn, 2015) and in cattle as well (Wang et al., 2011). However, the bacterium may adopt strategies to avoid the immune system, firstly at the expense of macrophages – inhibiting the maturation and acidification of the phagolysosomes and promoting apoptosis (Zhai et al., 2019) – and then of CD8+ and CD4+ T lymphocytes, delaying their activation (Urdahl et al., 2011). In this second phase, *Mycobacterium* survives and the disease shows a chronic evolution characterized by a continuous blocking of the host immune-clearance mediated by the microorganism with the final formation of nodular granulomatous lesions - especially in lymph nodes and lungs – and the consequent spread of the disease by aerosol droplets (Palmer and Waters, 2006). Paradoxically, the granuloma

structure, composed of microorganisms surrounded by fibroblasts, epithelial cells and immune cells (e.g. macrophages, T cells, dendritic cells and B cells), represents a strategy to limit the bacillus transmission to other animals (Palmer and Waters, 2006; Salgame, 2011b). During the chronic phase, the CD4+ T polarization shifts from Th1 to Type 2 lymphocytes (Th2), which favor humoral immunity and the production of anti-inflammatory cytokines (e.g. IL4, IL13). The same profile has also been observed after *M. bovis* challenge in vaccinated cattle as compared to those not vaccinated (Widdison et al., 2006a). As reported in humans, Type 17 (Th17) and regulatory (Treg) T cells are also involved in tuberculosis immune response (Agrawal et al., 2018), where the pro-inflammatory activity mediated by Th17 can be antagonized by Treg (Saini et al., 2018). In cattle, IL17A has been evaluated as a possible biomarker for *M. bovis* tuberculosis, demonstrating a strong correlation with IFNG level in TB positive animals (Waters et al., 2016).

Chapter 5: Peripartum disease in dairy water buffalo

5.1. Transition period and metabolic syndrome in ruminants

In cattle, the period between 3 weeks before and 3 weeks after calving is called transition period (Grummer, 1995; Drackley, 1999). This is a critical physiological stage, characterized by a metabolic and endocrine adjustment. One of the main diseases after calving is postpartum uterine infection and endometritis (Sheldon and Owens, 2017); also metabolic disorders, such as ketosis, are associated with peripartum diseases (Dann et al., 2005) affecting the production and the reproductive performances (Ferguson, 2005). In fact, on one side, the higher demand of nutrient and energy is required for the synthesis of colostrum and milk; on the other side, the feed intake is decreased, leading cows to undergo to negative energy balance (NEB) (Wankhade et al., 2017). In other words, after calving, the output of nutrients with milk exceeds the input by voluntary feed intake, resulting in NEB condition, which is common in mammals, but the magnitude and the duration in dairy cows represent a biological extreme (Ceciliani et al., 2018). As the required input of nutrient is higher during NEB, the liver must adapt quickly to support the milk production and to process the non-esterified fatty acids (NEFA) derived from the extensive mobilisation of adipose triglycerides stimulated by NEB (Drackley et al., 2001).

The metabolic syndrome and the associated diseases are particularly studied in cows, where the pushing dairy cow selection towards higher performances led to an increase in milk production but challenged the animal metabolism and welfare (Gross and Bruckmaier, 2019). In cows, the main diseases related to metabolic stress during early lactation are hypocalcemia, fatty liver syndrome and ketosis, while those related to infectious diseases the metritis and mastitis are the most frequent (Ceciliani et al., 2018).

The hypocalcemia, also called milk fever, is due to the great decrease of calcium (Ca) at the end of pregnancy and the onset of lactation. The Ca amount in milk is around 1.2 g/L; considering that a dairy cow can produce up to 60L of milk per day, the daily Ca loss is more than 70g/d. Metabolic adjustment is needed in order to avoid hypocalcemia (DeGaris and Lean, 2008), otherwise retained placenta, metritis and mastitis can easily occur, together with displaced abomasum, ketosis and fatty liver (Neves et al., 2017). The fatty liver consists of the build-up of fat in the liver and it is one of the metabolic disorders associated with NEB. During the positive energy balance, adipose tissue stores energy as fatty acids into

triacylglycerol in a process called lipogenesis; instead, during NEB, adipose tissue releases fatty acids from triacylglycerol through the lipolysis mechanism (Contreras and Sordillo, 2011; Ameer et al., 2014). The high concentration of plasma fatty acids, derived from lipolysis of adipose tissue depots, is significantly related to increased disease susceptibility and lower lactation performance. Lipolysis is also characterized by adipose tissue remodelling and macrophages infiltration. With moderate lipolysis, macrophage infiltration is limited and involves mainly M2 phenotype with anti-inflammatory properties; whereas, during excessive lipolysis most infiltrating macrophages are M1 with a pro-inflammatory role that is able to enhance lipolysis and reduce adipocyte insulin sensitivity with a consequent increased susceptibility to diseases (Contreras et al., 2017). During NEB, the feed intake is not enough to cope with the energy demand with the consequent low glucose amount in the blood (also the ruminal production of propionic acid, a glucose precursor, is decreased); therefore, the increase of lipolysis leads to the release of fatty acids and glycerol that are oxidized to Acetyl-CoA in the liver. Here, the excess of Acetyl-CoA, due to the lack of energy, is converted into keton bodies (e.g. acetoacetate and β-hydroxybutyrate), leading to their accumulation and excretion in milk and urine (Ceciliani et al., 2018).

At delivery, the opening of the cervix and the expulsion of the calf and the placental membranes may damage the anatomical barriers. This event, together with the immunosuppressive state of the animal leads to endometritis or metritis, an inflammation of the inner mucosal layer of the uterus, or of the entire uterine wall, respectively. It is quite common post-partum (Ambarus et al., 2012) and often causes a reduced fertility (Ambarus et al., 2012). The inflammatory reaction is appreciable in the serum of cows affected by endometritis, with an increase of positive APPs, including AGP and Hp (Ceciliani et al., 2018). Mastitis, defined as the inflammation of the mammary gland, is frequent during the transition period and its characteristics are fully reviewed in the appropriate chapter.

The majority of studies on the transition period and metabolic syndrome have been performed in cows. However, biochemical and hormonal changes in the serum of water buffalo during the transition period have been stated, as well as fat mobilization demonstrated by increased levels of NEFA and b-hydroxybutyrate (BHBA) (Fiore et al., 2018). The characterization of the peripartum disease in water buffalo by the evaluation of metabolites, hormones and APPs would be relevant in order to unravel possible biomarker helping to monitor the transition period.

5.2. Acute-phase proteins (APPs)

Infection, neoplasia but also trauma and stress can induce an increase of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα, leading hepatocytes to produce acute phase proteins (APPs) that characterize the so-called acute phase response (APR) (Figure 18) that, in turn, promotes leucocytosis, complement activation and opsonisation (Ceciliani et al., 2012). An inflammatory stimulus leads to the activation of monocytes and macrophages. The cytokines released by these cells stimulate the liver to produce acute-phase proteins. Acute phase proteins, together with cytokines, generate a systemic response at neuroendocrine, metabolic, hematologic and biochemical level (Anglin et al., 2010).

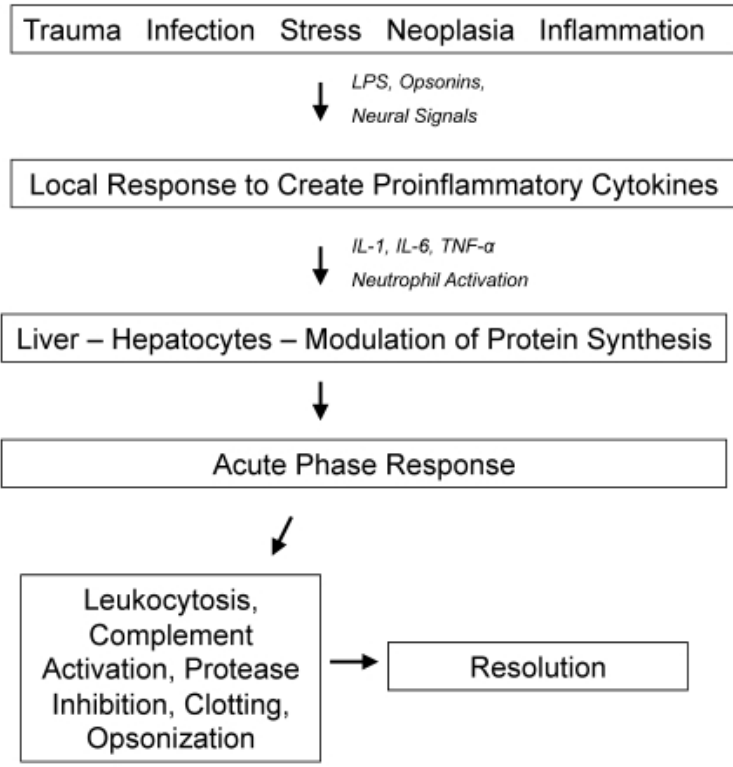

Figure 18 The acute phase response in animals.

From *(Cray et al., 2009)*

The physiological status can be restored by host defence against infection, tissue-repair response or adaptation to stress; on the contrary, if the causative agent persists, it leads to

chronic inflammation and pathological consequences such as autoimmunity, tissue damage and sepsis, fibrosis and tumour growth or auto-inflammatory diseases (Medzhitov, 2008). In cows, 3 proteins are regarded as major APPs (Hb, SAA and mammary associated serum amyloid A3 - MSAA3, this one in milk), 6 as moderate APPs (AGP, lipopolysaccharide-binding protein – LBP, Cp, Fb, inter alpha-trypsin inhibitor H4 – ITIH4, fetuin), and four proteins are regarded as negative APPs (albumin, lipoprotein, paraoxonase, retinol-binding protein) (Ceciliani et al., 2012). SAA is able to modulate the innate immunity (Ceciliani et al., 2012): in particular, it can bind cholesterol from dying cells, prevent atherosclerotic plaques formation (Manley et al., 2006), promote chemotaxis of polymorphonuclear cells (PMNs) and monocytes (Badolato et al., 1994), opsonize Gram- and Gram + bacteria (Molenaar et al., 2009). Hp–Hemoglobin (Hb) has an anti-inflammatory potential (Ceciliani et al., 2012): it can scavenge haemoglobin, prevents oxidative damages and stabilizes iron (Lim et al., 1998), stimulate angiogenesis and chaperone activity; it may also bind neutrophils, downregulating them by inhibiting lipoxygenase and cyclooxygenase activity (Saeed, SA; Ahmad, N; Ahmed, 2007). LBP is believed to be the sentinel against bacteria (Ceciliani et al., 2012): in fact, it is able to bind not only LPS but also other surface antigens of Gram – bacteria, working as an opsonin (Fan et al., 2002; Wright et al., 1989); furthermore, the complexity of the immunomodulatory activity is the results of pro- or anti-inflammatory properties, depending on its low or high concentration, respectively (Lamping et al., 1998). Alpha-1-acid glycoprotein is a dynamic APP (Ceciliani et al., 2012): it is able to bind more than 300 molecules and drugs under physiological conditions (Israili and Dayton, 2001), such as heparin and serotonin (Schmid, K; Kaufmann, H; Isemura, S; Bauer, F; Emura J; Motoyama, 1973), platelet-activating factor (Mcnamara, PJ; Brouwer, KR; Gillespie, 1986) and histamine (Chachaj, W; Bartecka, K; Malolepszy, 2001). Bovine AGP has anti-inflammatory and immunomodulatory roles, downregulating PMN activities by inhibiting reactive oxygen species (ROS) production and degranulation, increasing PMN chemotaxis and reducing monocytes chemotaxis (Lecchi et al., 2008); despite its anti-inflammatory functions, AGP shows a direct anti-bacterial activity (Meroni et al., 2019). AGP is also characterized by different post-translational modifications of oligosaccharides, which are associated with specific pathological states (Ceciliani et al., 2012). APPs are generally produced by the liver, but APPs expression was found also in the reproductive system, such as SAA, AGP, and Hb, in the respiratory tract (LBP), in the digestive tract (SAA, LBP, AGP) and adipose tissue (AGP, Hb, SAA, CRP). Focusing on mastitis, SAA3 and Hp were the major APP. SAA3, namely also MSAA3 in bovine, is mainly synthesized by bovine mammary gland epithelial

cells (McDonald et al., 2001), while SAA1 and SAA2 are the two major circulating forms and are produced by hepatocytes after stimulation by pro-inflammatory cytokines (for instance, IL-6 and TNFα) (Uhlar and Whitehead, 1999). MSAA3 and Hb were increased more than 100 times during mastitis caused by *Staphylococcus aureus* (Whelehan et al., 2011) and in both acute and chronic mastitis (Grönlund et al., 2003), in serum and milk. Interestingly, extra-mammary diseases were able to increase SAA concentration, without affecting MSAA3 and Hb amounts (Nielsen et al., 2004). In conclusion, APPs are promising as disease or inflammation biomarkers, even though very specific assays have to be developed; further investigation is needed in order to clearly differentiate the patho-physiological status of the animal on the basis of APPs concentration in ruminants (Schrödl et al., 2016).

5.3. The acute phase proteins during water buffalo peripartum

The periparturient phase is characterized by an acute phase reaction with the increase of positive acute phase proteins in the circulation (Ceciliani et al., 2012; Gross and Bruckmaier, 2019). In cattle, haptoglobin was found to be increased after calving in those cows affected by more than one disorder, through which retained placenta, displaced abomasum, subclinical ketosis (Huzzey et al., 2011). Also, the amount of SAA, together with Hp, was higher after parturition and significantly correlated with the values of NEFA (Tóthová et al., 2014). Ceruloplasmin and Hp were negatively correlated with the probability of pregnancy, suggesting that the acute phase response has a detrimental role in reproductive performance (Cooke et al., 2009).

To my knowledge, no APP has been measured in water buffalo to monitor the transition period. The transition period in water buffalo has also been poorly addressed so far. Changes in production performances and metabolic and biochemical parameters have been stated. The percentage of milk fat was found to be greater after calving, while a decrease of the daily milk production after 40 days post-partum and a decrease in milk yield in animals over the sixth lactation were reported (Fiore et al., 2017). Fiore and colleagues found that milk yield was positively correlated with insulin and Thyroid-stimulating hormone (TSH) values, while was negatively correlated with the percentage of lactose and Somatic Cell Count (SCC), demonstrating biochemical and hormonal changes in the serum of water buffalo during the transition period (Fiore et al., 2018). Fat mobilization after water buffalo calving is confirmed by the increased values of NEFA and b-hydroxybutyrate (BHBA) from -80 days prepartum

to 1 day post-partum (Fiore et al., 2017). The relevance in monitoring the physiological adjustments, including changes in metabolites, hormones and APPs, is fundamental in order to unravel possible biomarker during the transition period in dairy cows as well as in water buffaloes.

Chapter 6. Experimental designs and results (See Papers)

6.1 Water buffalo mastitis and microbiota (Papers 1,2,3,4 and Manuscript draft 5)

We investigated for the first time the milk microbiota from water buffalo species in relation to mastitis. Even though water buffalo are believed to be less sensitive to mastitis due to the anatomical features of the mammary gland (Thomas et al., 2004), mastitis still represents an economic and welfare issue, in particular for what concerns subclinical mastitis. In water buffalo as well, the development of mastitis may be related to both the presence of the specific pathogen and the modification of the microbial community of milk (Vayssier-Taussat et al., 2014). As milk is considered a low biomass samples, the experimental design of all microbiota studies included all recommendations described in Chapter 2.4. In particular, a special attention was dedicated to milk collection (cleaning the teat and discarding the first streams of milk), DNA extraction and PCR (negative controls from both procedures were included) and bioinformatic analysis (when quantifiable, negative samples were sequenced and analysed in order to assess and analyse the contamination effect).

- On the background that no information on water buffalo milk microbiota was available, our first aim was to unravel the microbiota structure and composition of milk from healthy, clinical and subclinical mastitis affected animals through next-generation sequencing technique (**Paper 1**). We found that the milk microbiota differs depending on the pathological status and mastitis-affected quarter milk samples showed a lower diversity as compared to the healthy ones, as already demonstrated in the cow (Oikonomou et al., 2012). The core microbiota of water buffalo milk, defined as the asset of microorganisms shared by all healthy milk samples, includes 15 genera, namely *Micrococcus*, *Propionibacterium*, *5-7N15*, *Solibacillus*, *Staphylococcus*, *Aerococcus*, *Facklamia*, *Trichococcus, Turicibacter*, *02d06*, *SMB53*, *Clostridium*, *Acinetobacter*, *Psychrobacter* and *Pseudomonas*. Only two genera (*Acinetobacter* and *Pseudomonas*) were present in all the samples from sub-clinical mastitis, and no genus was shared across all in clinical mastitis milk samples. The core microbiota, that was also partly shared with cow milk (e.g. *Staphylococcus*, *Propionibacterium*, *Pseudomonas*) (Addis et al., 2016b), confirmed the higher similarity in healthy quarters as compared to the others.

- Conventional therapy against mastitis includes the treatment of the mammary gland with antibiotics. The number of antimicrobials used in food-producing animals decreased by 10% from 2015 through 2016, accounting for 14 million kilograms; among medically important antimicrobials, the 70% was composed of tetracyclines, whose almost 50% was intended to use in cattle (https://www.fda.gov). In particular, the preventive use of antibiotics in healthy animals at the dry-off result is increasing the antimicrobial resistance of the bacterial population and the consequent more difficult mastitis treatment. Alternative strategies have been studied including the use of bacteriophages, vaccines, nanoparticles, cytokines and plant-, animal- or bacteria-derived antimicrobials (Gomes and Henriques, 2016). Lactic acid bacteria have been demonstrated to have a beneficial effect on human gut microbiota (Pessione, 2012). However, their use at mammary gland in the context of mastitis treatment/prevention is still under investigation (Malvisi et al., 2016). So, our second aim was to unravel alternative strategies to reduce the antibiotic use, inoculating inactivated culture of *Lactobacillus rhamnosus* in water buffalo quarters affected by subclinical mastitis and evaluating the change in milk microbiota together within the microbiological culture (**Paper 2**). Our study demonstrated that in subclinical mastitis samples, no improving at milk microbiota or microbiological culture level was found after *Lactobacillus rhamnosus* treatment; *Pseudomonas* belonging to *Pseumonadaceae* family, already found in subclinical mastitis water buffalo quarter milk (Catozzi et al., 2017b), increased its relative abundance almost four times more, up to 4% after treatment. As *Pseudomonas* is already known as mastitis pathogen in the cow (Hertl et al., 2011), sheep (Wright et al., 2015) and goats (Scaccabarozzi et al., 2015), microbiota balance may be affected. On the contrary, the antibiotic treatment changed the quarter milk microbiota, making all samples from microbiological culture (MC) positive to MC negative, as expected. Interestingly, we found that no paracrine effect was present within the mammary glands: in all animals with subclinical mastitis quarters treated with antibiotic and other subclinical mastitis quarters treated with *Lactobacillus rhamnosus* or PBS, only the antibiotic-treated quarters became MC negative, while the others, within the same mammary gland, did not change or became MC positive after treatment. As each quarter responded independently to the treatment, it suggests that the pathophysiological status of every single quarter is not influenced by the others.

- In addition to the results from the previous experiment, the independence of the mammary gland quarters has been demonstrated from the anatomical point of view (Berry and Meaney, 2006; Akers and Nickerson, 2011). However, it has been shown that infection of one udder

quarter also influences other uninfected quarters (Mitterhuemer et al., 2010; Jensen et al., 2013), suggesting the presence of communication among quarters at the immunological level. This interdependence based on the milk microbiota has not been determined so far. Therefore, the aim of the study was to investigate the intra- and inter-individual variability at microbiota level in healthy and infected water buffalo quarters (**Paper 3**). This is the first experiment evaluating the relationship between udder quarters at milk microbiota level in ruminants. In fact, the evaluation of the inter-dependence of mammary gland quarters have been investigated only at immunological level do far, demonstrating that the mammary gland quarters do not act independently during mastitis (Merle et al., 2007) and that the infection of one udder quarter influences also other uninfected quarters (Mitterhuemer et al., 2010; Jensen et al., 2013). More recent studies demonstrated that quarters of infected udders influence the percentage of B cells and the expression of adhesion molecules in neutrophils of uninfected quarters (Blagitz et al., 2015). Regarding milk microbiota, we pointed out that the quarters within the same mammary gland are more similar than those between different mammary gland and that individual signature may explain the higher similarity between milk microbiota of quarters within the same udder. However, the possible interdependence of milk quarters has to be taken into account when performing microbiota studies, considering that each individual may have specific microbiota signatures, shared within the same individual but not with the others.

- Next-generation sequencing technique is the most used method to study the microbiota. However, it is worth to comment that the sequencing length limitation is between 35-700bp (Goodwin et al., 2016). As longer amplicon sequencing, through third-generation machines (e.g. Nanopore technology), could improve the taxonomic resolution, we reported a methodology paper in order to investigate the whether the sequencing of the full-length 16S (1500pb) and the rrn operon (4500pb) by MinION could increase the taxonomic resolution (**Paper 4**). The samples included in the study were a known community (mock community), a simple community (culture of *Staphylococcus pseudintermedius*) and a complex community (skin sample). We found that the microbiota profiling with long amplicons improved the taxonomic resolution up to species level that it is not possible using short-length reads sequencing. The sequencing of the entire operon rrn is a better choice as compared with the full-length 16S sequencing in terms of resolution, even if the database selection can interfere with the results. We tried to perform the full-length 16S sequencing also on milk samples (**Manuscript draft 5**) and we improved the taxonomy resolution as well. In fact, the main

microbial agents detected after the full-length 16S rRNA gene sequencing in subclinical mastitis samples were in accordance with microbiological culture results, suggesting that Nanopore sequencing could provide a supportive role for microbiological culture, in particular in the field.

6.2 Water buffalo brucellosis and miRNA (Paper 6)

Brucellosis is a zoonotic disease causing more than 500,000 new human infections annually. Decreased animal welfare and production are reported (MCDERMOTT et al., 2013; Singh et al., 2015). Routine screening and animal vaccinations have led to the absence of brucellosis cases in western regions, although it remains endemic in developing regions (e.g. Middle East, Asia, Africa and South America and in some areas of Italy) (Garofolo et al., 2017). Ruminants are highly susceptible to brucellosis. *B. melitensis* and *B. abortus* are the main infectious agents for small and large ruminants, respectively. *B. abortus biovar 1* is the main species causing brucellosis in water buffalo, with the consequent invasion of placenta and fetus in pregnant females and abortion during the last third of the pregnancy (Fosgate et al., 2002). The diagnosis of brucellosis consists of serological tests; however, even if the bacterium is present, results can be negative, mainly during the early disease. The hypothesis was to identify novel biomarkers for diagnosis and prophylaxis protocols, through which miRNA could be possible candidates due to their involvement in pathogen-host interaction networks (Eulalio et al., 2012). No information is present regarding circulating miRNAs during Brucella infection in water buffaloes (*Bubalus bubalis*). Therefore, the aim of the study was to (a) assess miRNA expression profiles in the blood sera of water buffaloes infected by *B. abortus*; (b) extract and measure miRNA expressions in vaginal fluid during B. abortus infection; (c) determine whether miRNAs can be used as biomarkers to assess brucellosis, and (d) integrate miRNAs to their target genes and relative biological processes (**Paper 6**). The miRNAs sequencing identified a total of 469 miRNAs, of which, 20 were differentially expressed in seropositive animals compared with seronegative animals. The validation in qPCR of 12 selected target resulted in 10 miRNAs (miR-let-7i, miR-150, miR-320a, miR-191, miR-let-7f, miR-339b, miR-30e, miR-151, miR-126-5p, and miR-92a) significantly altered between healthy and brucellosis-affected animals in vaginal fluids. Interestingly, no statistically significant differences were found at the serum level. To analyze the diagnostic potency of each miRNA, ROC curve analysis was performed, demonstrating that the area under the curve (AUC) was excellent for vaginal fluid miR-151 and miR-30e, with calculations of 0.957 and 0.931, respectively; good for miR-let-7f, miR-339b, miR-150 and miR-191 (AUC ≥ 0,799); fair for miR-let-7i, miR-92a and miR-320a; and poor for miR-126-5p. The AUC for the combined blood serum miRNAs was 0.753 (95% CI 0.620–0.857) with a cut-off value of 0.90 and 80% sensitivity and 56.25% specificity. The AUC for the combined vaginal fluid miRNAs was 0.88 (95% CI 0.742–0.959) with a cut-off value of 0.023 and

95.45% sensitivity and 85% specificity, suggesting that miRNAs from vaginal fluids could be useful in brucellosis screening. Gene Ontology analysis demonstrated that DE-miRNAs regulated mRNA coding for proteins involved in several molecular functions, cellular components and biological processes involved in *Brucella* pathogenesis. An example is the increase of PI3K (phosphatidylinositol 3-kinase) activities, highlighted by both GO and KEGG analysis, are involved in *Brucella* uptake and in regulating host cell pro-inflammatory responses. Furthermore, the deregulated miRNAs were associated with the apoptosis-related activity, promoting cell survival by targeting anti-apoptotic mRNA (BCL2 family), corresponding to the bacterial strategy in order to avoid its elimination.

6.3 Water buffalo tuberculosis: immune response within the wheal exudate after SIT and skin microbiota (Manuscript draft 7)

Tuberculosis (TB) is a zoonotic and emergent disease, which has led to 147,000 of new cases and more than 12,000 deaths globally in 2016 (www.who.int). It is caused by *Mycobacterium tuberculosis* complex (MTB), which includes genetically related species belonging to the genus *Mycobacterium* and is well adapted to both animals and humans (Malone and Gordon, 2017). Among MTB, *M. bovis* is the main infectious agent for ruminants (Pesciaroli et al., 2014), including water buffaloes (Cousins, 2001; Michel et al., 2007; de Garine-Wichatitsky et al., 2010). The *Mycobacterium* is mainly transmitted by inhalation (MENZIES and NEILL, 2000); as it is an obligate intracellular pathogen, it needs to adhere to the lungs alveolar surface, where phagocytosis occur by macrophages. Then, the immune response is triggered with the consequent activation of T lymphocytes. The infectious outcome, in humans and animal as well, depends on the balance between pro-inflammatory factors – mainly mediated by macrophage killing and CD4+ type 1 lymphocytes (Th1) functions – and anti-inflammatory activities that can promote the extension of tissues damages promoted by the bacterium and by the anti-inflammatory milieu of CD4+ type 2 lymphocytes (Th2) (Widdison et al., 2006b; Cicchese et al., 2018). The strategic control of the infection is mediated by the granuloma, composed of the bacteria surrounded by fibroblasts, epithelial cells and immune cells (e.g. macrophages, T cells, dendritic cells and B cells) (Palmer and Waters, 2006; Salgame, 2011b). If the immune system is not able to remove or restrain the pathogen, the *Mycobacterium* may adopt strategies to escape from the immune system, by inhibiting the maturation and acidification of the phagolysosomes and promoting apoptosis in macrophages (Zhai et al., 2019), and by delaying the activation of CD8+ and CD4+ T lymphocyte (Urdahl et al., 2011). If these adaptive reactions are successful, *Mycobacterium* survives and the disease evolves toward a chronic evolution (Palmer and Waters, 2006).

The single intradermal tuberculin skin test (SIT) is used to screen ruminants once per year. Protein purified derivates (PPD) from *M.bovis* are injected in the animal dermis and the reaction in terms of skin thickness, which features an inflammatory wheal, is evaluated after 72 hours (Good et al., 2018). In addition to SIT, supplemental tests (e.g. IFNγ and antibodies quantifications) are also performed. The diagnosis of tuberculosis is finally confirmed by pathology and/or microbiology after culling. The tests for TB control available for cows are also used for water buffalo. However, the efficacy of the SIT using *M. bovis* PPD in water buffaloes shows a reduced sensitivity and specificity (Kanameda et al., 1999); in fact, water

buffalo skin, due to the great thickness, makes the interpretation of SIT more difficult. Furthermore, *Mycobacterium avium,* a non-tuberculous *Mycobacterium* generally present in the environment, may also interfere with the *M. bovis* PPD test, on the background that the water buffalo skin is often covered with mud due to their attitude for frequent bathing. The first aim of the study was to investigate the mRNA and miRNA at wheal level, after SIT procedure (using *M. bovis* and *M. avium* PPD), in order to unravel the local immunity response (**Manuscript draft 7**). The identification of the mRNA (including cytokines and transcription factors) and miRNAs in the wheal after SIT could be useful for understanding the local immune outcome derived from *M. bovis* and *M. avium* infection.

Regarding the local response at the wheel, the investigation was carried out on mRNA extracted from wheal exudate of 36 animals of which 24 were *M. bovis* positive (*M.bovis*+) and 12 were *M. avium* positive classified (*M.avium*+). The polarization toward Th1, Th2, TReg and Th17 lineage was addressed by measuring the abundance of the respective cytokines, namely TBET, STAT4, IFNγ, IL1β for Th1, STAT5B, IL4 for Th2, FOXP3, IL10 for TReg and RORC, STAT3, IL17A for Th17. The microRNAs involved in the immune response against TB, namely miR-122-5p, miR-148a-3p, miR30a, miR-455-5p, were equally measured. We found that IFNγ was upregulated in *M.bovis*+ as compared to *M.avium*+ samples (fold change=2.54; $p = 0.037$), and positively correlated with two Th1 transcriptional factors, namely TBET (R2=0.43; $p=0.025$) and STAT4 (R2=0.42; $p=0.021$). Among miRNAs, only MiR-148a-3p was found to be increased in *M.bovis*+ animals ($p=0.03$). We demonstrated the presence of a pro-inflammatory Th1-associated profile in *M.bovis*+ animals. miR-148a has been known to positively regulate TBET, suggesting its role in maintaining the pro-inflammatory milieu mediated by Th1 subsets. Further investigation is needed to fully unravel the immune response during tuberculosis in water buffalo, especially in animals classified as *M. avium*+.

6.4 Water buffalo transition period and APPs (Manuscript draft 8)

The most critical phase during the lactation cycle in dairy ruminants, in particular in dairy cows, is the transition from late pregnancy to early lactation, with a focus on the peripartum period. The negative nutrient balance during the first weeks of lactation, during which feed intake cannot meet the need for the rapidly increasing milk production, imposes metabolic stress which may result in several diseases and decrease fertility (Esposito et al., 2014). During the peripartum period, dairy cows have compromised liver function together with increased inflammation and oxidative stress (Bionaz et al., 2007; Trevisi et al., 2012). For example, the increase of Acute Phase Proteins (APP), a family of proteins that are overexpressed during systemic inflammation, is believed to be related to the control of inflammation, given that the function of most of them is exquisitely anti-inflammatory (Ceciliani et al., 2012). If not properly balanced, and if the resolution of inflammation is delayed, the inflammatory status peripartum may result in impaired health and productivity of cows (Bradford et al., 2015). The physiological situation in terms of inflammation and oxidative stress is virtually unknown. Besides their anti-inflammatory properties, APPs are regarded as reliable biomarkers to monitor the inflammatory status of dairy cows during the peripartum period (Ceciliani et al., 2012). Little information about water buffalo acute phase proteins is available, both during peripartum and also in general. In this species, studies on APP are limited to acute diseases (Horadagoda et al., 2001, 2002), identifying Serum Amyloid A (SAA), Haptoglobin (Hp) and α1-acid glycoprotein as major acute reactants. We measured the abundance of three APPs, namely Hp, SAA and AGP, was measured by ELISA (**Manuscript draft 8**). A peak of SAA and Hp was detected in the first week after calving, as already demonstrated in the literature, while the concentration of AGP was measured herein for the first time and demonstrated to increase after parturition.

The buffalo cows in the present study exhibited an acute phase reaction around parturition which is likely necessary for the adaptation to lactation.

Chapter 7. Final remarks and conclusions

The findings of this thesis present an overview of the water buffalo microbiota and immunity during infectious diseases and transition period.

For dairy cows, concerns have been raised since more than 20 years that the increase in milk yield, mainly driven by genetic selection but also improved feeding and management strategies, is putting cows at risk for various metabolic but also infectious diseases (Gröhn et al., 1998; Fleischer et al., 2001; Ingvartsen and Moyes, 2013). The mammary gland defence against the insurgency of an intramammary infection relies on a combination of immune defences, which include somatic cells, cytokines and microRNA, and the microbiome environment. The balance of this equilibrium is believed to help to protect the animal from diseases (Liu et al., 2016). Water buffalo is regarded as more resistant to mastitis than the dairy cow, although data to support this statement are scarce. The background of this concept lies in the mammary gland anatomical features, that includes a long narrow teat canal, a teat skin less sensitive to chapping and sores, a streak canal with thicker epithelium and keratin layer, a tighter sphincter of streak canal and the absence of milk cistern (Thomas et al., 2004). Nevertheless, mastitis, in particular, subclinical mastitis, still represents an economic and health issue in less developed countries (Preethirani et al., 2015), not to mention the risk related to milk-borne pathogens. Furthermore, it might provide a potential issue also in countries where water buffalo is farmed in a more intensive way, such as Italy and Turkey if genetic pressure is directed toward the increase of milk production. The very concept of mammary gland microbiota has been recently challenged (Rainard, 2017), in spite of numerous studies in cow literature demonstrate the presence of microbial community in milk from the mammary gland (Oikonomou et al., 2012; Addis et al., 2016a; Lima et al., 2017) even when these microbes could not be cultivated on a selective terrain. We investigated for the first time the water buffalo milk microbiota derived from healthy, subclinical and clinical mastitis affected quarter milk samples. The core microbiota of water buffalo milk, defined as the asset of microorganisms shared by all healthy milk samples, includes 15 genera, namely *Micrococcus*, *Propionibacterium*, *5-7N15*, *Solibacillus*, *Staphylococcus*, *Aerococcus*, *Facklamia*, *Trichococcus, Turicibacter*, *02d06*, *SMB53*, *Clostridium*, *Acinetobacter*, *Psychrobacter* and *Pseudomonas*. Only two genera (*Acinetobacter* and *Pseudomonas*) were present in all the samples from sub-clinical mastitis, and no genus was shared across all in clinical mastitis milk samples. The microbiota structure was also investigated: we found a

higher diversity in healthy samples, which could be clearly differentiated from those affected by clinical mastitis, while subclinical mastitis samples were difficult to define due to the high heterogeneity among them.

In the background of the antibiotic resistance, we investigated the role of inactivated cultures of *Lactobacillus rhamnosus* on subclinical mastitis quarters. Antibiotic may negatively impact on animal health over time and antibiotic residues may remain in animal products, including milk. Moreover, antibiotic use promotes bacteria to become resistant to antimicrobial drugs (Ceniti et al., 2017; Yang et al., 2016). The use of probiotics to treat mastitis in ruminants have been tried but results are confusing (Crispie et al., 2008; Mignacca et al., 2017). After treatment with *Lactobacillus rhamnosus*, water buffalo quarters did not show any clinical lesions, but no change nor in microbiota neither in the microbiological culture was detected. Milk microbiota was restored only after antibiotic treatment, as expected. The main finding of this study was that the introduction of whatever substances, inactivated bacteria or mock inoculation of apparently non-inflammatory liquid, may induce an inflammatory reaction as assessed by the increase of somatic cells influx into the mammary gland.

In another study, we tried to unravel the interdependence of water buffalo milk quarters at microbiota level. Although the independence of ruminants' mammary gland quarters has been hypothesized from an anatomical point of view, the existence of communication and relationship between them has been demonstrated at immunological level (Merle et al., 2007). We found that the quarters within the same mammary gland were more similar than those between different mammary gland, suggesting that individual signatures may explain the higher similarity between milk microbiota of quarters within the same udder. Especially, the independence of milk quarters needs to be taken into account when performing microbiota studies.

We also explored a way to improve the taxonomic resolution that we know to be limited to family and in some cases genus level when considering NGS technique. We used the full-length 16S gene as a marker in comparison to the V4-16S region to unravel the milk microbiota until species level. The long amplicon sequencing through third-generation machines (e.g. Nanopore technique) allowed to improve the taxonomic resolution, reaching the right species level as confirmed by the microbiological culture result. However, the targeted metagenomic still remains cheaper and the obtained information is able to be processed in an easier way, as compared to metagenomics.

We investigated the immunity associated with the other two major infectious diseases in water buffaloes, namely brucellosis and tuberculosis. For both diseases, the diagnosis is difficult and late, making both diseases difficult to eradicate.

We reported for the first time the role of miRNA as a biomarker for brucellosis, in serum and vaginal fluids. After RNA sequencing, 20 miRNAs showed significantly altered expression in seropositive animals compared with seronegative animals. miRNAs were easily and efficiently extracted from vaginal secretion and the levels of 10 miRNAs were modulated in vaginal secretion of seropositive compared to seronegative animals. The diagnostic accuracy of the combination of six miRNAs was good (AUC= 0.88) with a sensitivity of 95.45 and a specificity of 85, suggesting that a multi-miRNAs panel would allow an early and fast procedure for *Brucella abortus* diagnosis. These results may provide a starting point for the identification of biomarkers in fluids that do not require invasive procedures for collection. However, no correspondence between RNA sequencing and qPCR was present in blood samples, indicating that the validation in qPCR is fundamental.

In tuberculosis, the immune system activity changes during the disease process, showing a pro-inflammatory milieu in the first phase that shifts to an anti-inflammatory environment in the chronic phase (Harris et al., 2009). Programs of control and eradication for tuberculosis have applied to manage and restrain the disease (Caminiti et al., 2016). The intradermal tuberculin test using *M. bovis* PPD is the first examination, followed by additional analyses that include IFNG and antibody quantification. The diagnosis is confirmed after animal culling by the presence of tuberculosis lesions and/or the positive microbiological culture of the pathogen. However, the efficacy of SIT in water buffalo is lower than in cows, with reduced sensitivity and specificity. In fact, the test result is difficult to be evaluated due to the great cutis thickness and the possible interference caused by *Mycobacterium avium*, a non-tuberculous *Mycobacterium* generally present in the environment. Among the measured expression of transcription factors and cytokines related to Th1 (TBET, STAT4, IFNγ, IL1β), Th2 (STAT5B, IL4), TReg (FOXP3, IL10) and Th17 (RORC, STAT3, IL17A) and miRNA (miR-122-5p, miR-148a-3p, miR30a, miR-455-5p), we found an upregulation of the IFNG in *M.bovis*+ as compared to *M.avium*+ samples, with a positive correlation with the corresponding transcription factors (TBET and STAT4). Furthermore, miR-148a resulted upregulated in TB positive samples and it is known to positive regulate TBET, suggesting its role in maintaining the pro-inflammatory milieu mediated by Th1 subsets (Haftmann et al., 2015).

The investigation of water buffalo peripartum was performed combining information from the inflammation status, assessed by APP quantification (Hp, SAA and AGP). The concentration of acute phase proteins was increased around calving: the pattern of Hp and SAA, both considered major APP (Ceciliani et al., 2012), was basically correspondent to those reported for dairy cows, indicating a peak during the period after the calving when infections are more frequent (Chan et al., 2010). AGP is a moderate APP able to interact with several inflammatory mediators and also showing anti-inflammatory properties in cows (Ceciliani et al., 2012). The AGP concentration in water buffaloes was assessed herein for the first time, indicating an increase starting from the 4th week after peripartum, with a peak at the 7th week that can support the hypothesis of no inflammatory status during the seventh weak from calving. This pattern clearly differs from bovine, where increase from the day – 7 to +7 with a decline in the following 2 weeks has been reported (Jafari et al., 2006). Taken together, we can conclude that water buffaloes were not or only slightly under metabolic stress but exhibited an acute phase reaction around parturition which is likely necessary for the adaptation to lactation.

In conclusion, the results of this thesis will hopefully provide data for improving the knowledge of dairy water buffaloes. Although not well adapted to dry climates, water buffalo provides a formidable source of milk, meat and work in developing countries such as Vietnam, Bangladesh and India, where water is the main element in agriculture, and where rains are abundant. In these countries, the introduction of highly productive dairy cows has not succeeded (and will likely not, given how these dairy breeds are sensitive to environmental changes). The very high nutritional quality of milk compensates for its reduced amount. Although probably more sensitive to mastitis than believed, and very much sensitive to zoonosis as Brucella and TB, water buffalo might represent an acceptable option for those countries where the use of dairy cows is not an option. This thesis tried to cover, at least in part, the gap in knowledge about water buffalo microbiota and immunity.

Open questions remain to be answered. For what concerns milk microbiota, further investigation is needed to assess the relationship between microbiota with parity and different stages of lactation, as well as the relationship between farming conditions and microbiota.

The relationship between microbiota and mammary gland immune system remains to elucidated. It is totally unknown what is the basis for immune tolerance of the microbes in the mammary gland. Another unaddressed question is how it is possible that microbiota is

established within a fluid, the milk, that is continuously ejected. Is there any resident microbiota, that is adherent to the membrane of epithelial mammary gland cells, such as that adherent to intestinal cells, that provides the bulk of the milk microbiota.

The source of milk microbiota is also unknown. The information about where it comes from is totally missing.

For what regards the validation procedure, this is one of the major weak points of the technique of identifying bacteria only by means of 16S. Further efforts have to be put into developing techniques to cultivate the widest number possible of bacteria. Combining proteomics analysis with NGS might also be useful for reciprocal validation of the two techniques. Furthermore, the four quarters of the mammary buffalo udder cannot be regardd as separated entities and cannot be considered as individual experimental units for in vivo studies regarding microbiome description. Even if more resistant to mastitis as compared to a cow, the antibiotic use still remains an open issue. The use of GRAS as alternative strategies is not an answer yet, considering their potential pro-inflammatory activity. Future experiments should also be performed concerning the improvement of full-length 16S sequencing by i) increasing the number of samples; ii) reassessing the V1-V9 primer choice, as unspecific host amplicons around 700 bp were detected in addition to the expected ones about 1,500 bp level. Moreover, iii) The full-length 16S sequencing could not be enough to reach species-level identification, within some genera. iv) Test other databases or even build up a specific database would be worth for milk microbiota analysis. The identification of the milk metagenome, including genes for antibiotic resistance, represents one of the future applications. In addition to mastitis research, future investigation is also needed for other infectious diseases (e.g. brucellosis, tuberculosis) and metabolic diseases. Even if findings were promising, the only finding of differential expression or abundance of miRNA or APPs, respectively, needs to be validated on further experiments involving more animals, in order to assess their potential use as a biomarker for that specific pathophysiological condition.

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List of papers published, submitted or in preparation during PhD

Published papers

Paper 1

Catozzi, C., Sanchez Bonastre, A., Francino, O., Lecchi, C., De Carlo, E., Vecchio, D., Martucciello, A., Fraulo, P., Bronzo, V., Cuscó, A., D'Andreano, S., Ceciliani, F., 2017. The microbiota of water buffalo milk during mastitis. PLoS One 12. doi:10.1371/journal.pone.0184710

Paper 2

Catozzi, C., Cuscó, A., Lecchi, C., De Carlo, E., Vecchio, D., Martucciello, A., D'Angelo, L., Francino, O., Bonastre, A.S., Ceciliani, F., 2019. Impact of intramammary inoculation of inactivated Lactobacillus rhamnosus and antibiotics on the milk microbiota of water buffalo with subclinical mastitis. PLoS One 14. doi:10.1371/journal.pone.0210204

Paper 3

Catozzi, C, Cuscó, A., Lecchi, C., Talenti, A., Martucciello, A., Cappelli, G., Bonastre, A.S., Francino, O., Ceciliani, F., 2019. Short communication: Intra- and inter-individual milk microbiota variability in healthy and infected water buffalo udder quarters. J. Dairy Sci. 102, 7476–7482. doi:10.3168/jds.2019-16352

Paper 4

Cusco, A., Catozzi, C., Vines, J., Sanchez, A., Francino, O., 2018. Microbiota profiling with long amplicons using Nanopore sequencing: full-length 16S rRNA gene and whole rrn operon. F100Reasearch 450734. doi:10.1101/450734

Paper 6

Lecchi, C., Catozzi, C., Zamarian, V., Poggi, G., Borriello, G., Martucciello, A., Vecchio, D., DeCarlo, E., Galiero, G., Ceciliani, F., 2019. Characterization of circulating miRNA signature in water buffaloes (Bubalus bubalis) during Brucella abortus infection and evaluation as potential biomarkers for non-invasive diagnosis in vaginal fluid. Sci. Rep. 9. doi:10.1038/s41598-018-38365-x

Accepted manuscripts

Manuscript draft 5

Carlotta Catozzi, Fabrizio Ceciliani, Cristina Lecchi, Andrea Talenti, Domenico Vecchio, Esterina De Carlo, Carlo Grassi, Armand Sánchez, Olga Francino, Anna Cuscó
Short-communication: Milk microbiota profiling on water buffalo with full-length 16S rRNA using Nanopore sequencing – Journal of Dairy Science

Manuscripts to be submitted

Manuscript draft 7

Carlotta Catozzi, Valentina Zamarian, Gabriele Marziano, Emanuela Dalla Costa, Alessandra Martucciello, Esterina De Carlo, Domenico Vecchio, Fabrizio Ceciliani, Cristina Lecchi
Evaluation of lymphocyte-related mRNA and miRNA in wheal exudates of water buffaloes (*Bubalus bubalis*) positive to the intradermal *M. bovis* and *M. avium* PPD test

Manuscript draft 8

Helga Sauerwein, Thomas Blees, Valentina Zamarian, Carlotta Catozzi, Ute Müller, Hassan Sadri, Sven Dänicke, Jana Frahm, and Fabrizio Ceciliani
Acute phase proteins and markers of oxidative status in water buffalos during peripartum

Other manuscripts not related to the present thesis project

Zamarian, V., Catozzi, C., Ressel, L., Finotello, R., Vilafranca, M., Altamira, J., Lecchi, C., 2019. MicroRNA Expression in Formalin-Fixed , Paraffin-Embedded Samples of Canine Cutaneous and Oral Melanoma by RT-qPCR. Vet. Pathol. doi:10.1177/0300985819868646 (Published)

Carlotta Catozzi, Gabriela Avila, Valentina Zamarian, Davide Pravettoni, Giulia Sala, Fabrizio Ceciliani, Nicola Lacetera, Cristina Lecchi 2019. In-vitro effect of heat stress on bovine monocytes lifespan and polarization. Immunobiology. doi.org/10.1016/j.imbio.2019.11.023 (Published)

Valentina Zamarian, Carlotta Catozzi, Anna Cuscó, Damiano Stefanello, Roberta Ferrari, Helena Ariño Bassols, Fabrizio Ceciliani, Olga Francino, Armand Sanchez, Valeria Grieco, Davide Zani, Andrea Talenti, Paola Crepaldi, Cristina Lecchi 2019
Characterization of skin surface and dermal microbiota in mast cell tumor-affected dogs (Submitted)

Activities during the PhD

Activity	Date	CFU	Notes
Open Access – open data (transferable skills)	13/12/2018		
Evaluation of scientific research (transferable skills)	01/02/2019		
How to write a scientific Project (transferable skills)	25/06/2019		
How to write a CV (transferable skills)	18/07/2019		
Communication 1	24/01/2017 – 21/02/2017	2	Approved exam
Bibliographic database	20/01/2017 – 09/02/2017	2	Approved exam
Medical statistics 1	26/10/2016 – 18/01/2017	4	Approved exam
Digital imaging	10/05/2017 – 12/05/2017	2	Approved exam
Benessere dell'animale da laboratorio e animal care – lagomorfi, modelli acquatici e roditori	08/03/2019 – 07/06/2019		Approved exam
Epigenetic mechanisms and their relevance for human pathology	20-21/02/2017	3	
SAS software course	26/06/2017		
ANGUS – analysis sequencing data	02/07/2018 – 14/07/2019		
Bioinformatic tools to study OMIC data	16/01/2019 – 23/01/2019	2	Approved exam
Basic and advanced techniques for optical microscopy in biological and preclinical sample	19/02/2019 – 20/02/2019	2	Approved exam
Pathology of laboratory animals	26/03/2019 – 16/05/2019	2	Approved exam
Winter school ISCCA – flow cytometry for beginners	06/02/2019 – 09/02/2019		

Congress	Date	Abstract title	Notes
EAAP Annual Meeting 2017.	28/08/2017 Tallinn, Estonia.	Development of an ELISA for the acute-phase protein alpha 1-acid glycoprotein (AGP) in cattle	Author in the abstract
PhD course on epigenetic mechanisms and their relevance for human pathology - presentation of the abstract	21-22/02/2017 Milano	Circulating miRNA as possible biomarkers of pain in horses	First author
SISVet congress- presentation of the abstract **Win of RNIV – ELANCO award.**	28/06/2017-01/07/2017 Napoli	Water buffalo milk microbiota related to health status	First author
SISVet congress	28/06/2017-01/07/2017 Napoli	Expression Analysis of MicroRNAs in FFPE samples of canine cutaneous and oral melanoma by RT-qPCR	Author in the abstract
Targeting Microbiota Congress – (poster)	25-27/10/2018 Berlino	Water buffalo milk microbiota during mastitis	First author
London Calling – Nanopore Congress – (poster)	24-25/05/2018 London	Nanopore sequencing of full-length 16S rRNA gene in low-biomass samples: subclinical	First author

		mastitis in water buffalo	
International Mastitis Conference – (poster):	11-12/06/2018 Milano	Comparison between second and third generation sequencing of water buffalo milk microbiota samples	First author
SisVet Congress – (oral presentation)	20-22/06/2018 Torino	Water buffalo subclinical mastitis: changes of milk microbiota after treatment with Lactobacillus rhamnosus	First author
ASPA congress	11-14/06/2019 Sorrento	Effect of heat stress on monocytes and lymphocytes in dairy cattle	Author in the abstract
SisVet Congress – (oral presentation)	19-22/06/2019 Olbia	Cytokine expression in water buffalo exudate affected by tuberculosis	First author
EEAP Congress	26-30/08/2019 Ghent	Effect of cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acid on bovine PBMC apoptosis and viability	Author in the abstract

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1 Paper 1

The microbiota of water buffalo milk during mastitis

RESEARCH ARTICLE

The microbiota of water buffalo milk during mastitis

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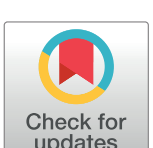

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Abstract

The aim of this study was to define the microbiota of water buffalo milk during sub-clinical and clinical mastitis, as compared to healthy status, by using high-throughput sequencing of the 16S rRNA gene. A total of 137 quarter samples were included in the experimental design: 27 samples derived from healthy, culture negative quarters, with a Somatic Cell Count (SCC) of less than 200,000 cells/ml; 27 samples from quarters with clinical mastitis; 83 samples were collected from quarters with subclinical mastitis, with a SCC number greater of 200,000 cells/ml and/or culture positive for udder pathogens, without clinical signs of mastitis. Bacterial DNA was purified and the 16S rRNA genes were individually amplified and sequenced. Significant differences were found in milk samples from healthy quarters and those with sub-clinical and clinical mastitis. The microbiota diversity of milk from healthy quarters was richer as compared to samples with sub-clinical mastitis, whose microbiota diversity was in turn richer as compared to those from clinical mastitis. The core microbiota of water buffalo milk, defined as the asset of microorganisms shared by all healthy milk samples, includes 15 genera, namely *Micrococcus*, *Propionibacterium*, *5-7N15*, *Solibacillus*, *Staphylococcus*, *Aerococcus*, *Facklamia*, *Trichococcus*, *Turicibacter*, *02d06*, *SMB53*, *Clostridium*, *Acinetobacter*, *Psychrobacter* and *Pseudomonas*. Only two genera (*Acinetobacter* and *Pseudomonas*) were present in all the samples from sub-clinical mastitis, and no genus was shared across all in clinical mastitis milk samples. The presence of mastitis was found to be related to the change in the relative abundance of genera, such as *Psychrobacter*, whose relative abundance decreased from 16.26% in the milk samples from healthy quarters to 3.2% in clinical mastitis. Other genera, such as *SMB53* and *Solibacillus*, were decreased as well. Discriminant analysis presents the evidence that the microbial community of healthy and clinical mastitis could be discriminated on the background of their microbiota profiles.

Introduction

The development of culture-independent techniques by means of high-throughput DNA sequencing has just begun to unravel the impact of large community of micro-organisms, the so called microbiota, on human and animal health [1]. Microbiota establishes mutual relationship with its hosts and the resulting cross-talk extends beyond the balance between tolerance to commensal micro-organisms and developing protection against pathogens [2].

Metagenomic techniques have also revealed how "healthy" microbiota, e.g. the microbial community belonging to healthy individuals, includes potential pathogens. Recent studies on gut microbiota have provided the evidence that the onset of a disease can be the result of a change in the interaction with other microorganisms [3]. A new concept of pathobiome, which can be defined as the microbiota environment integrating also pathogenic agents, is taking shape and has been recently discussed and thoughtfully reviewed [4].

In cows, most of the studies has been carried out on ruminal microbiota [5–9]. A metagenomic approach has also been applied to the relationship between resident microbiomes and the development of reproductive diseases [10–14].

Although the relevance of different bacterial pathogens in mastitis has been known for a long time, the impact of complex community of microbes and their interaction in the development of intramammary infection or mastitis has been only recently, and partially, described [15, 16], and recently reviewed [17]. Milk harbours a wide range of bacteria, many of which cannot be identified by culturing of samples on selective media, leaving therefore as undetected those microorganisms that cannot be cultured. As a consequence, for example, it has been reported that 25% of clinical mastitis caused by bacteria are routinely not detected by means of bacterial culture [18], as confirmed by the finding that bacterial species may be present also in culture-negative samples collected from animals with clinical mastitis [19].

The microbial content of raw and pasteurized milk revealed the presence of a rich and diverse bacterial population [20]. Metagenomic pyrosequencing techniques of bacterial 16S rRNA were applied to investigate milk samples from mastitic and healthy dairy cows, revealing that microbiota were different [15, 19]. Although the concept of milk microbiota as determined by culture independent techniques has been very recently challenged [21], the pyrosequencing of bacterial 16S rRNA could discriminate healthy from sub-clinically and clinically affected quarters [16]. Major pathogens such as *Streptococcus uberis* and *Staphylococcus aureus* were also found in milk from animals with no evidence of inflammatory reaction, suggesting the hypothesis that the development of mastitis can be regarded more as a dysbacteriosis than a primary infection [16].

Water buffaloes provide the most important source of non-cattle milk worldwide (13.2%) [22]. In some countries, such as India, water buffalo milk accounts for the 55% of the total milk produced [23]. The effects of environmental factors and management practices, as well as the stage of lactation, parity and calving season, on physical-content and somatic cell counts (SCC) were recently described [24–26]. Dairy water buffaloes can be affected by mastitis with a frequency only slightly lower as compared to cows [27–29]. Mastitis could therefore have negative impacts on water buffalo dairy economy equal to that on cow dairy farms in term of reducing milk yield, premature culling and cost of therapy [30]. Information about pathogens involved in mastitis occurrence in water buffalo is limited. Culture dependent approaches demonstrated that most frequently isolated bacteria during mastitis are coagulase negative, causing 78% of intramammary infections cases of mastitis [31, 32], *Prototeca* spp. and *Streptococcus pluranimalium* being found occasionally [33, 34].

Culture independent techniques have been applied to the study of mozzarella production, focusing on raw milk, natural whey cultures and curd to the final cheese product [35, 36]. Milk

microbiota associated with the health status of water buffalo mammary gland has not been investigated yet.

The aim of the present study is to bridge this gap by providing insights into the microbiota of dairy water buffalo milk related to healthy status by means of high-throughput DNA sequencing of the 16S rRNA genome milk samples from healthy and clinical and sub-clinical mastitis affected quarters in dairy water buffaloes.

Materials and methods

Sample collection

One hundred thirty-seven quarter milk samples derived from 88 dairy water buffalo cows belonging to 14 farms, homogeneously distributed in Campania area (Italy), were collected from January to February 2016. The samples were collected after owner permission and the collection methods were consistent with recommendations according to standard procedure by National Mastitis Council [37].

Samples were collected after teat ends have been disinfected with 70% ethylic alcohol and the first strain of milk was discarded. Microbial diversity was analysed after classification of quarter milk as follows: 27 samples were collected from healthy quarters with no clinical signs of mastitis during the present lactation, with two consecutive Somatic Cell Counts (SCC) values lower than 200,000 cells/ml and aerobic culture negative for udder pathogens (H); 27 samples with clinical mastitis (CM) were collected from quarters showing signs of clinical mastitis and aerobic culture positive. Three animals with negative microbiological culture but with very high SCC ($>$ 2400,000 cells/ml) were also included in this group. For 14 samples it was not possible to carry out a reliable SCC due to the very high density of milk. Finally, 83 samples with sub-clinical mastitis (SM) were collected from quarters showing no signs of clinical mastitis but with aerobic culture positive for udder pathogens. Fifteen samples with SCC number greater of 200,000 cells/ml but with negative microbiological culture were also included in this group.

Samples were refrigerated and delivered within 12 hours for SCC and microbiological analysis. Animals that were treated in lactation with antibiotics within the previous 90 days were excluded from the experiment.

Somatic cells count and microbiological culture

Somatic cells count (SCC) was measured in milk samples using Fossomatic (Foss) apparatus by means of the UNI EN ISO 13366–2: 2007 technique for electronic optical fluorometric counters [38].

Microbiological culture tests were performed for each milk sample using different media: cultures were incubated at 37˚C for 24h in aerobic conditions on blood agar (Trypticase Soy Agar with 5% sheep blood), MacConkey agar and Baird Parker Agar; at 37˚C for 72h in aerobic conditions on *Prototheca* Isolation Medium (PIM) at 37˚C in micro-aerobic conditions on *Mycoplasma* agar. Gram staining, coagulase and oxidase tests were performed on cultures with mastitis pathogens; in particular, *Staphylococcus* spp. culture coagulase detection was carried out using rabbit plasma and then for *Streptococcus* spp. Streptokit-BioMérieux test was employed using Lancefield grouping, in order to identify antigen differences between species.

DNA extraction

One ml of milk was centrifuged for 10 min at room temperature at 16,100 rcf [16]. The supernatant was discarded and the remaining pellet was resuspended in 250μl of the Power Bead

Tube solution of the PowerSoil™ DNA isolation kit (MO BIO), which was used to extract bacterial DNA, according to the manufacturer's instructions. DNA samples were eluted in 50 μl of C6 solution and stored at -20˚C until further processing. Therefore, DNA concentration and purity were analyzed using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A) at wavelengths 230, 260 and 280 nm.

Amplification of the hypervariable V1-V2 region of bacterial 16S rRNA gene by PCR and barcoding

V1-V2 regions of 16S rRNA gene were amplified for each sample [16, 19]. The forward primer was 5′–CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNN*GATA*AGAGTTTGATCCTGGCTCAG–3′, composed of the adapter linker, the Key, the barcode that is different from each sample, the spacer and the conserved bacterial F27 forward primer, respectively. The reverse primer was 5′–CCTCTCTATGGGCAGTCGGTGATTGCTGCCTCCCGTAGGAGT– 3′, composed of the adapter linker and the R338 reverse primer. PCR was carried out following the instructions of Thermo Scientific Phusion Hot Start II High-Fidelity DNA Polymerase Kit; each PCR reaction contained RNAse and DNAse free water, 5x Phusion Buffer HF (5 μl), dNTPs 2mM (2.5 μl), Primer Fw 10mM (1.25 μl), primer Rv 10 mM (1.25 μl) and Phusion High Fidelity Taq polymerase (0.25 μl), and 5 ng of DNA sample in a final volume of 25 μl. The lack of amplification of a negative control for each PCR reaction demonstrated the absence of contamination by reagents that could interfere with the analysis [39]. The thermal profile used for the amplification consisted of an initial denaturation of 30 sec at 98˚C, followed by 30 cycles of 30 sec at 98˚C, 15 sec at 55˚C, 20 sec at 72˚C and a final extension of 7 min at 72˚C. Each PCR plate included samples derived from each group. Quality and quantity of PCR products were determined using Agilent Bioanalyzer 2100 and Qubit™ fluorometer. All 137 quarter milk samples (27 H, 27 CM and 83 SM) were used for the downstream analysis.

High-throughput sequencing, bioinformatics and statistical analysis

Sequencing was carried out using Ion Torrent Personal Genome Machine (PGM) with the Ion 318 Chip Kit v2 (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A) under manufacturer's conditions. The raw sequences have been submitted to NCBI under the Bioproject accession number PRJNA384692. Raw reads or FASTA sequences were de-multiplexed, quality-filtered and analysed using Quantitative Insights Into Microbial Ecology (QIIME) 1.9.1 software [40].

As parameters for the analysis, we considered a sequence length greater than 300 bp, a mean quality score above 25 in sliding window of 50 nucleotides, no mismatches on the primer and default values in the split libraries script. VSearch (version 1.11.1) was used to dereplicate sequences, cluster them by de novo approach at 97% of similarity and detect and remove chimeras [41]. Taxonomy was assigned by the Ribosomal Database Project (RDP) classifier [42] using Greengenes database 13.8 [43] as reference, and then sequences were aligned through PyNAST method [44]. Reads were also filtered removing chloroplast and low abundance sequences (less than 0.005% of total Operational Taxonomic Units (OTUs)) [45].

The filtered OTU table was used to perform downstream analyses. Taxonomy showed the composition of OTUs for each sample or group of samples. Alpha and beta diversity, which analyse differences within and among samples, respectively, were carried out with a depth of 9300 sequences. Alpha diversity outputs were represented using two different metrics, describing how many taxa are present in the samples: observed species that considers only the richness or the total number of OTUs and Shannon index that estimates the evenness or the relative abundance of OTUs in addition to the richness. As the definition of subclinical mastitis is not homogeneous, an alternative classification of non-mastitic samples was carried out

for the purpose of statistical analyses of alpha diversity, using four different grouping based on SCC, independently from the microbiological culture, namely: a total of 22 samples derived from clinically healthy quarters with a SCC of less than 100,000 cells/ml (class 1); 33 samples derived from clinically healthy quarters with a SCC ranging from 100,000 to 499,000 cells/ml (class 2); 14 samples derived from clinically healthy quarters with a SCC ranging from 500,000 to 100,000,000 cells/ml (class 3); 40 samples derived from clinically healthy quarters with a SCC greater than 100,000,000 cells/ml (class 4). Beta diversity, which evaluates how many taxa are shared among samples, was calculated using weighted and unweighted UniFrac distance matrices, where quantitative and qualitative approach is respectively considered in addition to the phylogenetic analysis derived from UPGMA trees. Distance matrices were plotted using the Principal Components Analysis (PCA).

Taxonomical analysis, due to the not-normal distribution of data assessed by Shapiro-wilk test, was evaluated with the non-parametric Kruskal-Wallis method and Dunn's post-hoc multiple comparison test; Bonferroni correction was also performed.

Statistical significance of alpha diversity was assessed using the non-parametric Monte Carlo test (999 permutations).

Beta diversity statistics was performed with the non-parametric Adonis and ANOSIM methods, which reflects the ANOVA test for not normally distributed samples. Statistical significance is determined by *p*-value, R^2 value or percentage of variation explained by the variable (for Adonis method) and R value (for ANOSIM method) where more the value is close to 1, more the dissimilarity is high.

Results

Diagnosis of mastitis by bacterial culture and SCC

In order to identify and classify samples for Next Generation Sequencing (NGS) characterization of microbiota, milk was collected and tested for microbiological culture. Results are presented in Table 1.

All milk samples from healthy quarters had negative microbiological cultures and a SCC $<$ 200,000 cells/ml.

Among SM affected quarters, bacteria that are potentially associated with mastitis were recovered in 67 samples (81%), whereas the others 15 were negative after microbiological culture with SCC $>$ 200.000 cells/ml. For 1 sample, microbiological results were missing.

All the samples collected from quarters with CM contained bacteria that are associated with mastitis, as detected under standard growing conditions, except for 3 samples that were negative, and 4 samples whose microbiological results were missing (nr. 2) or contaminated (nr.2). No sample was found positive for Mycoplasma.

Ion torrent output: Sequence results after filtering processes

The sequencing of 137 milk samples produced 31,777,423 total reads with an average read length of 217.5 nucleotides, a median of 259.5 nucleotides and a mode of 346 nucleotides. Before removing chloroplast sequences, 16,231 OTUs were found. After chloroplast, low abundance filtering and removal of two samples as previously described, 1,398 OTUs were obtained.

Core microbiota and taxonomic profile analysis

Water buffalo milk microbiota is composed of 9 main phyla, namely *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Spirochaetes*, *TM7* and *Tenericutes* (Fig 1 and Table 2).

Table 1. Microbiological culture results: Prevalence of cultured bacteria species in each group of milk samples.

Cultured bacteria	CM	CM%	H	H%	SM	SM%	Total
Negative	**3**	**11.1**	**27**	**100**	**15**	**18.1**	**55**
Trueperella pyogenes	4	14.8	0	0	1	1.2	5
Escherichia coli	0	0.0	0	0	1	1.2	1
Pseudomonas aeruginosa	2	7.4	0	0	0	0.0	2
Streptococcus agalactiae	1	3.7	0	0	1	1.2	2
Staphylococcus aureus	5	18.5	0	0	37	44.6	42
Staphylococcus aureus-Streptococcus agalactiae	3	11.1	0	0	4	4.8	7
Staphylococcus chromogenes	0	0.0	0	0	2	2.4	2
Streptococcus dysgalactiae	4	14.8	0	0	3	3.6	7
Staphylococcus. spp.	0	0.0	0	0	18	21.7	18
Staphylococcus. spp. -*Escherichia coli*	1	3.7	0	0	0	0.0	1
Contaminated and/or missing	4	14.8	0	0	1	1.2	5
Total	**27**	100.0	**27**	100	**83**	100.0	**137**

Only samples used for microbiota determination were included.

https://doi.org/10.1371/journal.pone.0184710.t001

The healthy milk microbiota is dominated by *Firmicutes*, representing the 57.70% of the bacteria, followed by *Proteobacteria* (23%), *Actinobacteria* (12%), *Bacteroidetes* (6%) and *Fusobacteria* (1%).

As compared to milk from H animals, SM milk presents a decrease of *Firmicutes (48%) and Actinobacteria (6%)* and a relative increase in *Bacteroidetes* (11%) and *Proteobacteria (33%)*. In CM milk, the relative abundance of *Bacteroidetes* increases to 24% and *Fusobacteri*a to 8%, whereas *Proteobacteria*, *Tenericutes* and *Actinobacteria* were decreased. Statistical differences are presented in Table 2. Only *Fusobacteria* phylum was found to be statistically significantly different between SM and CM samples. Results were also analysed at family level: relative abundances and statistical differences ($p \leq 0.05$) are presented in S1 Fig and S1 Table, considering the main families (relative frequency at least at 1%). *Peptostreptococcaceae*,

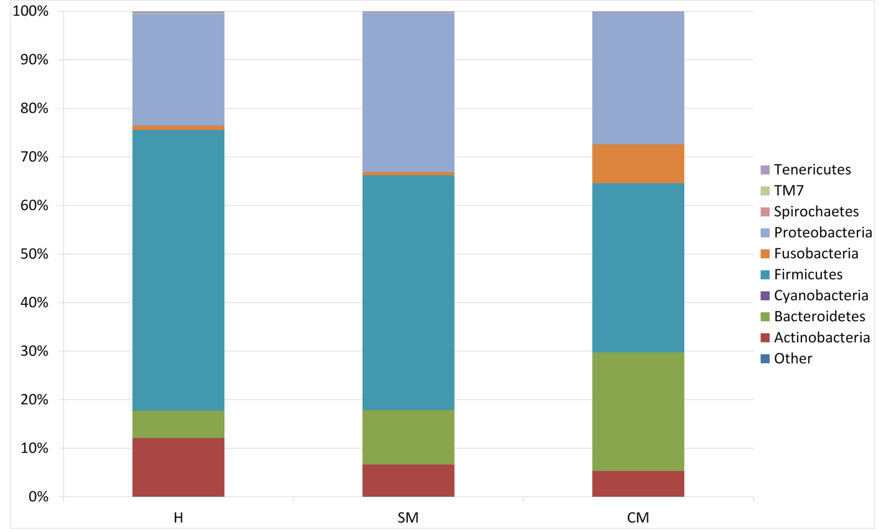

Fig 1. Water buffalo milk taxonomic profile at phylum level. Microbiota composition at the phylum level for the 16S rRNA. H = Healthy samples; SM = Sub-Clinical mastitis samples; CM = Clinical mastitis samples

https://doi.org/10.1371/journal.pone.0184710.g001

Table 2. Relative abundance of microbiota taxa at phylum level.

	Relative abundance frequences			*p-value (where p<0.05)*		
	H	**SM**	**CM**	**H vs SM**	**H vs CM**	**SM vs CM**
Other	0.08%	0.11%	0.06%	ns	ns	Ns
Actinobacteria	12.04%	6.55%	5.26%	0.043	0.053	Ns
Bacteroidetes	5.66%	11.22%	24.44%	ns	ns	Ns
Cyanobacteria	0.03%	0.00%	0.00%	ns	ns	Ns
Firmicutes	57.70%	48.33%	34.83%	ns	ns	Ns
Fusobacteria	0.94%	0.66%	8.00%	ns	0.001	<0.0001*
Proteobacteria	22.93%	32.71%	27.11%	ns	ns	Ns
Spirochaetes	0.01%	0.02%	0.02%	ns	ns	Ns
TM7	0.14%	0.05%	0.02%	0.039	0.004	Ns
Tenericutes	0.47%	0.35%	0.25%	ns	0.011	Ns

H = Healthy samples; SM = Sub-Clinical Mastitis samples; CM = Clinical mastitis samples. Significance at $\leq$ 0.05.

* Bonferroni correction was applied.

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Aerococcaceae, *Staphylococcaceae*, *Clostridiaceae*, *Moraxellaceae* and *Corynebacteriaceae* accounted for 69% of the families of H milk. Among the major families (> 8%), *Peptostreptococcaceae*, *Aerococcaceae* and *Staphylococcaceae* decreased in a statistically significant way in SM milk. Together with *Staphylococcaceae* and *Moraxellaceae*, *Aerococcaceae*, *Clostridiaceae*, *Corynebacteriaceae* and *Peptostreptococcaceae* are decreased in CM milk as well. As compared with SM milk, CM milk presented an increase of *Porphyromonadaceae*, *Fusobacteriaceae* and *Leptotrichiaceae*, and a decrease of *Staphylococcaceae* and *Moraxellaceae*.

The modifications at family level reflect on those at genus level (Fig 2 and Table 3) (relative frequency at least at 1%).

The water buffalo core microbiota at genus level, defined as the asset of genera shared by all healthy milk samples, included 15 genera, namely *Micrococcus*, *Propionibacterium*, *5-7N15*, *Solibacillus*, *Staphylococcus*, *Aerococcus*, *Facklamia*, *Trichococcus*, *Turicibacter*, *02d06*, *SMB53*, *Clostridium*, *Acinetobacter*, *Psychrobacter and Pseudomonas*. As compared to H quarters, milk from SM presents a statistically significant decrease of *Propionibacterium*, *Solibacillus*, SMB53, and *Clostridium*, and an increase of *Porphyromonas*. Milk obtained from CM evidenced a further decrease of most of the genera found with a relative abundance more than 1%, and an increase of *Bacteroides*, *Porphyromonas*, *Aerococcus*, *Lactococcus*, *Peptoniphilus*, *Fusobacterium*, *Sneathia* and *SM853*. As compared to SM, CM milk samples present a decrease of *Staphylococcus*, *Turicibacte*r, *02d06*, *SMB53*, *Clostridum* and *Psychrobacter*, and an increase of *Bacteroides*, *Porphyromonas*, *Aerococcus*, *Peptoniphilus*, *Fusobacterium* and *Sneathia*. Fig 3 presents the microbial relative abundance at genus level in H, SM and CM milk samples. A classification of samples independent on microbiology and based on SCC was also carried out. The samples were grouped in four SCC classes: Class 1, with a SCC < 100,000 cells/ml, Class 2, with a SCC between 100,000 cells/ml and 500,000 cells/ml, Class 3, with a SCC between 500,000 cells/ml and 1,000,000 cells/ml and Class 4, with a SCC > 1,100,000 cells/ml. Results of relative abundance of genera are reported in Fig 4 and Table 4.

No “core microbiota” could be defined following a classification of samples in SCC classes. As compared to SCC class 1, the relative abundance of *Jeotgalicoccus* was decreased from 0.85% of Class 1 to 0.56% of class 4. The relative abundance of *Corynebacterium*, *Solibacillus*, *SMB53* and *Clostridium* was decreased as well from class 1 to class 4. On the contrary, the relative abundance of *Lactococcus* was increased, from 0.24% of class 1 to 14.35% of class 4,

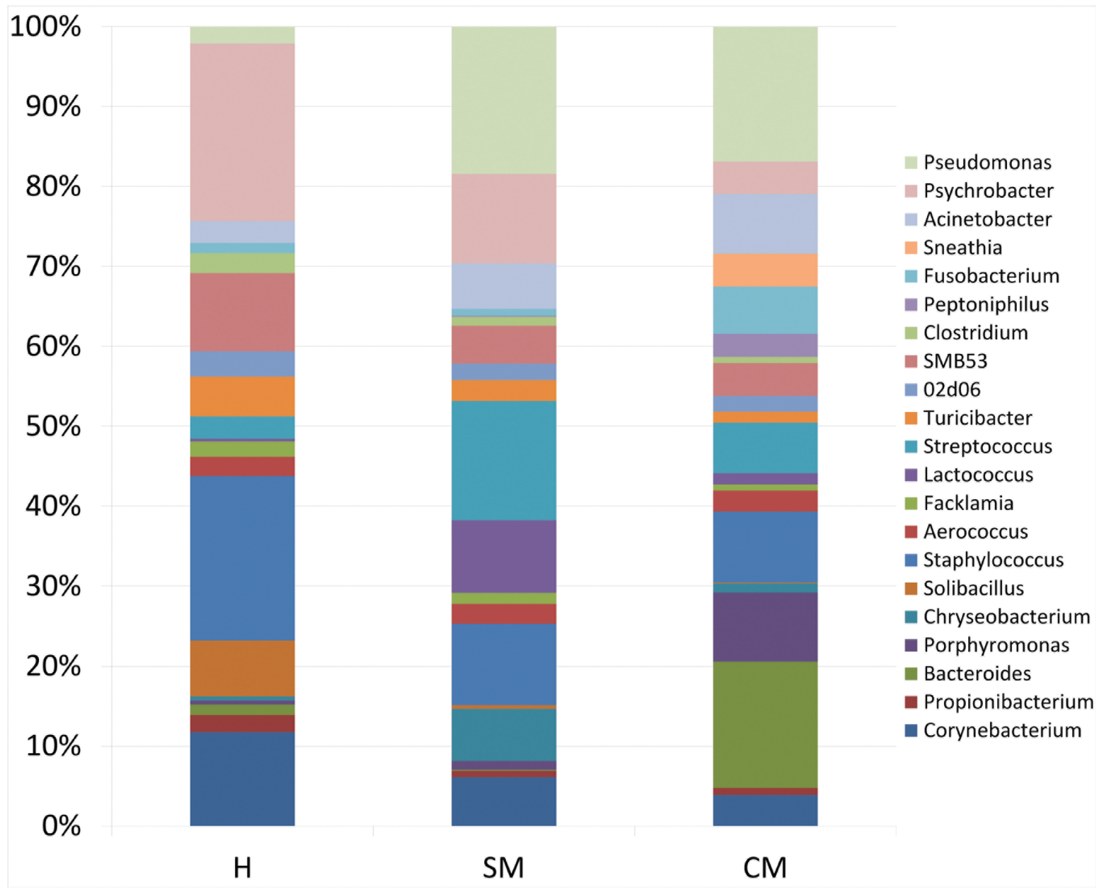

Fig 2. Water buffalo milk taxonomic profile at genus level. Microbiota composition at the genus level for the 16S rRNA gene Microbiota composition at the genus level for the 16S rRNA gene. H = Healthy samples; SM = Sub-Clinical mastitis samples; CM = Clinical mastitis samples.

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although it must be said that only differences between class 1 and class 3, and 3 to class 4 were statistically significant.

Discriminant analysis and clustering of samples

Alpha diversity analysis showed that H and CM samples were statistically different with 445.76 (STD = ± 140.82) and 198.89 (STD = ± 186.28) observed OTUs ($p = 0.006$) and 5.72 (STD = ± 1.33) and 4.08 (STD = ± 2.05) Shannon index ($p = 0.03$), respectively. Statistical differences were also found comparing H with SM group ($p = 0.018$) with 445.76 and 317.15 (STD = ± 178.70) observed OTUs, respectively. Alpha diversity is plotted in Fig 5 using Shannon index. On the contrary, it is not possible to discriminate between SM and CM samples. As the definition of subclinical mastitis is not homogeneous, an alternative classification of non-mastitic samples was carried out, using parameters that are independent from microbiological results, alternatively classifying the healthy and sub-clinical mastitis samples in four different grouping based on SCC. Results are presented in S2 Fig. Class 1 (SCC < 100,000 cells/ml) and 4 (SCC > 1,000,000 cells/ml) were statistically different with 468.19 (STD = ± 126.31) and 266.93 (STD = ± 159.87) observed OTUs ($p = 0.006$) and 6.61 (STD = ± 1.11) and 4.91 (STD = ± 1.6) Shannon index ($p = 0.006$), respectively. Also class 1 and 3 (SCC between 500,000 and 1,000,000 cells/ml) were statistically different, with 6.61 (STD = ± 1.11) and 4.29 (STD = ± 1.3) Shannon

Table 3. Relative abundance frequencies at genus level. Grouping following mastitis diagnosis.

	Relative abundance frequencies			*p-value (where p<0.05)*		
Genera	**H**	**SM**	**CM**	**H vs SM**	**H vs CM**	**SM vs CM**
Corynebacterium	8.61%	4.80%	3.10%	ns	0.012	ns
Propionibacterium	1.57%	0.61%	0.71%	0.002	0.004	ns
Bacteroides	0.95%	0.11%	12.60%	ns	0.018	<0.001
Porphyromonas	0.37%	0.86%	6.88%	0.051	<0.0001*	<0.0001*
Chryseobacterium	0.39%	5.12%	0.92%	ns	ns	0.016
Solibacillus	5.12%	0.37%	0.10%	<0.0001	<0.0001*	0.025
Staphylococcus	15.09%	7.98%	7.05%	ns	0.017	0.005
Aerococcus	1.76%	1.97%	2.10%	ns	0.007	0.006
Facklamia	1.41%	1.08%	0.60%	0.053	<0.001	0.018
Lactococcus	0.25%	7.11%	1.13%	ns	ns	ns
Streptococcus	2.04%	11.70%	5.04%	ns	ns	ns
Turicibacter	3.66%	2.06%	1.11%	ns	<0.0001	0.001
02d06	2.31%	1.63%	1.56%	ns	<0.0001	0.002
SMB53	7.18%	3.70%	3.28%	0.018	0.001	ns
Clostridium	1.82%	0.88%	0.63%	0.009	<0.0001	<0.001
Peptoniphilus	0.00%	0.10%	2.28%	ns	<0.0001	<0.001
Fusobacterium	0.94%	0.66%	4.74%	ns	<0.0001	<0.0001*
Sneathia	0.00%	0.00%	3.26%	ns	0.007	<0.0001
Acinetobacter	2.03%	4.47%	5.97%	ns	ns	ns
Psychrobacter	16.26%	8.79%	3.22%	ns	0.002	0.027
Pseudomonas	1.57%	14.45%	13.48%	ns	ns	ns
Micrococcus	0.53%	0.14%	0.21%	<0.0001	<0.001	NS
Flavobacterium	0.06%	0.51%	0.49%	ns	Ns	ns
Jeotgalicoccus	0.82%	0.46%	0.26%	<0.001	<0.0001	ns
Trichococcus	0.82%	0.74%	0.48%	0.03	<0.001	0.007
Helcococcus	0.16%	0.21%	1.84%	ns	ns	0.014
Roseomonas	0.02%	0.55%	0.00%	ns	ns	ns
Erwinia	0.01%	0.51%	0.00%	ns	ns	0.016

*Bonferroni-corrected p-value at 0.0001

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index (p = 0.006), respectively. Class 2 (SCC between 100,000 and 499,000 cells/ml) and 4 were statistically different for observed OTUs, 391.15 (STD = ± 166.03) and 266.93 (STD = ± 159.87), respectively (p = 0.03).

Beta diversity analysis was carried out comparing milk samples from healthy and clinical and sub-clinical quarters, using the weighted and unweighted Unifrac distance metric. The results provided the evidence that it is possible to discriminate between the groups (Adonis: R^2 = 0.09 and p = 0.001; ANOSIM: R = 0.15 and p = 0.003 for weighted Unifrac; Adonis: R^2 = 0.09 and p = 0.001; ANOSIM: R = 0.09 and p = 0.0012 for unweighted Unifrac). Beta diversity is plotted in using unweighted Unifrac Fig 6 (Panel A): the first component (C1) explains the 31.9% of the variability and separate healthy from clinical mastitis milk samples, even if some overlaps are present. On the other hand, the second component (C2) explains the 8.9% and separates clinical mastitis samples from the others, although with some overlaps. Considering only H and CM groups, where C1 = 34.1% and C2 = 9.9%, the separation of healthy and clinical mastitis samples is improved as shown in Fig 6 (Panel B) (Adonis: R^2 = 0.09 and p = 0.001; ANOSIM: R = 0.15 and p = 0.003 for weighted Unifrac; Adonis: R^2 = 0.17 and p = 0.001;

Fig 3. Water buffalo milk taxonomic profile at genus level. The microbial relative abundance at genus level between: H = Healthy samples; SM = Sub-Clinical mastitis samples; CM = Clinical mastitis samples; * indicates statistical significance ($p \leq 0.05$).

https://doi.org/10.1371/journal.pone.0184710.g003

ANOSIM: R = 0.37 and $p = 0.001$ for unweighted Unifrac), showing that H quarters can be discriminated from CM quarters by C1. Box Plot representing C1 and C2 axes are presented in S3 Fig. Beta diversity analysis was also carried out comparing the four SCC groups derived

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

Class 1
Class 2
Class 3
Class 4

g__Pseudomonas
g__Psychrobacter
g__Moraxella
g__Acinetobacter
g__Fusobacterium
g__Clostridium
g__SMB53
g__Proteiniclasticum
g__02d06
g__Turicibacter
g__Streptococcus
g__Lactococcus
g__Trichococcus
g__Facklamia
g__Aerococcus
g__Staphylococcus
g__Jeotgalicoccus
g__Solibacillus
g__Vitellibacter
g__Porphyromonas
g__5-7N15
g__Propionibacterium
g__Corynebacterium
g__Brevibacterium

Fig 4. Water buffalo milk microbiota composition at the genus level for the 16S rRNA gene after classification of clinically healthy samples in SCC classes. Microbiota composition at the genus level for the 16S rRNA gene: Class 1, with a SCC < 100,000, Class 2, with a SCC between 100,000 and 500,000, Class 3, with a SCC between 500,000 and 1,000,000 and Class 4, with a SCC > 100,000,000.

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from all clinically healthy quarters, using the weighted and unweighted Unifrac distance metric (Adonis: $R^2 = 0.08$ and $p = 0.001$; ANOSIM: $R = 0.09$ and $p = 0.003$ for weighted Unifrac; Adonis: $R^2 = 0.08$ and $p = 0.001$; ANOSIM: $R = 0.06$ and $p = 0.017$ for unweighted Unifrac). Results are presented in S4 Fig and show that the distribution of class 3 (SCC between 500,000 and 1,000,000 cells/ml) and 4 (SCC $>$ 1,000,000 cells/ml) was more scattered in the plot compared to class 1 (SCC $<$ 100,000 cells/ml) and 2 (SCC between 100,000 and 499,000 cells/ml), which were more homogeneous, and better clusterized by C1 (component one) axis that explains the 36.4% of the variability. C2 (component two) axis cannot discriminate between groups. Box Plot representing C1 and C2 axes are presented in S4 Fig.

Discussion

We report here the first detailed characterization of milk microbiota in water buffaloes with clinical and sub-clinical mastitis as determined by 16S rRNA gene diversity profiling. Therefore, being the ribosomal 16S RNA gene domain restricted to bacteria and archaea [46] we did

Table 4. Relative abundance frequencies at genus level. Grouping following SCC classes.

	Relative abundance—SCC group				*p-values*					
Genus/Classes	**Class 1**	**Class 2**	**Class 3**	**Class 4**	**1 vs 2**	**1 vs 3**	**1 vs 4**	**2 vs 3**	**2 vs 4**	**3 vs 4**
Brevibacterium	0.02%	0.32%	0.92%	0.81%	ns	ns	ns	ns	ns	ns
Corynebacterium	8.97%	6.51%	3.45%	2.87%	ns	ns	0.036	ns	0.046	ns
Propionibacterium	1.33%	1.08%	0.23%	0.62%	ns	0.012	0.002	ns	ns	ns
5-7N15	0.80%	0.59%	0.12%	0.51%	ns	0.016	0.002	ns	0.029	ns
Porphyromonas	0.35%	0.48%	0.60%	0.82%	ns	ns	ns	ns	ns	ns
Vitellibacter	0.00%	0.16%	7.69%	7.49%	ns	ns	ns	ns	ns	ns
Solibacillus	3.29%	2.50%	0.03%	0.28%	ns	0.001	<0.0001	0.036	0.001	ns
Jeotgalicoccus	0.85%	0.46%	0.26%	0.56%	ns	<0.0001	<0.0001	0.006	0.009	ns
Staphylococcus	13.18%	11.04%	2.39%	3.61%	ns	ns	ns	ns	ns	ns
Aerococcus	1.60%	2.82%	0.70%	1.63%	ns	ns	ns	ns	0.05	ns
Facklamia	1.51%	1.23%	0.39%	0.98%	ns	ns	ns	ns	ns	ns
Trichococcus	0.98%	0.87%	0.13%	0.55%	ns	ns	0.027	ns	ns	ns
Lactococcus	0.24%	4.14%	7.21%	14.35%	ns	0.008	ns	ns	ns	0.009
Streptococcus	1.88%	1.28%	5.54%	4.31%	ns	ns	ns	ns	ns	ns
Turicibacter	4.92%	2.14%	1.47%	0.94%	ns	ns	0.039	ns	ns	ns
02d06	2.83%	1.80%	0.65%	0.90%	ns	ns	0.029	ns	ns	ns
Proteiniclasticum	0.04%	0.35%	0.98%	0.75%	ns	ns	ns	ns	0.05	ns
SMB53	8.98%	4.53%	1.94%	1.82%	ns	ns	0.004	ns	0.031	ns
Clostridium	2.38%	1.09%	0.53%	0.41%	ns	0.014	0.001	ns	0.018	ns
Fusobacterium	0.30%	0.89%	0.14%	0.70%	ns	ns	ns	ns	ns	ns
Acinetobacter	1.66%	3.32%	0.82%	1.17%	ns	ns	ns	ns	ns	ns
Moraxella	0.00%	1.87%	3.15%	2.66%	ns	ns	ns	ns	ns	ns
Psychrobacter	14.47%	13.01%	0.48%	5.16%	ns	ns	ns	ns	ns	ns
Pseudomonas	1.48%	8.03%	3.18%	3.48%	ns	ns	ns	ns	ns	ns

Class 1: SCC < 100,000 cells/ml; class 2: SCC between 100,000 cells/ml and 499,000 cells/ml; class 3: SCC between 500,000 cells/ml and 1,000,000 cells/ml; class 4: with a SCC > 1,000,000 cells/ml.

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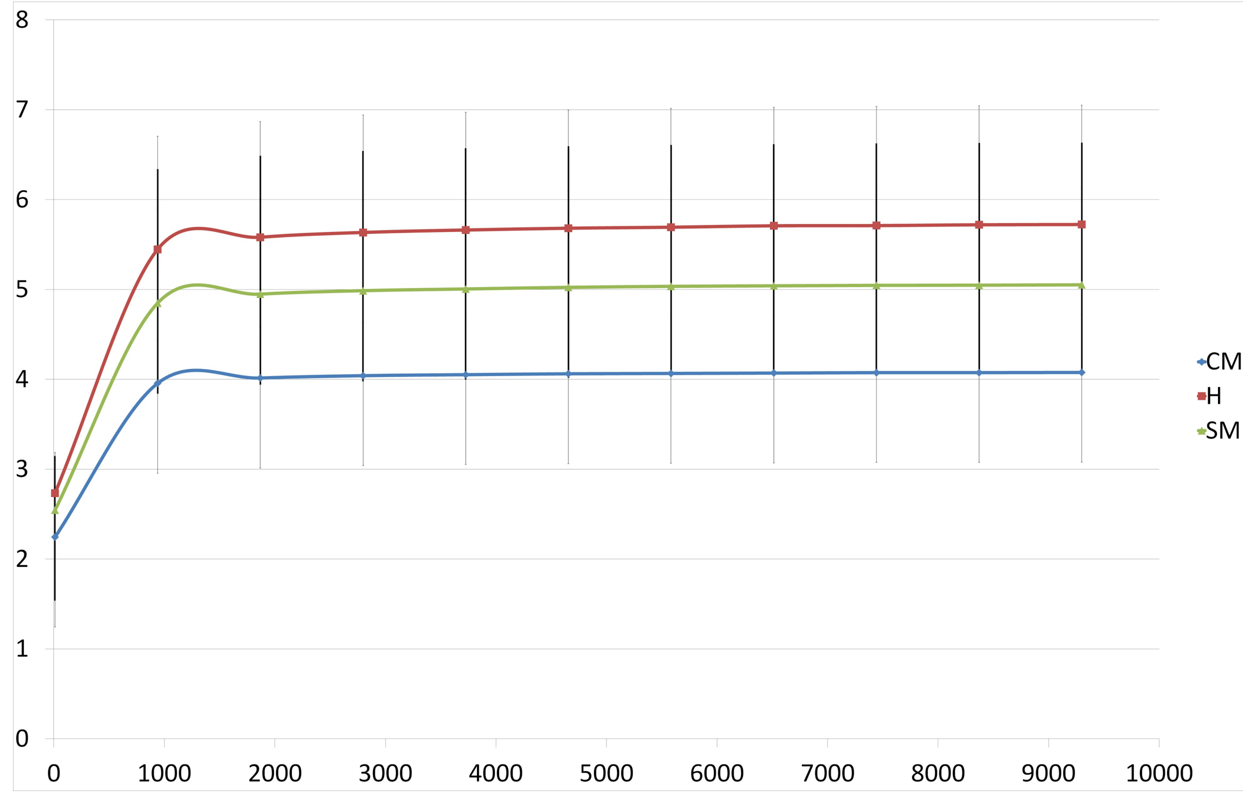

Fig 5. Alpha diversity analysis. Rarefaction curves of samples with regards to quarter patho-physiological status (CM: clinical mastitis; H: healthy; SM: sub-clinical mastitis), as defined by the Shannon index. Statistical difference is present between H and CM groups ($p = 0.03$).

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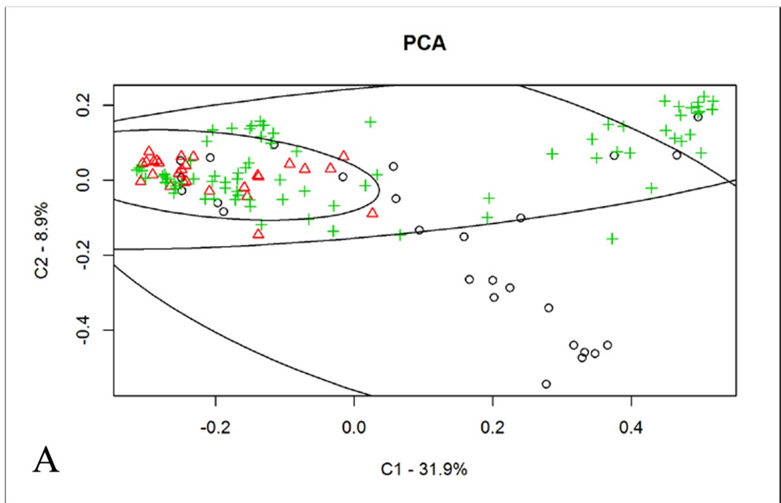
not address the eukaryote content of milk. The microbiota of milk from healthy quarters was determined as well, providing the evidence that the OTU diversity of milk from healthy quarters is much wider that samples with clinical and sub-clinical mastitis consistently with what has been already reported in bovine milk [16, 19], colostrum [47] and teat microbiota [48]. Discriminant analysis models of water buffalo milk showed that samples collected from healthy quarters can be discriminated from samples derived from clinical and sub-clinical mastitis, in agreement with what was observed in bovine milk [15, 19]. On the contrary it was not possible to discriminate in clusters samples derived from SM quarters. The clustering of H milk samples was improved removing SM quarters, which were more scattered in the plot; in fact, sub-clinical samples might share healthy or clinical mastitis features such as absence of inflammatory reaction or positive bacterial culture, respectively.

The water buffalo health milk core microbiota, i.e. the number and the identity of genera that are shared among different individuals, contained 15 genera, of which *Staphylococcus* and *Psycrobacter* were the most prevalent.

The microbiota from water buffalo healthy milk is different as compared with that of bovine milk, where the core microbiota includes *Faecalibacterium*, unclassified *Lachnospiraceae*, *Propionibacterium* and *Aeribacillus* [15], and human milk, where nine genera, namely *Staphylococcus*, *Streptococcus*, *Serratia*, *Pseudomonas*, *Corynebacterium*, *Ralstonia*, *Propionibacterium*, *Sphingomonas*, *Bradyrhizobiaceae*, were present [49].

Together with *Streptococcus*, which ranges from 2% in H samples to 11.7% in SM samples, *Staphylococcus* genus was already reported as being part of core microbiota of human [49] and



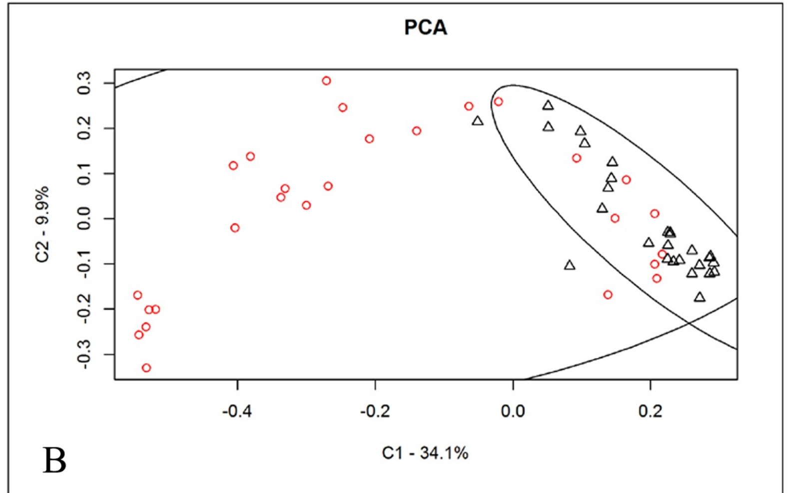


Fig 6. Beta diversity analysis. Unweighted Unifrac analysis including H (Healthy) and CM (Clinical mastitis) samples. Adonis: R = 0.17 p = 0.001 ANOSIM: R = 0.37 *p* = 0.001. Panel A: results including H, SM and CM quarters. Panel B: results including only H and CM. o = CM; + = SM; Δ = H

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bovine milk [15, 19, 50, 51]. Although found in all healthy milk samples, *Pseudomonas* genus relative abundance in water buffalo milk was limited (1.5%) as compared to bovine non-mastitic milk (18.75%) [19].

The finding of *Psychrobacter* has already been reported in milk from dairy cows [19, 52], although in cow's healthy milk the average relative abundance of *Psychrobacter* is limited (4.9%) as compared to what found in water buffalo milk (16.26%). The relative abundance of *Psychrobacter* in water buffalo milk is related to the healthy status of the mammary gland, decreasing to 3.2% in SM milk, and absent in 22% of the CM milk samples. No species belonging to *Psychrobacter* has been so far associated to mastitis. Cold-adapted *Psychrobacter* genus has been recently related to anti-biofilm activities against *Staphylococci* and *Pseudomonas aeruginosa* bacteria [53]. This finding is remarkable, since provides clues to potentiate non-antibiotic relying resistance against mammary gland pathogens. Of the other most prevalent genera that were found in all the H samples, *SMB53* and *Solibacillus* were present with the highest

relative abundance, 7.17% and 5.11%, respectively. This is the first time that these two genera were found in milk. *SMB53* belongs to the family of *Clostridiaceae* and was found within the ileal bacterial community in grazing goats [54]. *Solibacillus* genus was identified among faecal bacterial community in dairy cows during subacute ruminal acidosis [55], but its presence in milk is reported here for the first time. Both *SMB53* and *Solibacillus* are regarded as faecal contaminants. On the background that all the farms included in the present study were equipped with bathing pools, that are of paramount importance for water buffaloes, in order to mitigate thermal stress, we may hypothesize that, due to the immersion of the teats in water, faecal contaminants were included in the microbiota of healthy water buffalo mammary gland. Furthermore, a recently published investigation highlighted differences between samples obtained directly from the udder cistern using a needle and vacuum and samples collected conventionally [56]. The authors suggested that contamination from teat skin, or environmental sources, may occur, interfering with the microbiological analysis and PCR-based bacteriological results. It could not be ruled out that contamination from skin and environmental sources could have occurred, affecting the taxonomic composition. It must also be said that both *Solibacillus* and *SMB53* decreased in a statistically significant way in CM milk, and therefore it is unlikely that their presence in healthy milk is related to contamination during collection of samples.

In milk from sub-clinical affected quarters, two genera, namely *Acinetobacter* and *Pseudomonas*, with a relative abundance of 4.47% and 15.09%, respectively, were present in all the samples. The presence of *Acinetobacter* was previously found in a cultured-independent study on the teat apex [57]. The involvement of *Acinetobacter* in the development of mastitis is unfrequent [58]. The presence of *Pseudomonas* was already reported in water buffalo milk [35, 59]. *Pseudomonas* is a known agent of mastitis pathogen in ruminants including cow [60], sheep [61] and goats [62], but little information is available in water buffalo species. The relative abundance of *Pseudomonas* genus was found as prevalent (18.75%) in milk from healthy cows, and decreased to 3.84% in clinical mastitis [19]. In the present study the relative abundance of *Pseudomonas* in healthy milk samples was found be limited (1.5%) as compared to SM (14.74%) and CM (13.48%) milk, but this increase was not found to be statistically significant.

No common genus was present in milk from quarters with clinical mastitis. In several samples the bacteria identified by aerobic microbiological culture corresponded to the most frequent bacterial species found after 16s rRNA gene sequencing. Moreover, many anaerobic bacterial sequences including those belonging to *Bacteroides*, *Porphyromonas* and *Fusobacterium*, were also identified. In some samples the relative abundance of these genera, such as for examples *Bacteroides* in C44 (r.a. = 92%), C56 (r.a. = 87%), C57 (r.a. = 82%), C48 (r.a. = 61%), *Porphyromonas* in C55 (r.a. = 62%) and *Fusobacterium* in C49 (r.a. = 42%), was predominant. This finding is consistent with others reported in previous studies on bovine milk [15], which detected anaerobic bacteria in both healthy and clinical mastitis affected samples, in particular those caused by *Trueperella* pyogenes. Anaerobic genera have been already found in bovine milk, although they were more frequently included in the list of gut microbes [20], and in summer mastitis [63], [64]. The presence of anaerobic genera was also found in teat microbiota as correlated to mastitis history [48]. The relative abundance of *Bacteroides* and *Porphyromonas* in healthy water buffalo is limited, and no traces of Fusobacterium sequences were found at all. In clinical mastitis samples, on the contrary, *Bacteroides*, *Fusobacterium* and *Porphyromonas* were found to be associated with mastitis where the main pathogen identified after microbiological culture was *Trueperella* (C44, C45, C57 and C58) or *Streptococcus dysgalactiae* (C49), suggesting in water buffalo as well a synergistic action between these genera, in particular where *Trueperella* is involved [64]. Discrimination between clinical and healthy quarters is significant, even if the not homogeneous microbiota profile of clinical samples needs to be deeply

investigated. A different criterion to classify non clinical mastitic samples was also considered, aiming to relate microbiota profiles with inflammatory parameters, such as SCC. Therefore, on this background, samples were classified in four classes depending on SCC. Following this classification, considering alpha-diversity, and independently on microbiological culture, did not allow to cluster samples, since an overlapping between class 1 with class 2, class 2 with class 3 and class 3 with class 4 were demonstrated. This results confirm the hypothesis that classifications of water buffalo mastitis following SCC need further investigations, as previously suggested [31].

Conclusion

The present study investigated the milk water buffalo microbiota from healthy quarters and sub-clinical and clinical mastitis, following a culture-independent metagenome approach, providing a first step in the evaluation of the microbial population in water buffalo milk, and contributing to identify the core microbiota in healthy milk. Our findings revealed the presence of genera that could not be assessed by culture-based analysis, such as *Psychrobacter*, SMB53 and *Solibacillus* whose relative decrease was associated with clinical mastitis. Open questions remain to be answered, including the relationship between microbiota with parity and different stages of lactation, as well as the relationship between farming conditions and microbiota.

Supporting information

S1 Fig. Water buffalo milk taxonomic profile at family level. Microbiota composition at the family level for the 16S rRNA gene. H = Healthy samples; SM = Sub-Clinical mastitis samples; CM = Clinical mastitis samples
(TIF)

S2 Fig. Alpha diversity analysis after classification of clinically healthy samples following SCC grouping. Rarefaction curves of the four SCC groups (Class 1, with a SCC $< 100{,}000$, Class 2, with a SCC between 100,000 and 500,000, Class 3, with a SCC between 500,000 and 1,000,000 and Class 4, with a SCC $> 100{,}000{,}000$), as defined by the Shannon index. Statistical differences are present between class 1 and ($p = 0.006$) and between class 1 and 3 ($p = 0.006$).
(TIF)

S3 Fig. Beta diversity analysis presented as box plot. Panel A and B presents the C1 and C2 boxplots derived from Fig 5, Panel A, including H, SM and CM quarters. Panel C and C presents the C1 and C2 derived from Fig 5, Panel B, including H, and CM quarters.
(TIF)

S4 Fig. Beta diversity analysis after classification of clinically healthy samples following SCC grouping. Unweighted Unifrac analysis including SCC groups derived from all clinically healthy quarters: class 1 with SCC of less than 100,000 cells/ml; class 2 with SCC ranging from 100,000 to 499,000 cells/ml; class 3 with SCC ranging from 500,000 to 100,000,000 cells/ml; class 4 with SCC greater than 100,000,000 cells/ml. Adonis: $R^2 = 0.08$ and p = 0.001; ANOSIM: R = 0.06 and p = 0.017.
Panel A: beta diversity plot. Panel B: C1 and C2 boxplots derived from Panel A.
O = group 1; Δ = group 2; + = group 3; x = group 4.
(TIF)

S1 Table. Relative abundance of microbiota taxa at family level. H = Healthy samples; SM = Sub-Clinical Mastitis samples; CM = Clinical mastitis samples.

* Bonferroni correction was applied.
(DOCX)

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2 Paper 2

Impact of intramammary inoculation of inactivated *Lactobacillus rhamnosus* and antibiotics on the milk microbiota of water buffalo with subclinical mastitis

RESEARCH ARTICLE

Impact of intramammary inoculation of inactivated *Lactobacillus rhamnosus* and antibiotics on the milk microbiota of water buffalo with subclinical mastitis

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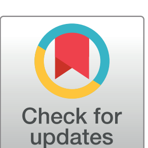

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Abstract

Water buffalo mastitis represents a major issue in terms of animal health, cost of therapy, premature culling and decreased milk yeld. The emergence of antibiotic resistance has led to investigate strategies to avoid or reduce antibiotics' based therapies, in particular during subclinical mastitis. The use of Generally Regarded As Safe bacteria (GRAS) such as *Lactobacillus rhamnosus* to restore the unbalance in mammary gland microbiota could provide potential corrective measures. The aim of this study was to investigate the changes in milk microbiota after the intramammary treatment with inactivated cultures of *Lactobacillus rhamnosus* of mammary gland quarters naturally affected by subclinical mastitis as compared to antibiotic therapy.A number of 43 quarters affected by subclinical mastitis with no signs of clinical inflammation and aerobic culture positive for pathogens were included in the study. The experimental design was as follows: 11 quarters were treated with antibiotics, 15 with inactivated cultures of *Lactobacillus rhmnosus* and 17 with PBS as negative control, by means of intrammary injection. Samples were collected at eight time points, pre- (T-29, T-21, T-15, T-7, T0 days) and post- treatment (T1, T2, and T6 days). Microbiological culture and Somatic Cell Count (SCC) were perfomed on all the samples, and microbiota was determined on milk samples collected at T0 and T6 by amplifying the V4 region of 16S rRNA gene by PCR and sequencing using next generation sequencing technique. Treatment with *Lactobacillus rhamnosus* elicited a strong chemotactic response, as determined by a significant increase of leukocytes in milk, but did not change the microbiological culture results of the treated quarters. For what concerns the analysis of the microbiota, the treatment with *Lactobacillus rhamnosus* induced the modification in relative abundance of some genera such as *Pseudomonas* and *5-7N15*. As expected, antibiotic treatment caused major changes in microbiota structure with an increase of *Methylobacterium* relative abundance. No changes were detected after PBS treatment. In conclusion, the present findings demonstrated that the

additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

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in vivo intrammmary treatment with *Lactobacillus rhamnosus* has a transient pro-inflammatory activity by increasing SCC and is capable to modify the microbiota of milk after six days from inoculation, albeit slightly, even when the bacterial cultures were heat inactivated. Further studies are necessary to assess the potential use of this GRAS as supportive therapy against mastitis.

Introduction

The domestic water buffalo (*Bubalus bubalis*) contributes to a significant share of global milk production and is the major milk producing animal in several countries, such as India and Pakistan [1]. Water buffaloes are resistant to most of the disease affecting dairy cows, even in a context of low feeding and environmental stress [2–4]. The background of this resistance lies in mammary gland anatomical features, including a long narrow teat canal, a teat skin less sensitive to chapping and sores, a streak canal with thicker epithelium and keratin layer, a tighter sphincter of streak canal and the absence of milk cistern [5]. These distinct features of the buffalo mammary gland are believed to prevent the invasion of micro-organisms. The few studies on water buffalo mastitis presented the evidence that somatic cell score in quarters with intramammary infection is low, and a limited decrease in milk production was found among infected animals as compared to healthy ones [6]. However, mastitis is still occurring in dairy buffaloes in intensive dairy farming [7], with an impact that might be comparable to that of dairy cows concerning production losses, culling and treatment costs [8], beside decreasing animal health and welfare [9,10].

The conventional therapy against mastitis includes the treatment of the mammary gland with antibiotics. Although necessary for both therapeutic and prophylactic purposes, treatment with antibiotics is not fully efficient, and presents several drawbacks. The extended use of antibiotics is at the background of the development of anti-microbial resistance that can persist in the bacterial community [11, 12], as demonstrated for *Streptococcus agalactiae* [13] and *Staphylococcus aureus* [14]. Furthermore, the massive use of antibiotics in dairy animals is at the origin of antibiotic residues' pollution in the environment and contamination of milk and other animal-derived products, causing antibiotic resistance in humans as well [15].

Alternative strategies are investigated, aiming to reduce the use of antibiotics. New therapeutic approaches, such as, among the others, Generally Recognized As Safe (GRAS) bacteria, including Lactic Acid Bacteria (LAB), have been developed [16]. The *in vitro* and *in vivo* effects of treatment with LAB produced different and opposite results in cows. *In vitro* studies on the effects of *Lactococcus lactis* as potential anti-mastitis therapeutics have shown promising results on bovine mammary epithelial cells by producing nisin A, a polycyclic antibacterial peptide [17]. The different strains of lactobacilli that have been investigated so far included *Lactobacillus perolens*, *Lactobacllus rhamnosus*, *Lactobacillus brevis* and *Lactobacillus plantarum* [18–21]. *In vitro* results were encouraging, and an overall reduction of bacterial load together with an anti-inflammatory activity were demonstrated. On the contrary, the *in vivo* use of GRAS produced contradictory results and their activity remains inconclusive. *Lactococcus lactis* stimulates the intramammary immune system of cattle, as determined by polymorphonuclear cells (PMN) recruitment and increasing of haptoglobin and serum amyloid A concentrations in milk [22]. Nonetheless, only in few studies the live cultures of *Lactococcus lactis* were effective in bovine mastitis treatment [23]. In a model of mouse mastitis, the experimental infection with *Staphylococcus chromogenes* and treatment with live cultures of *Lactococcus lactis* induced an increased

level of IL-1β and TNFα, in addition to tissues damages, suggesting that these GRAS strains cannot be used for mastitis treatment in rodents. Recent findings on ewes affected by subclinical mastitis confirmed that the infusion of *Lactococcus lactis* into the mammary gland leads to a transient clearance of the pathogens, but also increases the inflammatory status of the mammary gland [24]. Similarly, treatment with different strains of *Lactobacillus* failed to decrease cow mastitis and caused a local inflammatory response [25,26]. Among GRAS, *Lactobacillus rhamnosus* was found to possess the strongest antibacterial activity against *Salmonella enterica* [27], and also capable of preventing the *Escherichia coli*–induced changes in epithelial barrier functions [28]. Similar results were demonstrated in cows as well, where the potential of *Lactobacillus rhamnosus* against *Escherichia coli*-infection in vagina and endometrium [29,30], intestine [31] and respiratory apparatus [32] was also reported. Information about the activity of *Lactobacillus rhamnosus* on mammary gland is, one the contrary, very limited. *In vitro* studies provided evidence that *Lactobacilus rhamnosus* pretreatment was able to attenuate the pro-inflammatory effects of an *E.coli* challenge on primary bovine mammary epithelial cells by suppressing TLR and inflammasome related gene expression [33,34]. To the best of the knowledge of the authors, no study was carried out to investigate the *in vivo* effects of *Lactobacillus rhamnosus* in the mammary gland, in particular for what concerns how *Lactobacillus* treatment can influence the delicate equilibrium between bacterial communities.

Culture-independent techniques relying on high-throughput DNA sequencing of 16S provided an in-depth knowledge of bacterial communities, and are currently applied to unravel the relationship between resident microbial population and the development of mastitis[35–38]. The results of these studies demonstrated that bacterial species are present in culture-negative samples collected from animals with clinical mastitis [39] and that major pathogens, such as *Streptococcus uberis* and *Staphylococcus aureus*, can be found in milk from clinically healthy animals [35]. On this background, the insurgency of mastitis may be related to both the presence of specific pathogen and the modification of the microbial community of milk [40]. This observation was confirmed in water buffalo, whose milk microbiota has been recently published [41].

The aim of this study was to investigate the effect of an intramammary inoculation of *Lactobacillus rhamnosus* on the milk SCC and microbiota of water buffaloes naturally affected by sub-clinical mastitis. The effect of antibiotics, that were also used as positive control for antibacterial activity on milk microbiota, were characterized as well. *Lactobacillus rhamnosus* was selected on the background of its *in vitro* antibacterial activity in the epithelial mammary gland cellular model.

Materials and methods

Bacterial strain, culture conditions and inactivation of *Lactobacillus rhamnosus* inocula

The probiotic *Lactobacillus rhamnosus* strain GG (LMG 18243) from the BCCM/LMG Bacteria Collection (Belgium) was prepared as follows: the bacterium was grown at 37˚C for 48h in Trypticase Soy Broth (TSB, BD, Italy) in a Gaspak jar using the commercial gas-generating AnaeroGen AN25 kit (Oxoid, England) for anaerobic growth. The probiotic culture was then centrifuged at 3000 x g for 20 min, washed twice with sterile pyrogen-free saline solution (NaCl 0.9%) and suspended in the solution used for the inoculum, namely sterile PBS (Sigma-Aldrich, Milano). This bacterial suspension (approximately 10^9 CFU x mL^{-1}) was inactivated after boiling at 100˚C for 15 min. The absence of viable cells was verified by culturing on TBS medium. Five mL of the heat-inactivated suspension were used for each intramammary injection.

Study design and intramammary challenge

The experimental protocol was approved by Italian Ministry of Health (Protocol No.982/2015PR). The study was carried out on 20 multiparous water buffaloes (*Bubalus bubalis*) homogeneous for parity (2nd to 4th lactation) and in mid lactation (from 60 to 160 DIM). The animals were housed in a commercial farm and left 29 days to become familiar with the experimental conditions. During that time, animal health status was diagnosed clinically and quarter milk samples were collected for bacteriological analysis and Somatic Cell Count (SCC).

For the purpose of this study, quarters affected by sub-clinical mastitis were defined as those with no evidence of clinical signs, but positive to microbiological culture for three times before T0 (included). Following these criteria, a total number of 43 samples were included in the study as affected by sub-clinical mastitis. Milk samples were collected weekly at T-29, T-21, T-15, T-7 and T0 and then intramammary inoculated following this protocol: at T0, 15 quarters were inoculated with 5 ml of inactivated cultures of *Lactobacillus rhamnosus* (LAB) (LAB-T0), 11 quarters were inoculated with amoxicillin-clavulanic acid (Synulox Lactating Cow Intramammary Suspension, Pfizer, Italy) (Ab-T0), and 17 quarters were inoculated with 5 ml of sterile PBS (Sigma-Aldrich, Milano) (PBS-T0). After challenging, samples of milk were further collected at time T1, T2 and T6.

Milk samples were collected after disinfection of teat ends with a 2% povidone-iodine (Betadine Solution) and discarding of the first three strains of milk. Gloves were changed each time and 150 ml of milk were collected in sterile containers. After collection, milk samples were immediately refrigerated and delivered to the laboratory for microbiological analysis and SCC. Milk samples were finally aliquoted and stored at -80 C for microbiota identification, which was carried out on milk samples at T0 and T6.

Clinical observation and animal care

Clinical signs were monitored throughout the experiment by a veterinary practitioner, every 8 hours during the first 24 hours from the challenge and then every time the water buffaloes were milked. Rectal temperature was measured every 24 hours. General attitude, and appetite were evaluated, and the udders were palpated to identify soreness, swelling hardness and heat, to assess the development of clinical signs.

Microbiological culture (MC) and Somatic Cell Count (SCC)

Microbiological culture tests were performed for each milk sample using different media as previously reported [41]. Briefly, samples were incubated at 37˚ for 24h in aerobic conditions on Trypticase soy agar (with 5% sheep blood), MacConkey agar and Baird Parker agar; at 37˚ for 72h in aerobic conditions on Prototheca isolation medium (PIM); at 37˚ in microaerobic conditions on Mycoplasma agar. Gram staining, coagulase and oxidase tests were performed on cultures with mastitis pathogens; in particular, in *Staphylococcus* spp. positive culture were tested for coagulase activity using rabbit plasma, and *Streptococcus* spp positive cultures were evaluated with Streptokit-BioMérieux test for Lancefield grouping. Somatic cell count was measured in milk samples at T-29, T-21, T-15, T-7, T0, T1, T2, and T6 (days) using Fossomatic (Foss) apparatus by means of the UNI EN ISO 13366–2:2007 technique for electronic optical fluorimetric counters.

DNA extraction

One ml of milk was centrifuged at room temperature at 16,100 rcf [36,41]. Fat and supernatant were discarded and the remaining pellet was resuspended with 250ul of the Power Bead Tube

of the DNEasy Power Soil Kit (QIAGEN) used to extract bacterial DNA, according to the manufacturer's instructions. After the DNA elution in 50 μl of DNAse and RNAse free water, DNA concentration and purity were analysed using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Weltham, Massachusetts, U.S.A.) at wavelength 230, 260 and 280 nm and DNA samples were stored at -80˚ until further processing. The reagents included in the kit, without any bacterial DNA, were used as blank control for each DNA extraction batch.

Amplification of the hypervariable V4 region of bacterial 16S rRNA gene by PCR and barcoding

V4 region of 16S rRNA gene was amplified for each sample [37]. The forward primer was 5′-CCATCTCATCCCTGCGTGTCTCCGAC**TCAG***NNNNNNNNNNNNNNNNN***GAT**GTGYCAGCMGCCGCGGTAA- 3′, composed of the adapter linker, the key, the barcode, different for each sample, and the forward primer 515F. The reverse primer was 5′-CCTCTCTATGGGCAGTCGGTGATGGACTACNVGGGTWTCTAAT- 3′, composed of the adapter linker and the R806 reverse primer. The Thermo Scientific Phusion Hot Start II High-Fidelity DNA polymerase kit was used to perform V4 PCR; each PCR reaction contained RNAse and DNAse free water, 5x Phusion Buffer HF (5 μl), dNTPs 2mM (2.5 μl), Primer Fw 10μM (1.25 μl), Primer Rv 10μM (1.75 μl), Phusion High Fidelity Taq Polymerase 2 U/μl (0.25 μl) and 5 ng of DNA. When DNA samples quantification was too low (less than 5 ng/μl), 5 μl of the samples were used to perform PCR. The thermal profile consisted of an initial denaturation of 30 sec at 98˚C, followed by 32 cycles of 15 sec at ˚98 C, 15 sec at 50˚C, 20 sec at 72˚C, and a final extension of 7 min at 72˚C. Each PCR plate included samples derived from each group. After DNA purification using Agencourt AMPure XP kit with a ratio 1:1, quality and quantity of PCR products were determined using Agilent Bioanalyser 2100 and Qubit fluorometer.

For 17 samples showing DNA concentration lower than 1 ng/μl at Qubit quantification, PCR was repeated using the same PCR condition and increasing the number of cycles up to 36. The lack of amplification of extraction and PCR negative controls was confirmed for all PCR.

Next-generation sequencing, bioinformatics and statistical analysis

Sequencing was performed using Ion Torrent Personal Genome Machine (PGM) with the Ion 318 Chip Kit v2 (Thermo Fisher Scientific, Weltham, Massachusetts, U.S.A.), by the Centre for Research in Agricultural Genomics (CRAG, Bellaterra, Barcelona), following manufacturer's instructions. The raw sequences have been submitted to NCBI under Bioproject accession number SUB4205063—Bioproject number: PRJNA477950. After sequencing, reads were demultiplexed in order to have sequence file for each barcode/sample and Primer Rv was removed. Then, sequences were imported in the Quantitative Insight Into Microbial Ecology 2 (QIIME 2) software [42] (https://qiime2.org), which was used to analyze data. After obtaining a unique file with all sequencing data, DADA2 was used as quality filtering method in order to denoise, dereplicate single-end sequences and remove chimeras [43]. Afterward, the primer Fw was removed and a truncation length of 245 bases was used, taking into account the quality plot result and the mean V4 length of around 250 bases. After that, the units of observation, composed of unique sequences namely Amplicon Sequence Varians (ASVs), were used to classify them and assign taxonomy, using Greengenes 13.8 [44] at 99% of Operational Taxonomic Units (OTUs) identity and trimmed to V4 region, as reference database. Finally, chloroplasts were removed from the sequences.

The filtered feature table was used to perform the downstream analysis. The taxonomic analysis was performed for each sample or group of samples at phylum, family and genus level.

Diversity analysis was assessed using 9500 sequences per sample. Alpha diversity that analyses differences within samples was performed using qualitative and quantitative approaches (richness or Observed species and evenness or Shannon index, respectively); beta diversity that analyzes differences among samples estimating how many taxa are shared among samples, was performed using qualitative and quantitative approaches as well (unweighted and weighted UniFrac distances matrices, respectively).

As data presented in this study were not-normally distributed and composed of pre- and post-treatment samples (T0 vs T6 within the same group), non-parametric paired test was applied. To compare the effect of the treatment on the microbiota (PBS-T6 vs LAB-T6; PBS-T6 vs Ab-T6; LAB-T6 vs Ab-T6), non-parametric unpaired test was applied. Taxonomic statistical analysis was performed using Wilcoxon signed pairwise test (pairwise.wilcox.test in coin package) and Kruskal Wallis test followed by Dunn pairwise test (dunn.test package) in R version 3.4.3 (http://www.R-project.org), for paired and unpaired comparisons, respectively. A specific QIIME 2 plugin for longitudinal studies was used for alpha diversity and beta diversity principal coordinates analyses: as two time points were considered for this experiment, Wilcoxon rank sum pairwise test was used for paired data, while Kruskal Wallis and Wilcoxon Mann-Whitney U pairwise test were applied for unpaired data [45]. Workflow details are available at dx.doi.org/10.17504/protocols.io.ucpesvn.

Results

Diagnosis of sub-clinical mastitis, intramammary inoculation of inactivated *Lactobacillus rhamnosus*, collection of samples

The diagnosis of sub-clinical mastitis was carried out according to microbiological culture results and SCC. None of the animals included in this study evidenced any clinical signs related to the development of an acute mastitis. Results of microbiological culture are presented in Tables 1 and S1 showing that, at T6, bacteria associated with mastitis were found in all the samples included in the study, except those collected from quarters treated with antibiotics, all of which became negative at microbiological culture at T6, with only one exception.

Somatic Cell Counts were measured at T-29, T-21, T-15, T-7 and T0, with the aim to monitor the microbial status of each quarter and identify those that would be included in the study, and T1, T2, and T6, to assess the effects of the treatment. All quarters challenged with inactivated *Lactobacillus rhamnosus* showed an increase in SCC. In individual quarters, elevation of SCC median reached its peak 24h post inoculation and then decreased afterward (Fig 1). PBS-infused control quarters showed a significant increase in SCC as compared with prechallenge levels starting from T1, and increased after T2. Antibiotic treated quarters showed an increase in SCC starting from T1 and further increases at T2. At T6, the SCC were decreased at the T0 level in all the three groups of samples.

Ion Torrent output: Sequences results after filtering procedures

The sequencing of 43 samples produced a total of 9,468,300 sequences and 4,039 features were obtained (with a mean of 112,717.85, a minimum of 9,778 and a maximum of 500,775 sequences) after filtering.

Core microbiota and taxonomic profile analysis before and after the treatment

The core microbiota of milk from water buffaloes affected by subclinical mastitis is composed of eight main phyla, namely *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*,

Table 1. Microbiological culture results for each treatment group.

	T0	T6
PBS treated	**17**	**17**
Staphylococcus aureus	9	7
Coagulase-negative Staphylococci	4	3
Streptococcus agalactiae	1	0
Staphylococcus aureus / Streptococcus agalactiae	2	3
Coagulase-negative Staphylococci / Streptococcus agalactiae	1	1
Negative	0	3
LAB treated	**15**	**15**
Staphylococcus aureus	9	9
Coagulase-negative Staphylococci	3	2
Streptococcus agalactiae	3	0
Staphylococcus aureus / Streptococcus agalactiae	0	2
Coagulase-negative Staphylococci / Streptococcus agalactiae	0	2
Negative	0	0
Ab treated	**11**	**11**
Staphylococcus aureus	8	1
Coagulase-negative Staphylococci	2	0
Streptococcus agalactiae	0	0
Staphylococcus aureus / Streptococcus agalactiae	0	0
Coagulase-negative Staphylococci / Streptococcus agalactiae	1	0
Negative	0	10

PBS: quarters treated with sterile PBS only, LAB: quarters treated with inactivated culture of *Lactobacillus rhamnosus* only, Ab: quarters treated with antibiotics, as described in Material and Methods. T0: pre-treatment time; T6: time at 6 days post treatment.

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Firmicutes, *Proteobacteria*, *Verrucomicrobia* and *[Thermi]*. Results are presented in Fig 2 and Table 2. The milk microbiota was dominated by Firmicutes (mean of 60.9% at T0) and Proteobacteria (mean of 18.8% at T0). Treatment with *Lactobacillus rhamnosus* and PBS did not induce any major change from T0 to T6, with the exception of *[Thermi]*. On the contrary, treatment with Ab induced a decrease of Firmicutes (from 61.8% at T0 to 26.7% at T6), and an increase of Proteobacteria (from 13.8% at T0 to 30.8% at T6) and Actinobacteria (from 13.8% at T0 to 28.5% at T6). Comparing the relative abundance of bacterial phyla at T6 between different treatments, no differences were found between treatment with PBS and treatment with *Lactobacillus rhamnosus*. On the contrary, several differences were found between milk microbiota from quarters treated with *Lactobacillus rhamnosus* and Ab in the relative abundance of *Acidobacteria*, *Cyanobacteria and Firmicutes*. Differences were also found between milk quarters treated with PBS and Ab, in the relative abundance of *Actinobacteria* and *Firmicutes*.

No core microbiota was present at family level. The main families found in milk microbiota at T0 were *Staphylococcaceae* (mean of 40.3%), followed by *Streptococcaceae* (mean of 5.8%), *Moraxellaceae* (mean of 5.2%), *Ruminococcaceae* (mean of 3.2%) and *Corynebacteriaceae* (mean of 4.4%). Taxonomic and statistical results at family level are shown in S1 Table and S1 Fig (relative abundance of almost 1%). PBS treatment did not cause significant milk microbiota alterations except for *Enterobacteriaceae* and *Rhodobacteriaceae*. Similarly, *Lactobacillus rhamnosus* treatment induced only an increase of *Pseudomonadaceae* (from 1.5% at T0 to 5.1% at T6). The main changes were present in antibiotic group at T6, where an increase of *Micrococcaceae* (from 0.9% at T0 to 3% at T6), *Bradyrhizobiaceae* (from 0.2% at T0 to 1.4% at

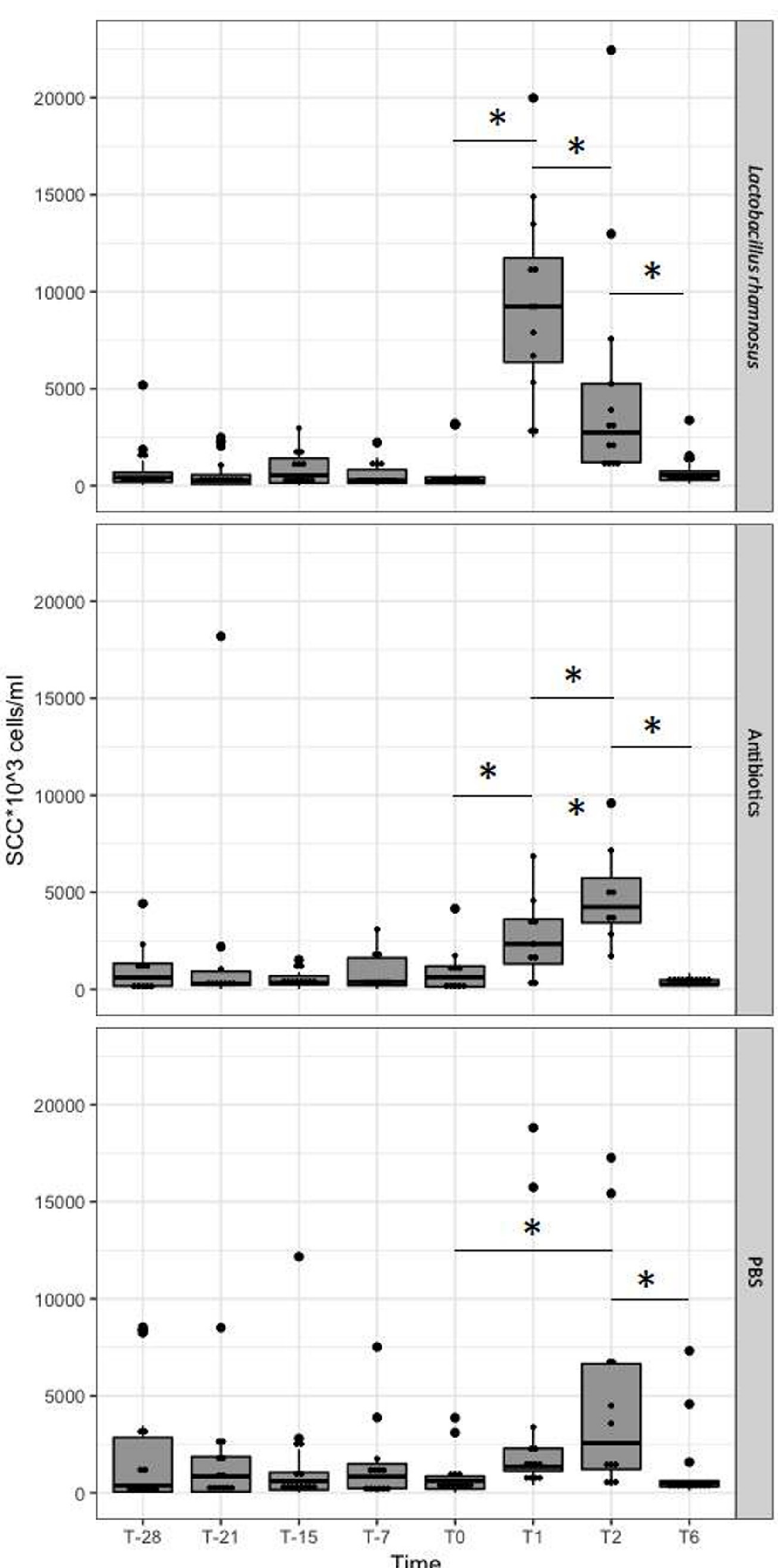
20000
15000
10000
5000
0
*
*
*
Lactobacillus rhamnosus
SCC*10^3 cells/ml
*
*
*
*
Antibiotics
*
*
PBS
T-28
T-21
T-15
T-7
T0
T1
T2
T6
Time

Fig 1. Somatic cell count in response to challenge with LAB, Ab and PBS. Median (line into the box), upper and lower quartiles (ends of the box) and highest and lowest values (extreme lines) are shown for challenged quarters. The bigger black points indicate outliers. * indicates statistical significance at $p < 0.05$.

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T6), *Methylobacteriaceae* (from 1.1% at T0 to 6.7% at T6) and *Rhodocyclaceae* (from 0.1% at T0 to 1.2% at T6) was observed. A decrease of *Staphylococcaceae* (from 42.5% at T0 to 7.5% at T6) was also observed, even if the difference was not statistically significant. Comparing the relative abundance of taxa at family level among groups at T6, no differences were found between Ab and PBS groups except for *Microbacteriaceae* and *Cytophagaceae* and no major changes between *Lactobacillus rhamnosus* and PBS groups, except for *Peptostreptococcaceae* and *Comamonadaceae*. On the contrary, several changes were present between *Lactobacillus rhamnosus* and Ab microbiota at T6 showing differences in relative abundance (RA) of *Micrococcaceae*, *Propionibacteriaceae*, *Staphylococcaceae*, *Peptostreptococcaceae*, and *Comamonadaceae*.

It was not possible to identify any core microbiota at genus level. Taxonomic and statistical results at genus level were shown in Table 3 and Fig 3 (relative abundance of almost 1%). Family level was indicated where genus level could not be reached. Milk samples at T0 were dominated by *Staphylococcus* (mean of 40%), followed by *Streptococcus* (mean of 9%), *Acinetobacter* (mean of 4.6%), *Corynebacterium* (mean of 4%) and *Propionibacterium* (mean of 2.3%). No changes were detected after PBS treatment. The main statistically significant changes after *Lactobacillus rhamnosus* treatment were identified as an increase of the RA of *Pseudomonas* from 1% at T0 to 4% at T6 and a minor increase of 5-7N15. As expected, major changes were found after Ab treatment, which induced a decrease of *Staphylococcus* from 41% at T0 to 3% at T6. A statistically significant increase of *Methylobacterium* was also found (from 1% at T0 to 6% at

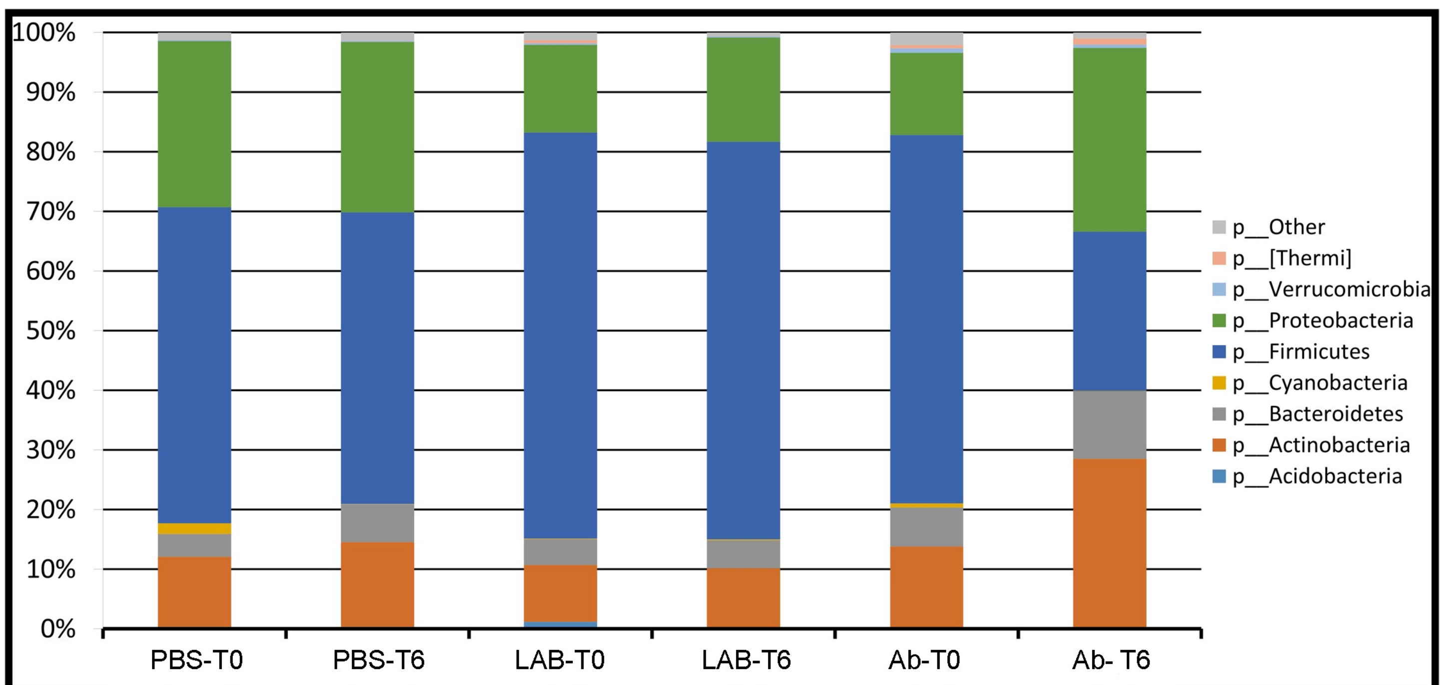

Fig 2. Water buffalo milk taxonomic profile at phylum level. LAB: quarters treated with inactivated culture of *Lactobacillus rhamnosus* only, Ab: quarters treated with antibiotics, PBS: quarters treated with sterile PBS only. T0: time zero; T6: time at 6 days post treatment.

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Table 2. Relative abundance of microbiota taxa at phylum level.

	Relative abundance frequencies											
	Quarter treated with PBS		Quarters treated with LAB		Quarters treated with antibiotics		PBS	LAB	Ab	T6		
	PBS-T0	PBS-T6	LAB-T0	LAB-T6	Ab-T0	Ab-T6	T0 vs T6	T0 vs T6	T0 vs T6	PBS vs LAB	PBS vs Ab	LAB vs Ab
Acidobacteria	0,3%	0,3%	1,2%	0,2%	0,0%	0,0%	ns	ns	ns	ns	ns	ns
Actinobacteria	11,7%	14,2%	9,5%	9,9%	13,8%	28,5%	ns	ns	0,01	ns	0,002	<0,001
Bacteroidetes	3,9%	6,3%	4,3%	4,7%	6,5%	11,4%	ns	ns	ns	ns	ns	ns
Cyanobacteria	1,8%	0,0%	0,1%	0,1%	0,7%	0,0%	ns	ns	ns	ns	ns	0,01
Firmicutes	53,0%	48,9%	68,1%	66,7%	61,8%	26,7%	ns	ns	0,005	ns	0,01	0.01
Proteobacteria	27,9%	28,6%	14,7%	17,5%	13,8%	30,8%	ns	ns	0,04	ns	ns	ns
Verrucomicrobia	0,3%	0,2%	0,3%	0,2%	0,7%	0,5%	ns	ns	ns	ns	ns	ns
[Thermi]	0,0%	0,0%	0,5%	0,0%	0,6%	1,0%	ns	0,03	ns	ns	ns	ns
Other	1,1%	1.5%	1,3%	0,6%	2,1%	1,0%	ns	ns	ns	ns	ns	ns

LAB: quarters treated with inactivated culture of *Lactobacillus rhamnosus* only, Ab: quarters treated with antibiotics, PBS: quarters treated with sterile PBS only. T0: time zero; T6: time at 6 days post treatment

https://doi.org/10.1371/journal.pone.0210204.t002

T6). Comparing the relative abundance of taxa at genus level among groups at T6, negligible changes were detected between PBS and *Lactobacillus rhamnosus* groups as well as between PBS and Ab groups. More genera differed between *Lactobacillus rhamnosus* and Ab microbiota, namely *Staphylococcus*, *Propionibacterium* and *5-7N15*.

Discriminant analysis following treatment

Considering the effect of the treatment on microbiota, alpha diversity showed differences between Ab- and LAB-treated groups at richness level, where a decrease of 85.4 and an increase of 80.3 observed species was observed, respectively ($p = 0.03$). No modification of richness or evenness was observed comparing microbiota T0 vs T6 within the same group.

Beta diversity analysis showed differences on the basis of the weighted UniFrac distance matrix. Modification in microbiota was observed only after Ab treatment, whose groups at T0 and T6 were discriminated by the axis 2 from PCoA plot ($p = 0.04$): samples moved across the axis 2 in the same direction between T0 and T6, suggesting that these samples experienced the same directional shift in terms of microbiota structure, even if the magnitude or the final composition could not be the same. The effect of the treatment, plotted in Fig 4, showed that the Ab effect on the microbiota structure was greater than the *Lactobacillus rhamnosus* effect ($p = 0.001$), which was in turn smaller than the PBS effect ($p = 0.003$).

Discussion

Probiotics have been used as a corrective measure to re-equilibrate the microbiota during mastitis, with contradictory results. Remarkably, the effects of GRAS on microbiota as determined by culture independent methods has not been investigated so far. In this study, we reported the effects of an *in vivo* treatment on mammary glands with inactivated cultures of *Lactobacillus rhamnosus* of water buffaloes affected by subclinical mastitis in order to analyze the change in microbiota structure and evaluate the use of this GRAS as alternative strategy to the use of antibiotics. To the best of the knowledge of the authors, this was the first study using *Lactobacillus rhamnosus* in an *in vivo* study on mammary gland. The scientific background behind the experimental design was that *Lactobacillus rhamnosus*, in combination with other Lactic acid bacteria, was able to modulate the pathogenic environment in the vaginal tract by regulating

Table 3. Relative abundance of microbiota taxa at family/genus level.

	Relative abundance frequencies						*p-value (where p < 0.05)*					
	Quarter treated with PBS		Quarters treated with LAB		Quarters treated with antibiotics		PBS	LAB	Ab	T6		
	PBS-T0	PBS-T6	LAB-T0	LAB-T6	Ab-T0	Ab-T6	T0 vs T6	T0 vs T6	T0 vs T6	PBS vs LAB	PBS vs Ab	LAB vs Ab
Deinococcus	0%	0%	0%	0%	1%	0%	ns	ns	ns	ns	ns	ns
Corynebacterium	3%	5%	3%	4%	6%	7%	ns	ns	ns	ns	ns	0,04
Dietzia	0%	1%	1%	0%	1%	1%	ns	ns	ns	ns	ns	ns
Nesterenkonia	1%	1%	0%	0%	0%	1%	ns	ns	ns	ns	ns	ns
Rhodococcus	0%	0%	0%	0%	0%	1%	ns	ns	ns	ns	ns	ns
Propionibacterium	3%	2%	2%	2%	3%	10%	ns	ns	ns	ns	ns	0,002
CF231	0%	0%	0%	0%	1%	0%	ns	ns	0.03	ns	ns	ns
5-7N15	0%	1%	0%	1%	0%	0%	ns	0.01	ns	ns	ns	ns
Hymenobacter	0%	0%	0%	0%	0%	6%	ns	ns	ns	ns	ns	ns
Chryseobacterium	0%	0%	0%	0%	0%	1%	ns	ns	ns	ns	0,04	ns
Natronobacillus	0%	1%	0%	1%	1%	1%	ns	ns	ns	ns	ns	ns
Lysinibacillus	0%	1%	0%	0%	0%	0%	ns	ns	ns	ns	ns	ns
Solibacillus	0%	2%	1%	1%	0%	1%	ns	ns	ns	ns	ns	ns
Jeotgalicoccus	0%	1%	1%	1%	1%	2%	ns	ns	ns	ns	ns	ns
Salinicoccus	0%	1%	0%	0%	1%	2%	ns	ns	ns	ns	ns	ns
Staphylococcus	33%	25%	43%	45%	41%	3%	ns	ns	0.03	ns	ns	0,01
Alkalibacterium	0%	1%	0%	0%	1%	1%	ns	ns	ns	ns	ns	ns
Facklamia	0%	0%	1%	0%	0%	0%	ns	ns	ns	ns	ns	ns
Granulicatella	0%	0%	0%	0%	0%	1%	ns	ns	ns	ns	ns	ns
Streptococcus	6%	6%	11%	8%	1%	2%	ns	ns	ns	ns	ns	ns
Bradyrhizobium	1%	0%	1%	0%	0%	1%	ns	ns	ns	ns	ns	ns
Methylobacterium	1%	5%	1%	1%	1%	6%	ns	ns	0,02	ns	ns	ns
Sphingomonas	0%	0%	0%	1%	1%	3%	ns	ns	ns	ns	ns	ns
Delftia	0%	1%	1%	0%	0%	1%	ns	ns	ns	ns	ns	ns
Hydrogenophilus	1%	1%	0%	1%	0%	1%	ns	ns	ns	ns	ns	ns
Escherichia	4%	0%	2%	0%	2%	1%	ns	ns	ns	ns	ns	ns
Halomonas	1%	1%	0%	2%	1%	2%	ns	ns	ns	ns	ns	ns
Acinetobacter	10%	6%	3%	2%	1%	2%	ns	ns	ns	ns	ns	ns
Psychrobacter	1%	0%	0%	0%	0%	0%	ns	ns	ns	ns	ns	ns
Pseudomonas	2%	3%	1%	4%	1%	2%	ns	0,007	ns	ns	ns	ns

LAB: quarters treated with inactivated culture of Lactobacillus rhamnosus only, Ab: quarters treated with antibiotics, PBS: quarters treated with sterile PBS only, as described in Material and Methods. T0: time zero; T6: time at 6 days post treatment

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Escherichia coli infection and inflammation of the bovine endometrium [29]. At least so far, live cultures of probiotic were not found to improve mouse [46], cow [18,22,47] or ewe [24] mastitis. On the contrary, most of the Lactobacilli and Lactococci strains used so far for *in vivo* studies have been demonstrated to exert a pro-inflammatory activity: *Lactococcus lactis*, for example, is regarded as a pathogen causing mastitis [48,49]. The cultures used for *in vivo* challenging were previously inactivated with heat. This procedure was carried out to prevent any potential proinflammatory activity related to *in vivo* treatment with GRAS, as previously reported [50] which would have probably induced an acute inflammation, eventually switching the clinical status from sub-clinical to clinical mastitis. Moreover, a potential interference on the microbiota analysis of an uncontrolled overgrowth of living lactobacilli culture after

Fig 3. Water buffalo milk taxonomic profile at family/genus level (relative abundance of almost 1%). LAB: quarters treated with inactivated culture of *Lactobacillus rhamnosus* only, Ab: quarters treated with antibiotics, PBS: quarters treated with sterile PBS only, as described in Material and Methods. T0: time zero; T6: time at 6 days post treatment.

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inoculation in the mammary gland was envisaged, which would probably prevail over the other bacterial species, interfering with the detection of other microbial species.

We found that, although inactivated, the intramammary inoculation of LAB had a significant chemotactic effect toward leukocytes, as shown by the increase of milk somatic cells after 1 day from the inoculation of LAB. Intramammary gland treatment with PBS induces an increase of SCC as well, but two days after inoculation: interestingly, this result is consistent with what has been previously reported in a similar study using sterile PBS as negative control [51]. Treatment with antibiotics also elicited a chemotactic effect as well, although the increase of SCC is more limited as compared with LAB. The present results confirmed that intramammary inoculation of either bacteria, PBS or antibiotics triggers an inflammatory response, as demonstrated by the increase of SCC.

The microbiota of milk from affected animals largely corresponds to what has been previously reported [41] with some exceptions; among the others, the relative abundance of *Psycrobacter* and *Pseudomonas*, which were at 8.79% and 14.45% in the previous study, ranged in the present study from 2% to 4% and from 1% to 2%, respectively. *SB53* was not found as well, whereas its RA was at 3.7% in previous reports. These differences may be explained by the fact that sub-clinical mastitis can be caused by intramammary infection by a heterogeneous group

Fig 4. Boxplots show quartile distribution of weighted UniFrac distances between each group diversity after LAB, Ab and PBS treatment. Statistical significant differences were found between Ab and LAB (p = 0.001) and LAB and PBS (p = 0.003). LAB: quarters treated with inactivated culture of *Lactobacillus rhamnosus* only, Ab: quarters treated with antibiotics, PBS: quarters treated with sterile PBS only, as described in Material and Methods.

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of microorganisms, and the relative abundance of each microbial population may therefore be heterogeneous as well. No families nor genera were shared among subclinical mastitis samples, confirming that microbiota varies more in sub-clinical mastitis than healthy individuals as previously reported [41,52].

No major changes in microbiological cultures were found in milk quarters treated with LAB and PBS. As expected, the milk from quarters treated with antibiotic became negative at microbiological count, with one exception.

After treatment with inactivated Lactobacillus *rhamnosus*, we found an increase of up to 4% in the relative abundance of *Pseudomonas*. This finding is interesting, because the relative

abundance of *Pseudomonas* genus was found to be associated to mastitis in water buffalo in our previous report [41], and is already known as mastitis pathogen in cow [53], sheep [54] and goats [55]. We may therefore hypothesize that the inoculation of LAB, though inactivated, may unbalance the microbiota of water buffalo by increasing the relative abundance of genus involved in the development of mastitis. It must also be said that the effects on microbiota of sterile PBS was more evident than the effects of inactivated cultures of LAB. This results may provide suggestions about the use of PBS as negative control for *in vivo* studies on mammary gland.

Other major finding was that the treatment with antibiotics increased at T6 the relative abundance of *Methylobacterium*, which was not found in milk treated with *Lactobacillus rhamnosus* and PBS. *Methylobacterium* forms biofilms and can develop resistance to high temperatures, drying, and disinfecting agents [56], which features may partially explain the growth capability of this genus after antibiotic treatment. These results supported in water buffalo the hypothesis that has been recently advanced in dairy cow that the mammary gland hosts a resilient microbiome that can reestablish after treatment with antibiotics [57].

Given the background that *Lactobacillus rhamnosus* culture was inactivated, and it induced an extravasation of leukocytes from blood toward the milk, we may speculate that the few modification of microbiota are determined by the intervening WBC, that are activated by the PAMP exposed on the surface of killed bacteria.

Interestingly, we found that no paracrine effect was present within the mammary glands: in all animals with subclincal mastitis quarters treated with antibiotic and other subclinical mastitis quarters treated with *Lactobacillus rhamnosus* or PBS, only the antibiotic-treated quarter became MC negative. About the others within the same mammary gland, they did not change or became MC positive at T6, suggesting the independence of every single quarter.

Conclusions

This is the first experiment on water buffaloes, and in ruminants in general, that aimed to investigate the effect of *Lactobacillus rhamnosus* on subclinical mastitis. We demonstrated that the *in vivo* intramammary treatment with *Lactobacillus rhamnosus* has a transient pro-inflammatory activity as assessed by the SCC and is capable to modify the microbiota of milk after six days from inoculation, albeit slightly, even when the bacterial cultures were heat inactivated. This study confirmed the potential pro-inflammatory activity of GRAS bacteria, and suggests that careful approaches are needed for its *in vivo* use.

Supporting information

S1 Table. Relative abundance ($>$ 1%) of microbiota taxa at family level. PBS: quarters treated with sterile PBS only, LAB: quarters treated with inactivated culture of *Lactobacillus rhamnosus* only, AB: quarters treated with antibiotics, as described in Material and Methods. T0: time zero; T6: time at 6 days post treatment.
(DOCX)

S2 Table. Sampling time, microbiological result, SCC and group for each quarter included in the study. MC: Microbiological count. SCC (Somatic Cell Count) is x 1000. NA: not Assessed.
(DOCX)

S1 Fig. Water buffalo milk taxonomic profile at family level (relative abundance of $>$ 1%). PBS: quarters treated with sterile PBS only, LAB: quarters treated with inactivated culture of *Lactobacillus rhamnosus* only, Ab: quarters treated with antibiotics, as described in Material and Methods. T0: time zero; T6: time at 6 days post treatment.
(TIF)

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3 Paper 3

Short-communication: intra- and inter-individual milk microbiota variability in healthy and infected water buffalo udder quarters

Short-communication: intra- and inter-individual milk microbiota variability in healthy and infected water buffalo udder quarters

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23 **Abstract**

24 The concept that ruminant mammary gland quarters are anatomically and physiologically
25 unrelated has been recently challenged by immunological evidences. How this
26 interdependence reflects on individual quarter milk microbiota is unknown. The aim of the
27 present study was to cover this gap by investigating the interdependence of quarters among
28 the same mammary gland at the milk microbiota level using next generation sequencing of
29 V4-16S rRNA gene. A total number of 52 samples was included in this study and classified
30 as healthy or affected by subclinical mastitis. DNA extraction, amplification of the V4-16S
31 rRNA gene and sequencing using Ion Torrent Personal Genome Machine were carried out.
32 We found that the intra-individual variability was lower than the inter-individual one. The
33 present findings further supports at milk microbiota level the hypothesis of the
34 interdependence of quarters, as previously demonstrated following immunological studies,
35 suggesting that individual factors (e.g. immunity, genetics) may have a role in modulating
36 milk microbiota.

37 Mammary gland quarters within dairy cows have been regarded as independent of each other,
38 given the background that each quarter has its own vascular system, nerve supply, and
39 suspensory apparatus (Berry and Meaney, 2006; Akers and Nickerson, 2011). Preliminary
40 investigations on immune related cells suggesting that mammary gland quarters do not act
41 independently during mastitis (Merle et al., 2007) were further confirmed by the evidence that
42 infection of one udder quarter influences also other uninfected quarters (Mitterhuemer et al.,
43 2010; Jensen et al., 2013). More recent studies demonstrated that quarters of infected udders
44 influence the percentage of B cells and the expression of adhesion molecules in neutrophils
45 of uninfected quarters (Blagitz et al., 2015).
46 To the best of our knowledge, the difference in bacterial taxonomy between quarters within
47 the same udder has not been investigated yet, except in human breast milk, where high intra-
48 individual similarity between individuals was demonstrated (Avershina et al., 2018). Culture-
49 independent methodologies relying on high-throughput DNA sequencing of 16S (Next
50 Generation Sequencing – NGS) are currently applied to describe the relationship between
51 resident microbial population and the development of mastitis and allow for an in depth
52 description of species that cannot be cultured (Oikonomou et al., 2012; Bicalho, 2014; Lima
53 et al., 2018), and are regarded as the ideal techniques to identify differences between quarter
54 milk microbiota.
55 The domestic water buffalo (*Bubalus bubalis*) provides a significant amount of global milk
56 production and is the major milk producing animal in several countries, such as India and
57 Pakistan (Fao, 2016). Water buffalo udder quarters are regarded as anatomically and
58 physiologically independent to the others within the same mammary gland, as in cow (Thomas
59 et al., 2004; Ambord et al., 2010). How this anatomical independence is related to
60 immunological and microbiological status is unknown. Starting from previous results about
61 water buffalo milk microbiota (Catozzi et al., 2017), this study aimed to elucidate the
62 interdependence of quarters by investigating the variability of milk microbiota in composition

63 and structure between healthy quarters within the same udder. In order to assess whether
64 modification in unhealthy status, such as mild inflammation, reflects on the other quarters, the
65 composition of microbiota in milk from animals affected by subclinical mastitis was also
66 determined.
67 Water buffalo quarter milk samples were collected from healthy (H) and subclinical mastitis
68 affected (SM) quarters, from 16 animals. Animals were enrolled from the same farm, in order
69 to reduce the microbiota variability due to different management and feeding regimen, and
70 were homogenous for parity (from second to fourth milking) and stage of lactation (mid
71 lactation). A total of 52 milk quarter samples, of which 18 healthy (from 6 animals) and 34
72 affected by subclinical mastitis (from 11 animals), were enrolled. Healthy quarters were
73 characterized by absence of clinical symptoms, negative microbiological culture for mastitis
74 pathogens and a somatic cell count (SCC) lower than 200,000 cells/ml; subclinical mastitis
75 samples were defined by absence of clinical symptoms, positive microbiological culture for
76 mastitis pathogens and/or SCC higher than 200,000 cells/ml. The list of samples enrolled is
77 presented in Supplementary Table 1
78
79 Mammary glands were disinfected and first strains of milk were discarded and gloves were
80 changed after every milk collection. Milk samples were collected, immediately refrigerated
81 and delivered to the laboratory for microbiological and SCC analysis.
82 Microbiological culture tests and SCC were performed as previously reported (Catozzi et al.,
83 2017).
84 The DNA extraction was carried out as previously reported as well (Catozzi et al., 2017).
85 Briefly, one ml of milk was centrifuged at room temperature at 16,100 rcf for 20 minutes. Fat
86 and supernatant were removed and the remaining pellet was resuspended with 250ul of the
87 Power Bead Tube of the DNeasy Power Soil Kit (QIAGEN) used to extract bacterial DNA,
88 according to the manufacturer’s instructions. V4 region of 16S rRNA gene was amplified for

89 each sample. The forward primer was 5' –
90 CCATCTCATCCCTGCGTGTCTCCGAC**TCAG***NNNNNNNNNNNNNNNNN***GAT**GTGY
91 CAGCMGCCGCGGTAA – 3', and composed of the adapter linker, the key, the sample-
92 specific barcode and the 515F forward primer. The reverse primer was 5' –
93 CCTCTCTATGGGCAGTCGGTGATGGACTACNVGGGTWTCTAAT – 3', composed of
94 the adapter linker and the R806 reverse primer. The Thermo Scientific Phusion Hot Start II
95 High-Fidelity DNA polymerase kit was used to perform V4 PCR (Catozzi et al., 2017). Next-
96 generation sequencing was carried out using an Ion Torrent Personal Genome Machine with
97 the Ion 318 Chip Kit v2 (Thermo Fisher Scientific, Weltham, Massachusetts, U.S.A.), by the
98 Centre for Research in Agricultural Genomics (CRAG, Bellaterra, Barcelona), following
99 manufacturer's instructions. Raw sequences have been submitted to NCBI under Bioproject
100 accession number PRJNA492401. Reads were demultiplexed and analysed using Quantitative
101 Insight Into Microbial Ecology 2 software (QIIME 2; https://qiime2.org) (Caporaso et al.,
102 2011). Briefly, DADA2 was used as quality filtering method in order to denoise, dereplicate
103 single-end sequences and remove chimeras (Callahan et al., 2016); a truncation length of 245
104 bases was used. After that, the units of observation, composed of unique sequences namely
105 Amplicon Sequence Variants (ASVs), were used to classify and assign taxonomy by
106 Greengenes 13.8 (DeSantis et al., 2006) at 99% of Operational Taxonomic Units (OTUs)
107 identity and trimmed to V4 region as reference database. The filtered feature table was used
108 to perform the downstream analysis. Taxonomic analysis was performed for each sample or
109 sample group at phylum and family level with a relative abundance of at least 1%. Results
110 and taxonomic classification are presented in Figure 1 at phylum (Panel A) and family (Panel
111 B) level and Table S2. It was found that *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and
112 *Proteobacteria* predominate the milk microbiota at phylum level (Table S2, Panel A), whereas
113 *Aerococcaceae*, *Corynebacteriaceae*, *Moraxellaceae*, *Staphylococcaceae* and
114 *Propionibacteriaceae* represented the most abundant taxa at family level (Table S2, Panel B).

115 The relative abundance of phyla found in healthy milk were largely comparable with those
116 previously reported (Catozzi et al., 2017), in particular for what concerns *Bacteroidetes* and
117 *Proteobacteria*. On the contrary, the relative abundance of *Actinobacteria* was found to be
118 increased (27.9% as compared to 12.04%) and *Firmicute*s were found to be decreased (37%
119 as compared to 57.7%). At family level, the relative abundance of *Aerococcaceae* and
120 *Corynebacteriaceae* were similar, whereas *Moraxiellaceae* and *Staphylococcaceae* were
121 decreased (7.6% and 9.2% as compared to 18% and 16%, respectively). On the contrary,
122 *Propionibacteria* were increased (8% as compared to 2%).
123 The number of studies carried out in milk water buffalo is too limited to identify a common
124 healthy and unhealthy microbiota. In bovine milk, beside variation related to inflammation
125 (Bicalho, 2014; Catozzi et al., 2017; Lima et al., 2017), variation in milk microbiota have
126 been linked not only to antibiotic treatment (Ganda et al., 2016, 2017), as expected, but also
127 to lactation stage, weather conditions and diet supplementation (Chaves Lopez et al., 2016; Li
128 et al., 2018), suggesting the presence of a wide range of factors and sources influencing the
129 milk microbial community (Derakhshani et al., 2018). Therefore, possible variations of the
130 relative abundance at phyla and family levels could be related to different management
131 conditions.
132 Beta diversity, which is a measure of the differences occurring between samples by estimating
133 how many taxa they share, was performed using qualitative and quantitative approaches
134 (unweighted and weighted UniFrac distances matrices, respectively). Diversity analysis was
135 assessed using a depth of 17500 sequences per sample. Abundancies for phyla and families
136 were represented using the CIRCOS software (http://circos.ca/software/) (Connors et al.,
137 2009). Wilcoxon signed pairwise test was performed for unpaired comparisons among beta
138 diversity matrices from quarters within the same animal and between different animals using
139 pairwise.wilcox.test function in R (http://www.R-project.org). After false Discovery rate

140 (FDR) correction, comparisons were considered statistically significant were $p < 0.05$.
141 Detailed workflow used in QIIME and in R is shown in Supplementary file 1.
142 The individual variation in the amount of the most abundant phyla (Panel A) and families
143 (Panel B) are reported in Figure 2 and Table S3. The violin plot indicates the range of standard
144 deviations (SD) of the main taxa, through which it is possible to evaluate the range of intra-
145 individual variability for all animals.
146 *Proteobacteria* and *Firmicutes* showed the highest variation in H (15% and 13%, respectively)
147 and SM samples (22% and 28%, respectively) (Table S2, Panel A). At individual level,
148 *Firmicutes* represented the most variable phylum in SM samples (SD mean of 19% ranging
149 from 3.1% to 30.6%), as compared to the healthy ones (SD mean of 9% ranging from 2.6%
150 to 21.2%), followed by *Proteobacteria* (SD mean of 12% and 9% for H and SM samples,
151 respectively). This result may potentially explain the differences found in microbiota from
152 previous reports (Catozzi et al., 2017). The other main phyla, namely *Acidobacteria* and
153 *Bacteroidetes*, were more stable, reaching a SD mean lower than 8% in H quarters (Table S3,
154 Panel A). *Staphylococcaceae* and *Moraxellaceae* were the most variable families for SM and
155 H samples with a SD of 34% and 16%, respectively (Table S2, Panel B). At individual level,
156 these families showed the greatest variability in H samples (*Moraxellaceae* with a SD mean
157 of 10% ranging from 0.9 to 37%) and SM samples (*Staphylococcaceae* with a SD mean of
158 28% ranging from 2.5 to 49%; Table S3, Panel B), whereas *Propionibacteriaceae,*
159 *Corynebacteriaceae* and *Aerococcaceae* were the most stable. We found that the most stable
160 phylum across healthy or subclinical mastitis affected quarters was represented by
161 *Bacteroidetes*. At family level, the relative abundance of *Propionibacteriaceae* showed the
162 greatest stability, followed by and *Corynebacteriaceae* and *Aerococcaceae*. On the contrary,,
163 *Firmicutes* and *Proteobacteria* were the most variable phyla in both healthy and subclinical
164 mastitis affected quarter milk samples; consequently, the families *Staphylococcaceae* and
165 *Moraxellaceae* showed the greatest variation in relative abundance. At family level, the mean

166 and median variability within animals was always lower than 6%, with the exception of
167 *Staphylococcaceae* and *Moraxellaceae*.
168 The comparison between quarter milk microbiota within the same individual and between
169 different individuals was performed using unweighted and weighted UniFrac distance
170 matrices. Results are presented in table S4; values close to 0 are representative of high
171 similarity; whereas, values close to 1 show a lower similarity. A box plot with statistical
172 significant differences is presented in Figure 3. Healthy and subclinical mastitis affected
173 quarters within individuals showed more similarity in terms of microbiota structure as
174 compared to those between individuals. Previous studies have demonstrated the
175 communication among quarters at immunological level (Burvenich et al., 2003; Merle et al.,
176 2007; Jensen et al., 2013; Blagitz et al., 2015). We presented the evidence that, in water
177 buffaloes as well, for what concerns the milk microbiota structure, the intra-individual
178 variability was lower than the inter-individual one in both healthy and subclinical mastitis-
179 affected quarters. The present finding is partially consistent with what has been recently
180 reported in human milk (Avershina et al., 2018), that demonstrated a high intra-individual
181 similarity between microbiota of milk collected by the two mammary glands. In fact, we found
182 that, in healthy samples, the similarity was greater in quarters within the same udder rather
183 that between different mammary glands. The same profile was also demonstrated also in
184 subclinical mastitis groups by means of the weighted Unifrac analysis.
185 Subclinical mastitis individual quarters showed a greater dissimilarity as compared to the
186 healthy ones, consistently with previous studies in water buffaloes, and cows, demonstrating
187 that the development of a disease destabilizes the microbiota rather than shifting to a
188 determined structure (Zaneveld et al., 2017). Nevertheless, the similarity of milk microbiota
189 structure within subclinical mastitis affected individuals was pretty much similar to that within
190 healthy individuals, demonstrating that the presence of the same major pathogens in the

191 quarters of the same mammary gland could influence the microbiota similarity, but also that
192 individual signatures could be responsible for that microbiota interdependence.
193 The new concept of 'hologenome', defined as the host-microbes genomes as a unit of
194 evolution, is taking shape (Shapira, 2016), meaning that selection processes involved the
195 genomes of both individual and microorganisms. Here, we support the presence of the
196 quarter's interdependence at milk microbiota level, showing that the intra-individual
197 similarity was greater than the inter-individual one.
198 In conclusion, the results provided in this preliminary study demonstrated that the four
199 quarters of the mammary buffalo udder cannot be regarded as separate entities, and cannot be
200 considered as individual experimental units for in vivo studies regarding microbiome
201 description. Further investigation is required to confirm the present results in bovine species.
202
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295

296 **Tables**

297

298 **Table S1**: Metadata of samples, including SampleId, Status, AnimalID, Microbiological

299 results and SCC (cells/ml x1000). H: healthy; SM: subclinical mastitis; SCC: Somatic Cell

300 Count; CNS: Coagulase Negative Staphylococci. Data not shown.

301

302 **Table S2**: Mean, standard deviation (SD), median, minimum (min) and maximum (max) of

303 the most abundant phyla (Panel A) and families (Panel B) with a relative abundance at least

304 of 1%.

305 H: healthy samples; SM: subclinical mastitis samples.

306 A

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Phylum	Status	mean	SD	median	min	max
p__Actinobacteria	H	27.9%	11.0%	27.0%	8.2%	53.1%
	SM	12.1%	8.7%	10.0%	1.2%	36.0%
p__Bacteroidetes	H	6.5%	4.6%	5.9%	0.0%	16.2%
	SM	4.5%	6.1%	1.2%	0.0%	21.8%
p__Firmicutes	H	37.0%	13.0%	35.8%	12.6%	55.2%
	SM	58.2%	28.4%	59.2%	8.3%	97.6%
p__Proteobacteria	H	24.9%	15.2%	22.2%	6.6%	73.2%
	SM	21.5%	21.9%	13.0%	0.8%	85.2%

313 B

Families	Status	mean	SD	median	min	max
f__Aerococcaceae	H	10.9%	7.4%	10.0%	0.0%	22.3%
	SM	5.7%	7.4%	2.0%	0.0%	24.8%
f__Corynebacteriaceae	H	9.4%	5.1%	8.8%	2.6%	19.1%
	SM	4.7%	4.6%	3.1%	0.0%	17.9%
f__Moraxellaceae	H	7.6%	16.3%	2.1%	0.0%	68.2%
	SM	6.4%	13.3%	1.5%	0.0%	65.5%
f__Propionibacteriaceae	H	8.1%	7.0%	5.8%	0.3%	23.9%
	SM	2.5%	2.5%	1.6%	0.2%	11.2%

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f__Staphylococcaceae	H	9.2%	5.7%	7.8%	1.7%	23.2%
	SM	37.8%	34.7%	30.2%	0.0%	96.6%

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318 **Table S3**: Minimum (Min), maximum (Max), mean and median of the standard deviation
319 (SD) of the most abundant phyla (Panel A) and families (Panel B) at individual level. Animal
320 identification is indicated between parentheses. H: healthy samples; SM: subclinical mastitis
321 samples.

322 A

Phylum	SD	H	SM
p__Actinobacteria	Min	3.3% (1)	1.5% (19)
	Max	10% (6)	14.4% (20)
	Mean	7.6%	6.8%
	Median	8.4%	6.9%
p__Bacteroidetes	Min	1.4% (6)	0.2% (19)
	Max	5.3% (1)	9.5% (18)
	Mean	3.0%	3.9%
	Median	2.4%	2.9%
p__Firmicutes	Min	2.6% (6)	3.1% (19)
	Max	21.2% (13)	30.6% (14)
	Mean	8.6%	19.0%
	Median	5.7%	19.8%
p__Proteobacteria	Min	2.7% (1)	1.9% (19)
	Max	32.1% (13)	32.4% (14)
	Mean	11.9%	9.0%
	Median	9.4%	6.9%

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325 B

Families	SD	H	SM
f__Aerococcaceae	Min	0.9% (7)	0.05% (17)
	Max	10.5% (13)	12.1% (20)
	Mean	5.7%	5.3%
	Median	6.2%	5.1%
f__Corynebacteriaceae	Min	2% (6)	0.9% (18)
	Max	8.5% (7)	10% (20)
	Mean	5.3%	3.9%
	Median	5.4%	4.3%
f__Moraxellaceae	Min	0.9% (16)	0.2% (8)
	Max	36.6% (13)	33% (14)
	Mean	10.3%	6.8%

	Median	3.4%	1.7%
f__Propionibacteriaceae	**Min**	1.1% (13)	0.2% (20)
	Max	5.3% (16)	4.9% (15)
	Mean	2.9%	1.6%
	Median	2.7%	0.8%
f__Staphylococcaceae	**Min**	2.6% (6)	2.5% (14)
	Max	10.1% (13)	48.9% (4)
	Mean	5.4%	27.8%
	Median	4.7%	26.7%

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328 **Table S4**: descriptive statistics of unweighted and weighted UniFrac distance
329 matrices. Minimum, maximum, mean, median and standard deviation (SD) are shown.

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Unweighted UniFrac distance matrices

	Healthy within	**Healthy between**	**Subclinical mastitis within**	**Subclinical mastitis between**
Min	0.35	0.41	0.24	0.34
Max	0.75	0.88	0.8	0.89
Mean	0.56	0.63	0.6	0.65
Median	0.57	0.6	0.64	0.66
SD	0.1	0.1	0.12	0.1

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Weighted UniFrac distance matrices

	Healthy within	**Healthy between**	**Subclinical mastitis within**	**Subclinical mastitis between**
Min	0.17	0.16	0.02	0.02
Max	0.36	0.45	0.43	0.56
Mean	0.24	0.28	0.26	0.31
Median	0.24	0.27	0.27	0.33
SD	0.05	0.06	0.1	0.12

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Figures

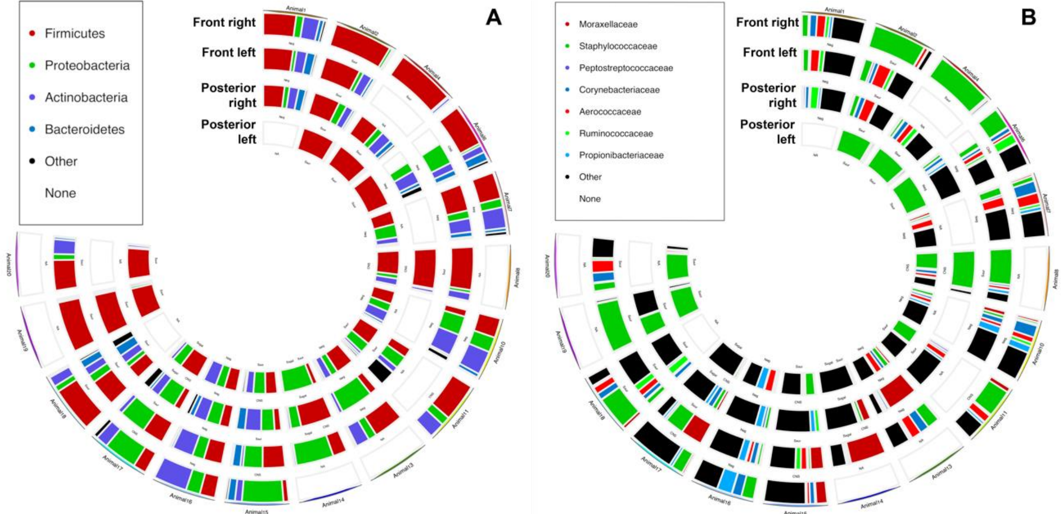
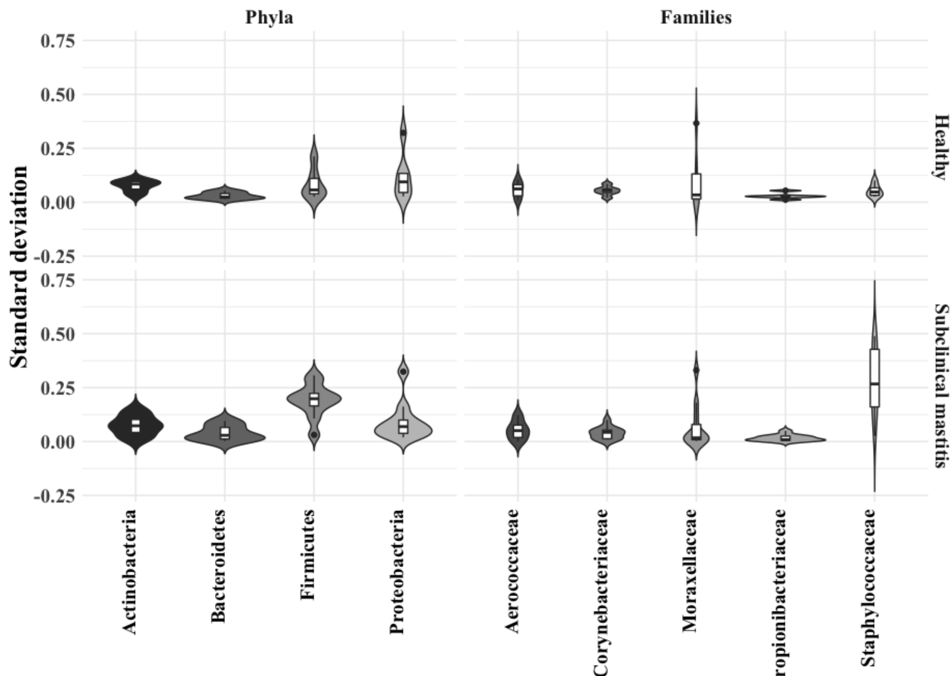


Figure 1: Taxonomic results at phylum (panel A, relative average abundance ≥1%) and family level (panel B, relative average abundance ≥2.5%) for all animal quarters. Each slice correspond to one animal and each circle section to a quarter. The microbiological culture result for mastitis pathogens is indicated below each quarter. White quarters indicate that sample is missing



347 **Figure 2:** Variation of the standard deviation at individual level for the main phyla (Panel A)
348 and families (Panel B). The relative average abundance was ≥1%.
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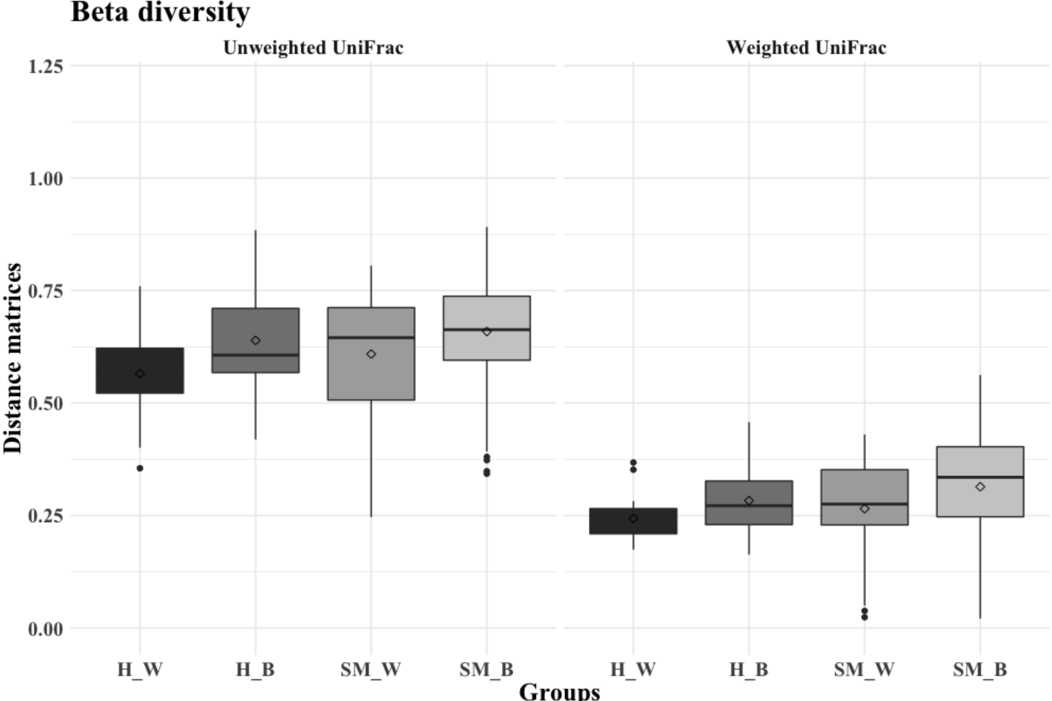

351
352 **Figure 3:** Box plots of unweighted and weighted UniFrac distance matrices. Median (line
353 into the box), mean (diamond shape), upper and lower quartiles (ends of the box) and
354 highest and lowest value (extreme lines) are shown. H: healthy samples; SM: subclinical
355 mastitis samples; W: comparison of samples within the same individual; B: comparison of
356 samples between different individuals. Statistical significance are presented where $0.05 < p$
357 < 0.001 (*) and $p < 0.001$ (**). False discovery rate correction was applied.
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4 Paper 4

Microbiota profiling with long amplicons using Nanopore sequencing: full-length 16S rRNA gene and the 16S-ITS-23S of the rrn operon

RESEARCH ARTICLE

REVISED Microbiota profiling with long amplicons using Nanopore sequencing: full-length 16S rRNA gene and the 16S-ITS-23S of the *rrn* operon [version 2; peer review: 2 approved, 3 approved with reservations]

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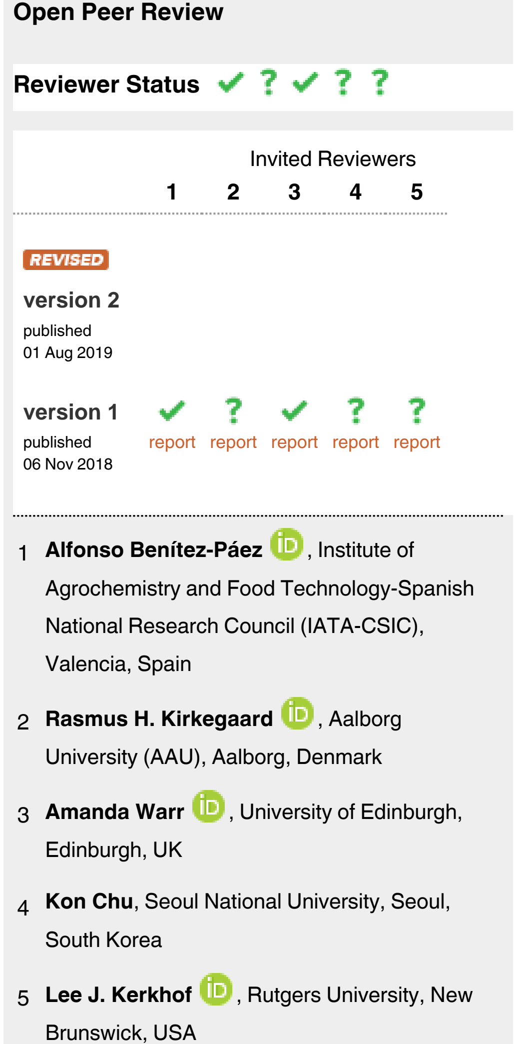
Abstract

Background: Profiling the microbiome of low-biomass samples is challenging for metagenomics since these samples are prone to contain DNA from other sources (e.g. host or environment). The usual approach is sequencing short regions of the 16S rRNA gene, which fails to assign taxonomy to genus and species level. To achieve an increased taxonomic resolution, we aim to develop long-amplicon PCR-based approaches using Nanopore sequencing. We assessed two different genetic markers: the full-length 16S rRNA (~1,500 bp) and the 16S-ITS-23S region from the *rrn* operon (4,300 bp).
Methods: We sequenced a clinical isolate of *Staphylococcus pseudintermedius*, two mock communities and two pools of low-biomass samples (dog skin). Nanopore sequencing was performed on MinION™ using the 1D PCR barcoding kit. Sequences were pre-processed, and data were analyzed using EPI2ME or Minimap2 with *rrn* database. Consensus sequences of the 16S-ITS-23S genetic marker were obtained using canu.
Results: The full-length 16S rRNA and the 16S-ITS-23S region of the *rrn* operon were used to retrieve the microbiota composition of the samples at the genus and species level. For the *Staphylococcus pseudintermedius* isolate, the amplicons were assigned to the correct bacterial species in ~98% of the cases with the16S-ITS-23S genetic marker, and in ~68%, with the 16S rRNA gene when using EPI2ME. Using mock communities, we found that the full-length 16S rRNA gene represented better the abundances of a microbial community; whereas, 16S-ITS-23S obtained better resolution at the species level. Finally, we characterized low-biomass skin microbiota samples and detected species with an environmental origin.
Conclusions: Both full-length 16S rRNA and the 16S-ITS-23S of the *rrn* operon retrieved the microbiota composition of simple and complex microbial communities, even from the low-biomass samples such as dog



skin. For an increased resolution at the species level, targeting the 16S-ITS-23S of the *rrn* operon would be the best choice.

Any reports and responses or comments on the article can be found at the end of the article.

Keywords
microbiome, microbiota, 16S, rrn operon, nanopore, canine, low-biomass, skin, dog

This article is included in the Nanopore Analysis gateway.

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Author roles: Cuscó A: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Catozzi C**: Investigation, Validation, Visualization, Writing – Review & Editing; **Viñes J**: Investigation, Visualization, Writing – Review & Editing; **Sanchez A**: Conceptualization, Funding Acquisition; **Francino O**: Conceptualization, Funding Acquisition, Methodology, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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REVISED **Amendments from Version 1**

We hereby present a revised version of our manuscript, based on the comments made by the referees and more results since version 1 was published.

The main changes of the manuscript when compared to version 1 are those stated here:

- We have assessed the performance of the mapping approach with the uncorrected long-amplicons strategy presented here.
- We have expanded the results adding a section of the *de novo* assembly of the 16S-ITS-23S genetic marker.
- We have provided more detail on the methodology used from the lab bench to the bioinformatics part, with supplementary file 1 providing the commands used for the mapping approach.
- The mapping results have been updated since in version 1 chimeras were computed differently for each amplicon and led to confusing results (more chimera for 16S rRNA than for 16S-ITS-23S). Now for both amplicons, we have performed base-level alignment using Minimap2 as the first step before yacrd (detailed in Supplementary File 1).
- The figures and tables have been simplified, re-ordered and completed to be more informative and clear. We got rid of the graphic bars and kept the heatmaps.

Finally, we want to thank all the referees for their time to evaluate our work and their invaluable feedback.

See referee reports

Introduction

The microbiota profile of low-biomass samples such as skin is challenging for metagenomics. These samples are prone to contain DNA contamination from the host or exogenous sources, which can overcome the DNA of interest[1,2]. Thus, the usual approach is amplifying and sequencing certain genetic markers that are ubiquitously found within the studied kingdom rather than performing metagenomics. Ribosomal marker genes are a common choice: 16S rRNA and 23S rRNA genes to taxonomically classify bacteria[3,4]; and ITS1 and ITS2 regions for fungi[5,6].

Until now, most studies of microbiota rely on massive parallel sequencing, and target a short fragment of the 16S rRNA gene, which presents nine hypervariable regions (V1-V9) that are used to infer taxonomy[7,8]. The most common choices for host-associated microbiota are V4 or V1-V2 regions, which present different taxonomic coverage and resolution depending on the taxa[9,10].

Apart from the biases derived from the primer choice, short fragment strategies usually fail to assign taxonomy reliably at the genus and species level. This taxonomic resolution is particularly useful when associating microbiota to clinics such as in characterizing disease status or when developing microbiota-based products, such as pre- or pro-biotics[11]. For example, in human atopic dermatitis (AD) the signature for AD-prone skin when compared to healthy skin was enriched for *Streptococcus* and *Gemella*, but depleted in *Dermacoccus*. Moreover, nine different bacterial species were identified to have significant AD-associated microbiome differences[12]. In canine atopic dermatitis, *Staphylococcus pseudintermedius* has been classically associated with the disease. Microbiota studies of canine atopic dermatitis presented an overrepresentation of *Staphylococcus* genus[13,14], but the species was only confirmed when complementing the studies using directed qPCRs for the species of interest[13] or using a *Staphylococcus*-specific database and V1-V3 region amplification[14].

With the launching of single-molecule technology sequencers (e.g. PacBio or Oxford Nanopore Technologies), these short-length associated issues can be overcome by sequencing the full-length 16S rRNA gene (~1,500 bp) or even the nearly-complete *rrn* operon (~4,300 bp), which includes the 16S rRNA gene, ITS region, and 23S rRNA gene.

Several studies assessing the full-length 16S rRNA gene have already been performed using Nanopore sequencing to: i) characterize artificial bacterial communities (mock community)[15–17]; ii) complex microbiota samples, from the mouse gut[18], wastewater[19], microalgae[20] and dog skin[21]; and iii) the pathogenic agent in a clinical sample[22–24]. Some studies have been performed using the nearly-complete *rrn* operon to characterize mock communities[25] and complex natural communities[26].

Here, we aim to assess these two long-amplicon approaches using MinION™ (Oxford Nanopore Technologies), a single-molecule sequencer that is portable, affordable with a small budget and offers long-read output. Its main limitation is a higher error rate than massive sequencing. We will test our approaches by sequencing several samples with different degrees of complexity: i) a clinical isolate of *Staphylococcus pseudintermedius*, ii) two bacterial mock communities; and iii) two complex skin microbiota samples.

Methods

Samples and DNA extraction

We first sequenced a pure bacterial isolate of *S. pseudintermedius* obtained from the ear of a dog affected by otitis.

Then, we used two DNA mock communities as simple and well-defined microbiota samples:

- HM-783D, kindly donated by BEI resources, containing genomic DNA from 20 bacterial strains with staggered ribosomal RNA operon counts (between 10^3 and 10^6 copies per organism per µl).
- ZymoBIOMICS™ Microbial Community DNA standard that contained a mixture of genomic DNA extracted from pure cultures of eight bacterial strains.

As a complex microbial community, we used two DNA sample pools from the skin microbiota of healthy dogs targeting two different skin sites: i) dorsal back (DNA from two dorsal samples from Beagle dogs); and ii) chin (DNA from five chin samples from Golden Retriever/Labrador crossed dogs). Skin microbiota samples were collected using Sterile Catch-All™ Sample Collection Swabs (Epicentre Biotechnologies) soaked in sterile SCF-1 solution (50 mM Tris buffer (pH 8), 1 mM

EDTA, and 0.5% Tween-20). DNA was extracted from the swabs using the PowerSoil™ DNA isolation kit (MO BIO) and blank samples were processed simultaneously (for further details on sample collection and DNA extraction see 27).

PCR amplification of ribosomal markers

We evaluated two ribosomal markers in this study: the full-length 16S rRNA gene (~1,500 bp) and the 16S-ITS-23S region of the ribosomal operon (*rrn)* (~4,300 bp). Before sequencing, bacterial DNA was amplified using a nested PCR, with a first PCR to add the specific primer sets tagged with the Oxford Nanopore universal tag and a second PCR to add the barcodes from the PCR barcoding kit (EXP-PBC001) (Supplementary Table 1). Each PCR reaction included a no-template control sample to assess possible reagent contamination.

For the first PCR, we targeted: i) the full-length 16S rRNA gene using 16S-27F[28] and 16S-1492R[29] primer set and ii) the 16S-ITS-23S of the *rrn* operon using 16S-27F and 23S-2241R[28] primer set (Supplementary Table 1). All the three primers contained the Oxford Nanopore tag, which is an overhang that allows barcoding the samples during the second PCR.

PCR mixture for the full-length 16S rRNA gene (25 µl total volume) contained 5 ng of DNA template (or 2.5 µl of unquantifiable initial DNA), 1X Phusion® High Fidelity Buffer, 0.2 mM of dNTPs, 0.4 µM of 16S-27F, 0.8 µM of 16S-1492R and 0.5 U of Phusion® Hot Start II Taq Polymerase (Thermo Scientific, Vilnius, Lithuania). The PCR thermal profile consisted of an initial denaturation of 30 s at 98°C, followed by 25 cycles of 15 s at 98°C, 15 s at 51°C, 45 s at 72°C, and a final step of 7 min at 72°C.

PCR mixture for the 16S-ITS-23S of the *rrn* operon (50 µl total volume) contained 5 ng of DNA template (or 2.5 µl of unquantifiable initial DNA), 1X Phusion® High Fidelity Buffer, 0.2 mM µl dNTPs 1 µM each primer and 1 U Phusion® Hot Start II Taq Polymerase. The PCR thermal profile consisted of an initial denaturation of 30 s at 98°C, followed by 25 cycles of 7 s at 98°C, 30 s at 59°C, 150 s at 72°C, and a final step of 10 min at 72°C.

The amplicons were cleaned-up with the AMPure XP beads (Beckman Coulter) using a 0.5X and 0.45X ratio for the 16S rRNA gene and the 16-ITS-23S of the *rrn* operon, respectively. Then, they were quantified using Qubit™ fluorometer (Life Technologies, Carlsbad, CA) and the volume was adjusted to begin the second round of PCR with 0.5 nM of the first PCR product or the complete volume when not reaching the required DNA mass (mostly in the samples that amplified with the 16S-ITS-23S genetic marker).

PCR mixture for the barcoding PCR (100 µl total volume) contained 0.5 nM of the first PCR product (50 ng for the 16S rRNA gene and 142 ng for the 16S-ITS-23S), 1X Phusion® High Fidelity Buffer, 0.2 mM µl dNTPs, and 2 U Phusion® Hot Start II Taq Polymerase. Each PCR tube contained the DNA, the PCR mixture and 2 µl of the specific barcode. The PCR thermal profile consisted of an initial denaturation of 30 s at 98°C, followed by 15 cycles of 7 s at 98°C, 15 s at 62°C, 45 s (for the 16S rRNA gene) or 150 s (for *rrn* operon) at 72°C, and a final step of 10 min at 72°C.

Again, the amplicons were cleaned-up with the AMPure XP beads (Beckman Coulter) using a 0.5X and 0.45X ratio for the 16S rRNA gene and the whole *rrn* operon, respectively. For each sample, quality and quantity were assessed using Nanodrop and Qubit™ fluorometer (Life Technologies, Carlsbad, CA), respectively. The samples with higher DNA concentrations were checked by agarose gel to see the size profile of the PCR products (Supplementary Figure 1).

The different barcoded samples were pooled in equimolar ratio to obtain a final pool (1,000–1,500 ng in 45 µl) to do the sequencing library. In few cases, 16S-ITS-23S amplicons did not reach the initial amount of required DNA and we proceeded with lower input material.

Nanopore sequencing library preparation

The Ligation Sequencing Kit 1D (SQK-LSK108; Oxford Nanopore Technologies) was used to prepare the amplicon library to load into the MinION™ (Oxford Nanopore Technologies), following the manufacturer's protocol. Input DNA samples were composed of 1–1.5 µg of the barcoded DNA pool in a volume of 45 µl and 5 µl of DNA CS (DNA from lambda phage, used as a positive control in the sequencing). The DNA was processed for end repair and dA-tailing using the NEBNext End Repair/dA-tailing Module (New England Biolabs). A purification step using 1X Agencourt AMPure XP beads (Beckman Coulter) was performed.

For the adapter ligation step, a total of 0.2 pmol of the end-prepped DNA were added in a mix containing 50 µl of Blunt/TA ligase master mix (New England Biolabs) and 20 µl of adapter mix and then incubated at room temperature for 10 min. We performed a purification step using Adapter Bead Binding buffer (provided in the SQK-LSK108 kit) and 0.5X Agencourt AMPure XP beads (Beckman Coulter) to finally obtain the DNA library.

We prepared the pre-sequencing mix (14 µl of DNA library) to be loaded by mixing it with Library Loading beads (25.5 µl) and Running Buffer with fuel mix (35.5 µl). We used two SpotON Flow Cells Mk I (R9.4.1) (FLO-MIN106). After the quality control, we primed the flowcell with a mixture of Running Buffer with fuel mix (RBF from SQK-LSK108) and Nuclease-free water (575 µl + 625 µl). Immediately after priming, the nanopore sequencing library was loaded in a dropwise fashion using the SpotON port.

Once the library was loaded, we initiated a standard 48 h sequencing protocol using the MinKNOW™ software v1.15.

Data analysis workflow

The samples were run using the MinKNOW software. After the run, fast5 files were base-called and de-multiplexed using Albacore v2.3.1. A second de-multiplexing round was performed

with Porechop v0.2.3[30], where only the barcodes that agreed with Albacore were kept. Porechop was also used to trim the barcodes and the adapters from the sequences, as well as 45 extra base pairs from each end that correspond to the length of the universal tags and custom primers (See Supplementary Figure 2 for a schematic overview of the process and Supplementary File 1 for the bioinformatics workflow of the mapping approach).

After the trimming, reads were selected by size: 1,200 bp to 1,800 bp for 16S rRNA gene; and 3,500 to 5,000 bp for the 16S-ITS-23S of the *rrn* operon. Afterwards, we removed chimeras with the following approach: i) we mapped each mock community to its mock database and the complex samples to the complete *rrn* database using Minimap2 v2.16 (with base-level alignment and z-score set to 70)[31]; ii) chimeras were detected and removed using yacrd v0.5[32].

To assign taxonomy to the trimmed and filtered reads we used to strategies: 1) a mapping-based strategy using Minimap2 v2.16[31] (with base-level alignment and z-score set to 70); or 2) a taxonomic classifier using What's in my Pot (WIMP)[33], a workflow from EPI2ME in the Oxford Nanopore Technologies cloud (based on Centrifuge software[34]).

For the mapping-based strategy, we performed Minimap2 again with the non-chimeric sequences. We applied extra filtering steps to retain the final results: we kept only those reads that aligned to the reference with a block equal or larger than 1,000 bp (for 16S rRNA gene) and 3,000 bp (for the 16S-ITS-23S of the *rrn* operon). For reads that hit two or more references, only the alignments with the highest Smith-Waterman alignment score (AS score) were kept.

The reference databases used in this study were:

- Mock DB: a collection of the complete genomes that were included in each mock community, as described by the manufacturer. The HM-783D database was retrieved from NCBI using the reference accession numbers, while Zymobiomics mock community has already its database online on the Amazon AWS server.
- *rrn* DB: sequences from the whole ribosomal operon from 22,351 different bacterial species retrieved from Genbank by Benitez-Paez *et al.*[25]. We have manually added a sequence of the *rrn* operon from *S. pseudintermedius*.

For assessing the mapping-based strategy, we have made a subset with the *rrn* DB to exclude all the operons that were representatives of Gammaproteobacteria class as an example to see how the alignment-based approach performs when missing main references within the database. These operons were identified by introducing a list of all the genera of the *rrn* DB as a batch in NCBI Taxonomy browser. The sequences belonging to Gammaproteobacteria (code 1236) were removed from the *rrn* DB.

For the taxonomic classification using the WIMP workflow, which uses the NCBI database, only those hits with a classification score >300 were kept[34].

Ampvis2 package in R was used to plot the heatmaps[35] and the Phyloseq package, to plot the alpha rarefaction curves[36].

An earlier version of this article can be found on bioRxiv (doi: https://doi.org/10.1101/450734)

Results

We have assessed the performance of the full-length 16S rRNA and the 16S-ITS-23S rRNA region of the ribosomal (*rrn*) operon to profile the microbial composition of several samples: a bacterial isolate, two mock communities and two complex skin samples (chin and dorsal back).

The samples amplified using the full-length 16S rRNA gene recovered a higher percentage of reads after the quality control when compared to 16S-ITS-23S of the *rrn* operon: 73–95% vs. 30–79%. For the 16S-ITS-23S of the *rrn* operon, the largest percentage of reads was lost during the length trimming step since some of the reads presented lengths that were shorter than expected (Supplementary Table 2).

Bacterial isolate analysis

We first sequenced an isolate of *S. pseudintermedius* obtained from a canine otitis. When using WIMP approach with the 16S-ITS-23S of the *rrn* operon, 97.5% of the sequences were correctly assigned at the species level as *S. pseudintermedius*. However, with the full-length 16S rRNA gene, 68% of the sequences were correctly assigned at the species level as *S. pseudintermedius,* while 13% at the genus one and ~20% were wrongly assigned, either by not reaching the species level or by giving an incorrect species (Table 1).

When using the mapping approach with the *rrn* DB, we obtained no hit to *S. pseudintermedius*. Instead, they were hitting mostly to *Staphylococcus schleiferi*, which is a closely related species; there were also few hits to *Staphylococcus hyicus* and *Staphylococcus agnetis*. This result was due to the *rrn* DB did not contain any representative of *S. pseudintermedius*. When including *S. pseudintermedius* sequence to the *rrn* DB (*rrn* + *S. pseudintermedius*) both markers retrieved the correct result with more than 97% of the assignments hitting the correct reference.

When comparing the alignment results obtained with Minimap2 and *rrn* DB vs *rrn* DB + *S. pseudinteremedius*, we found that the Smith-Waterman alignment score (AS) presented higher values in the correct alignments, especially when using 16S-ITS-23S marker gene (Figure 1). Thus, the AS score could

Table 1. Taxonomy assignments of *S. pseudintermedius* isolate. Taxonomic assignments were obtained i) using WIMP workflow with NCBI RefSeq database; ii) Minimap2 with *rrn* DB; and iii) Minimap2 with *rrn* DB including *S. pseudintermedius*.

	WIMP (NCBI RefSeq DB)		**Minimap2 (*rrn* DB)**		**Minimap2 (*rrn* DB + *S. pseudintermedius*)**	
Taxonomy	**16S**	**16S-ITS-23S**	**16S**	**16S-ITS-23S**	**16S**	**16S-ITS-23S**
Staphylococcus pseudintermedius	68.1%	97.6%	-	-	97.9%	97.5%
Staphylococcus sp	13.1%	0.3%	-	-	-	-
*Staphylococcus schleiferi**	2.2%	0.3%	94.9%	82.1%	1.94%	2.2%
*Staphylococcus aureus**	3.2%	0.2%	0.0%	0.0%	0.0%	0.0%
*Staphylococcus lutrae**	2.7%	0.1%	-	-	-	-
*Staphylococcus hyicus**	0.3%	0.1%	3.2%	8.2%	0.0%	0.2%
*Staphylococcus agnetis**	0.2%	0.0%	1.6%	9.7%	0.0%	0.1%
Other *Staphylococcus**	3.7%	1.4%	0.3%	0.0%	0.2%	-
Other species*	6.5%	0.1%	-	-	-	-

*Incorrect bacterial species assignment

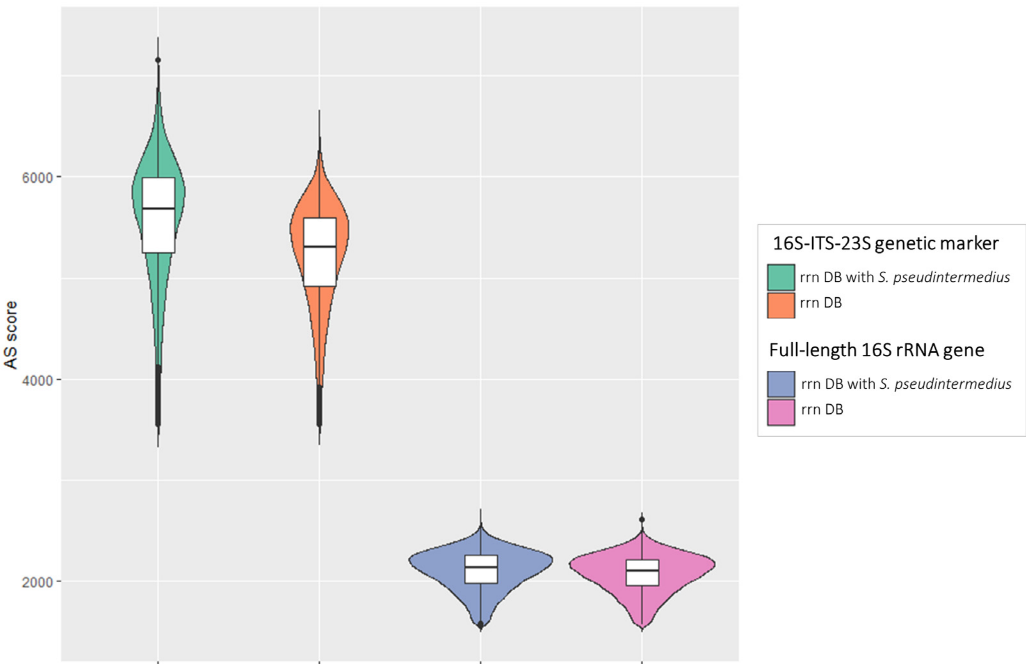


Figure 1. Violin plot representing the distribution of AS score for *S. pseudintermedius* isolate. Alignment scores for each genetic marker were obtained using Minimap2 and either *rrn* DB or *rrn* DB with a reference added for *S. pseudintermedius*. The first two plots are for 16S-ITS-23S, whereas the second ones are for 16S rRNA.

be a filter to identify a wrong taxonomic assignment due to the lack of a reference in the database.

Assessment of the mapping-based strategy

As we have already seen for *S. pseudintermedius*, in the mapping strategy a lack of a reference in the database led to an incorrect taxonomic assignment. Since both marker genes chosen for the microbiota profiling are highly conserved among bacteria, the mapping strategy (through Minimap2) will always align to some reference.

To check the behavior of the mapping approach when using an incomplete database, we have performed an example test using the mock communities. We have mapped the mock communities

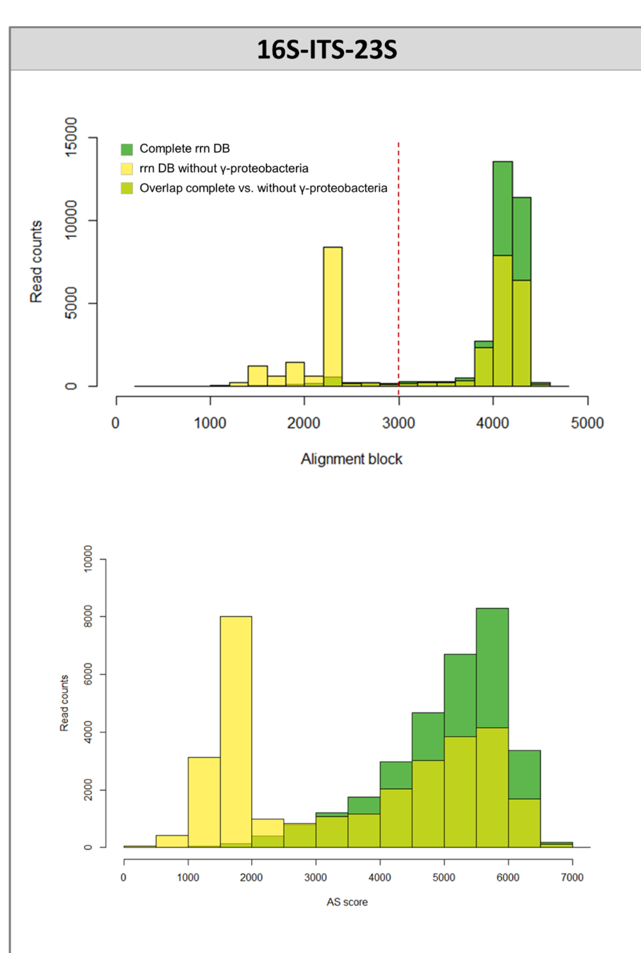
both against the complete *rrn* DB and against a subset of the *rrn* DB without any representative of the Gammaproteobacteria class. The *rrn* DB[25] contains 22,351 different bacterial species, including representatives of the species in both mock communities. We have chosen Gammaproteobacteria because each mock community contains three Gammaproteobacteria species, representing around 24% of the total microbial composition.

We checked the alignment score values and the alignment block length to detect any differences on the alignment performance when using complete or an incomplete database. We plotted two histograms: i) read counts distributed by the alignment block length; and ii) read counts distributed by the alignment score (AS) (Figure 2). For the 16S-ITS-23S genetic marker, we detected a clear pattern: when aligning to the *rrn* DB without Gammaproteobacteria, both histograms changed from a left-skewed distribution to a bimodal distribution with two peaks (Figure 2). A new peak appeared at the lower values that included the wrong taxonomic assignments, which are species not present in the mock community or the non-concordant hits when compared to the complete *rrn* DB results. Thus, for the 16S-ITS-23S marker, the initial filtering step by alignment block length will get rid of most of the incorrect taxonomic assignments. However, this pattern was not observed with the full-length 16S rRNA gene (Figure 2) or when closely-related references were present in the database, as seen above for the *S. pseudintermedius* isolate. So, to further confirm taxonomic results (especially for the 16S rRNA gene), we assigned taxonomy using two different bioinformatics approaches that work with different databases.

Mock community analyses

We analyzed two microbial mock communities to validate the ability of the presented approach: i) to quantify what is expected and detect biases of the technique; and ii) to reach a reliable taxonomic assignment at the species level.

For the first aim, we used the HM-783D mock community that contained genomic DNA from 20 bacterial strains with



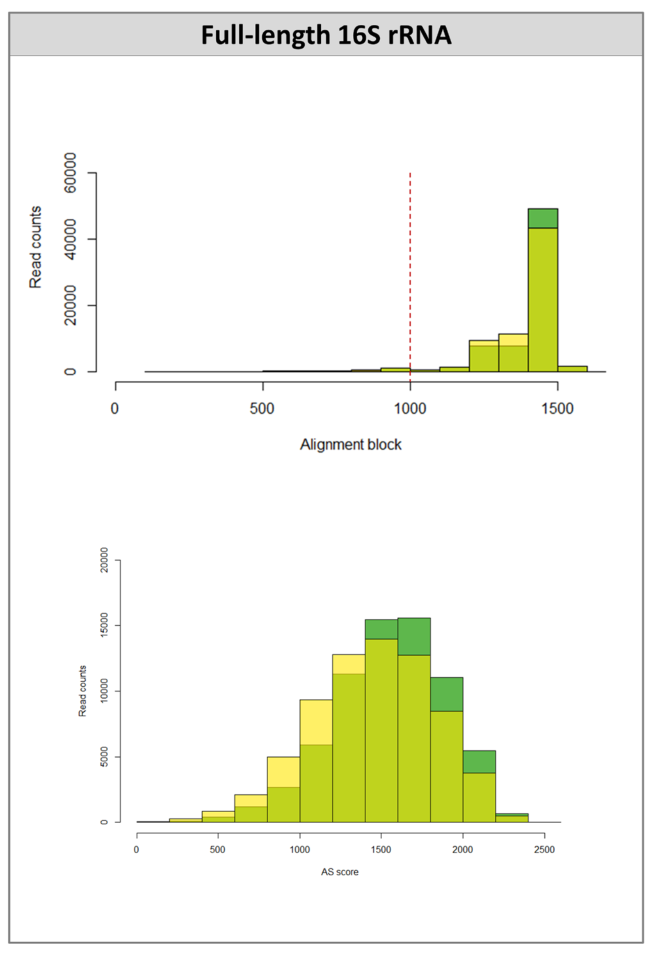


Figure 2. Assessment of the mapping-based strategy using a sample of Zymobiomics mock community. Top part, histograms of the read counts distributed by alignment block depending on the database used (complete *rrn* DB vs *rrn* DB without Gammaproteobacteria). Alignment threshold at 3,000 bp (for 16S-ITS-23S) and 1,000 (for 16S rRNA gene) are marked with a red dashed line. Below part, histograms of the read counts distributed by AS scores depending on the database used (complete *rrn* DB vs *rrn* DB without Gammaproteobacteria).

staggered ribosomal RNA operon counts (from 10^3 to 10^6 copies per organism per µl). This mock community would allow us determining if our approach reliably represents the actual bacterial composition of the community, especially considering the low-abundant species. We assigned taxonomy with Minimap2 and a database containing only the 20 representative bacterial genomes in the HM-783D mock community (Minimap2-mock DB).

On the one hand, using the full-length 16S rRNA gene we detected all the bacterial species present in the mock community, even the low-abundant ones. On the other hand, using the 16S-ITS-23S of the *rrn* operon we detected only the most abundant species (at least 10^4 operon copies) (Figure 3A). This could be due to the lower sequencing depth obtained with 16S-ITS-23S when compared with 16S rRNA (Supplementary Table 2). Moreover, the relative abundances of 16S-ITS-23S sequences were more biased than those obtained from 16S rRNA gene sequencing, which confirmed that the primers for 16S-ITS-23S of the *rrn* operon need to be improved for a better representation of the actual abundances (Figure 3B and 3C).

To assess if the technique and the analyses would give a reliable taxonomy at the species level we used Zymobiomics mock community, which contains equal quantities of 8 bacterial species. The expected 16S rRNA gene content for each representative is also known, so we were able to determine if the different analysis approaches reliably represented the actual bacterial composition of the community. We sequenced the Zymobiomics mock community twice per marker gene and found that the replicates presented equivalent results.

We assigned taxonomy with three different approaches: i) Minimap2 and a database containing only the 8 bacterial species of the correspondent mock community (mock DB); ii) Minimap2 and a database containing sequences for the *rrn* operon of 22,351 different bacterial species (*rrn* DB[25]) and iii) WIMP from EPI2ME and NCBI RefSeq database.

Similarly to what we have seen for the HM-783D mock community, when using the mapping strategy with Minimap2 and the mock database, we detected that the full-length 16S rRNA gene retrieved better the actual abundances of the mock community. The 16S-ITS-23S genetic marker over- and under-represented most of the bacterial species in the mock community. When using larger databases such as *rrn* DB and NCBI RefSeq, both the full-length 16S rRNA gene and the 16S-ITS-23S were able to detect 8 out of 8 bacterial species of the Zymobiomics mock community (Figure 4A). However, we also detected other taxa that included mostly higher taxonomic rank taxa (sequences not assigned to species level), but also not expected taxa (wrongly-assigned species), especially with WIMP and NCBI RefSeq database (see Supplementary Table 3 for complete taxonomic assignments).

On one hand, the mapping approach using the *rrn* DB provided highly similar results to the reference, despite the larger size of this database (Figure 4A), especially with 16S-ITS-23S marker (99% of the reads were correctly assigned at the species level with 16S-ITS-23S; near 90% for 16S). On the other hand, with the WIMP workflow and NCBI RefSeq database, a larger number of sequences are classified as “Other taxa”. Again, this is especially remarkable when using the full-length 16S rRNA gene, with > 50% of the taxonomic assignments not hitting the expected bacterial species. The results were also confirmed by alpha diversity analyses: WIMP strategy overestimated the actual bacterial diversity, when compared to *rrn* DB and the reference (Figure 4B).

Complex microbial community analyses

We profiled two complex and uncharacterized microbial communities from dog skin (chin and dorsal). We used both long-amplicon markers and the two bioinformatics approaches –Minimap2 and *rrn* DB and WIMP with NCBI RefSeq database– to corroborate the results.

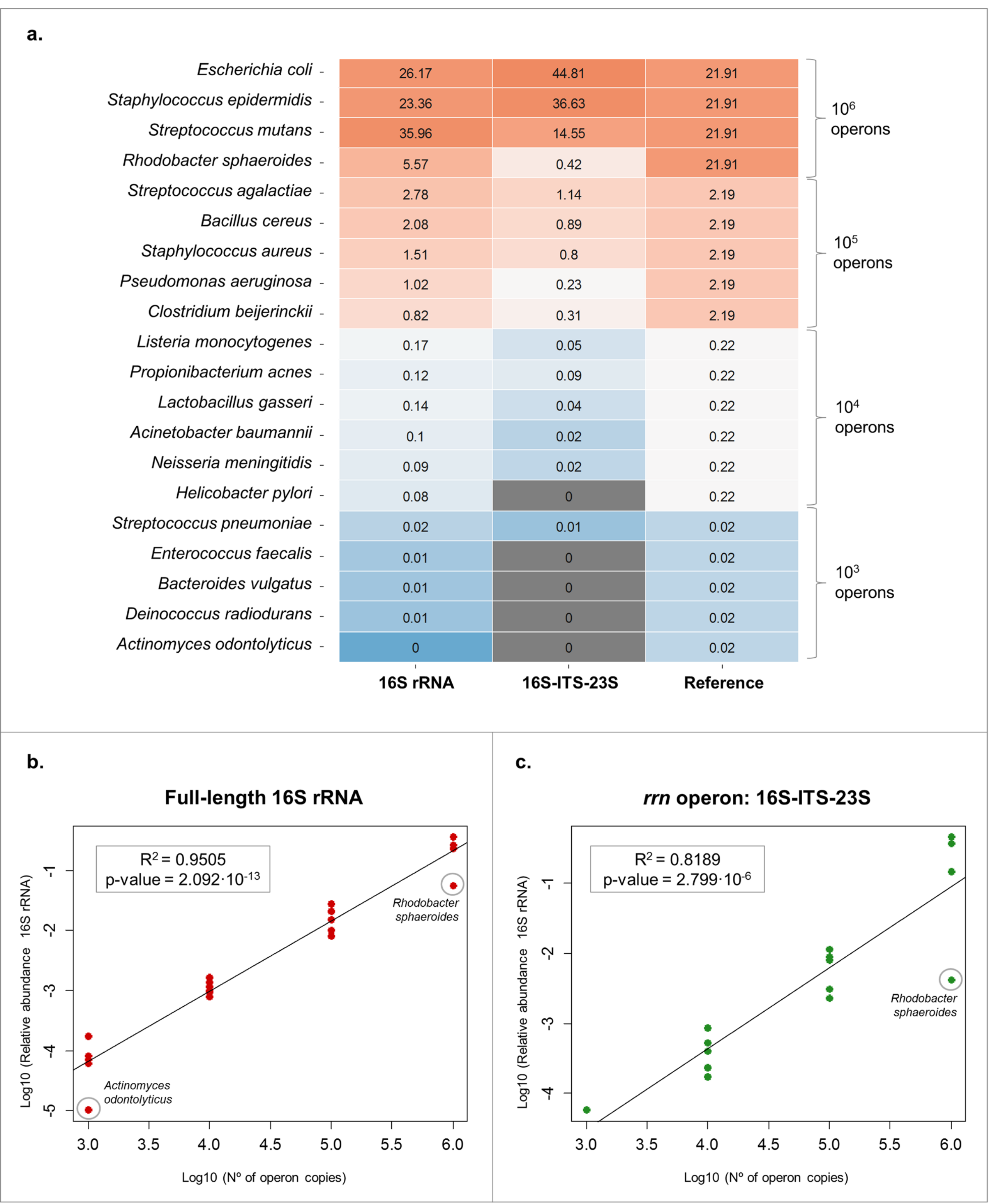
For chin samples of healthy dogs, we found a high abundance of *Pseudomonas* species (>40% of total relative abundance using 16S rRNA and >60% using 16S-ITS-23S) followed by other genus with lower abundances such as *Erwinia* and *Pantoea*. Focusing on *Pseudomonas*, at the species level we were able to detect that the most abundant species was *Pseudomonas koreensis*, followed by *Pseudomonas putida* and *Pseudomonas fluorescens* (Figure 5A and Supplementary Table 3). On the other hand, dorsal skin samples were dominated by bacteria from the genera *Stenotrophomonas*, *Sanguibacter*, and *Bacillus*. We reached species level for *Stenotrophomonas rhizophila* and *Sanguibacter keddieii*. It should be noted that *Glutamicibacter arilaitensis* is the same species as *Arthrobacter arilaitensis*, with newer nomenclature (Figure 5B and Supplementary Table 3). For both skin sample replicates, the results of the most abundant species converged using the two different methods and allowed for characterizing this complex low-biomass microbial community at the species level.

Finally, analyzing the dorsal skin samples, we also detected the presence of contamination from the previous nanopore run (Supplementary Table 2). We sequenced dorsal skin samples twice: one with a barcode previously used for sequencing the HM-783D mock community and another one with a new barcode. We were able to detect mock community representatives within the re-used barcode (Figure 5B). Some of them were found only in the sample that was using the re-used barcode (Sample_1); others were also present in the skin sample, such as *Bacillus cereus* or *Staphylococcus aureus*. In total, this contamination from the previous run was representing ~6% of the sample composition.

De novo assembly of the 16S-ITS-23S genetic marker

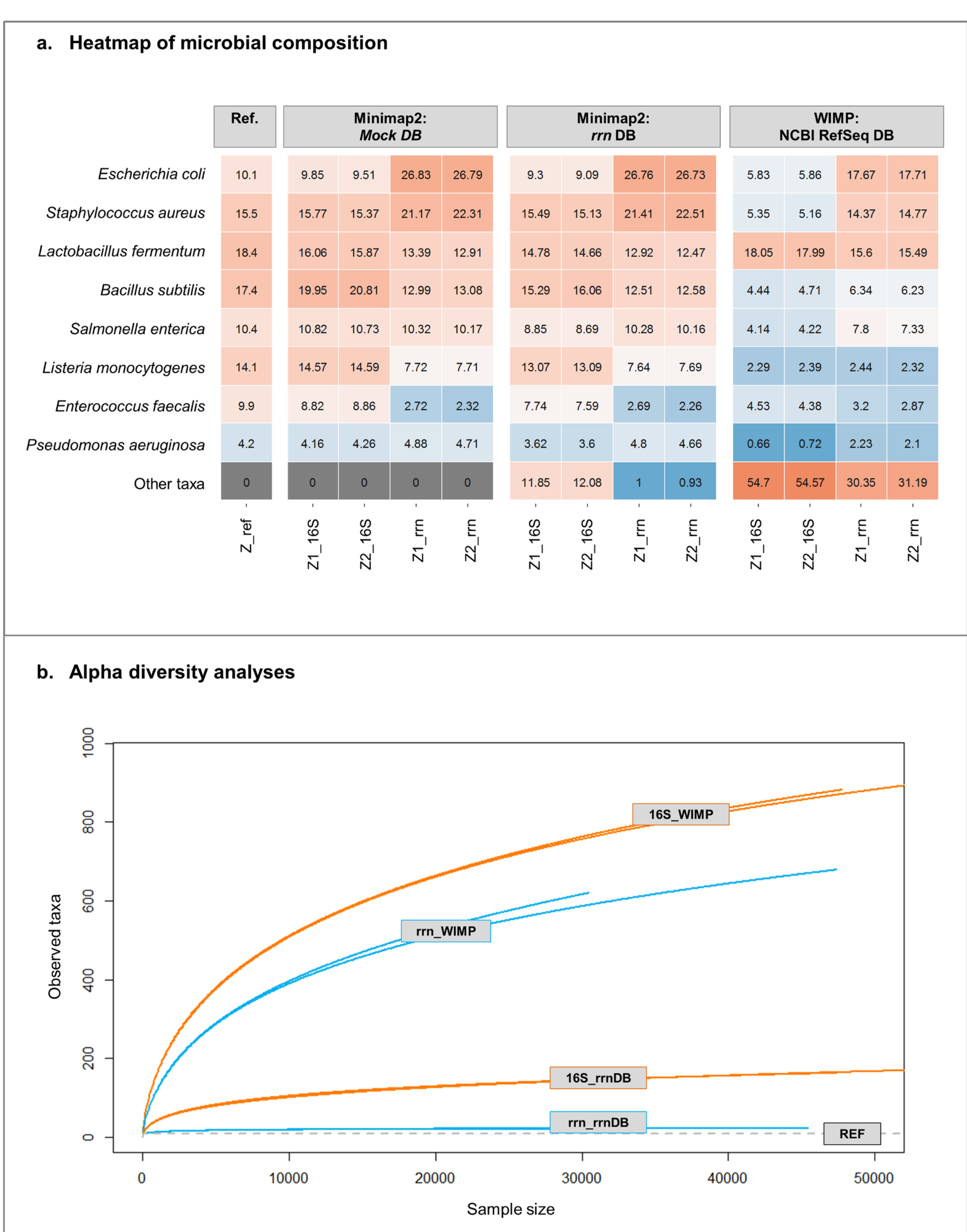
To further confirm the results obtained with the complex samples using directly the raw reads, we performed the assembly and consensus of the 16S-ITS-23S genetic marker using canu and we assigned taxonomy of the consensus sequences using BLAST.

For the HM-783D mock community, we were able to retrieve some of the most abundant bacterial species blasting with >99% of identity to their reference (*Escherichia coli*, *Staphylococcus*



	16S rRNA	16S-ITS-23S	Reference
Escherichia coli	26.17	44.81	21.91
Staphylococcus epidermidis	23.36	36.63	21.91
Streptococcus mutans	35.96	14.55	21.91
Rhodobacter sphaeroides	5.57	0.42	21.91
Streptococcus agalactiae	2.78	1.14	2.19
Bacillus cereus	2.08	0.89	2.19
Staphylococcus aureus	1.51	0.8	2.19
Pseudomonas aeruginosa	1.02	0.23	2.19
Clostridium beijerinckii	0.82	0.31	2.19
Listeria monocytogenes	0.17	0.05	0.22
Propionibacterium acnes	0.12	0.09	0.22
Lactobacillus gasseri	0.14	0.04	0.22
Acinetobacter baumannii	0.1	0.02	0.22
Neisseria meningitidis	0.09	0.02	0.22
Helicobacter pylori	0.08	0	0.22
Streptococcus pneumoniae	0.02	0.01	0.02
Enterococcus faecalis	0.01	0	0.02
Bacteroides vulgatus	0.01	0	0.02
Deinococcus radiodurans	0.01	0	0.02
Actinomyces odontolyticus	0	0	0.02

Figure 3. HM-783D mock community analysis. (**A**) Heat map representing the HM-783D mock community composition when mapped to its mock database. Grey colour represents the bacteria that were not detected (<10^4 copies with *rrn* operon). (**B**) Linear regression analysis of relative read proportions obtained using full-length 16S rRNA gene for all bacterial species present in HM-783D mock community and the actual operon copies (in log scale). (**C**) Linear regression analysis of relative read proportions obtained using the 16S-ITS-23S genetic marker for all bacterial species present in HM-783D mock community and the actual operon copies (in log scale).



	Z_ref	Mock DB Z1_16S	Mock DB Z2_16S	Mock DB Z1_rrn	Mock DB Z2_rrn	rrn DB Z1_16S	rrn DB Z2_16S	rrn DB Z1_rrn	rrn DB Z2_rrn	WIMP Z1_16S	WIMP Z2_16S	WIMP Z1_rrn	WIMP Z2_rrn
Escherichia coli	10.1	9.85	9.51	26.83	26.79	9.3	9.09	26.76	26.73	5.83	5.86	17.67	17.71
Staphylococcus aureus	15.5	15.77	15.37	21.17	22.31	15.49	15.13	21.41	22.51	5.35	5.16	14.37	14.77
Lactobacillus fermentum	18.4	16.06	15.87	13.39	12.91	14.78	14.66	12.92	12.47	18.05	17.99	15.6	15.49
Bacillus subtilis	17.4	19.95	20.81	12.99	13.08	15.29	16.06	12.51	12.58	4.44	4.71	6.34	6.23
Salmonella enterica	10.4	10.82	10.73	10.32	10.17	8.85	8.69	10.28	10.16	4.14	4.22	7.8	7.33
Listeria monocytogenes	14.1	14.57	14.59	7.72	7.71	13.07	13.09	7.64	7.69	2.29	2.39	2.44	2.32
Enterococcus faecalis	9.9	8.82	8.86	2.72	2.32	7.74	7.59	2.69	2.26	4.53	4.38	3.2	2.87
Pseudomonas aeruginosa	4.2	4.16	4.26	4.88	4.71	3.62	3.6	4.8	4.66	0.66	0.72	2.23	2.1
Other taxa	0	0	0	0	0	11.85	12.08	1	0.93	54.7	54.57	30.35	31.19

Figure 4. Zymobiomics mock community taxonomic analysis and diversity. (**A**) Heat map representing the relative abundance of the Zymobiomics mock community. "REF" column represents the theoretical composition of the mock community regarding the 16S rRNA gene content of each bacterium. (**B**) Alpha diversity rarefaction plot using observed taxa metrics.

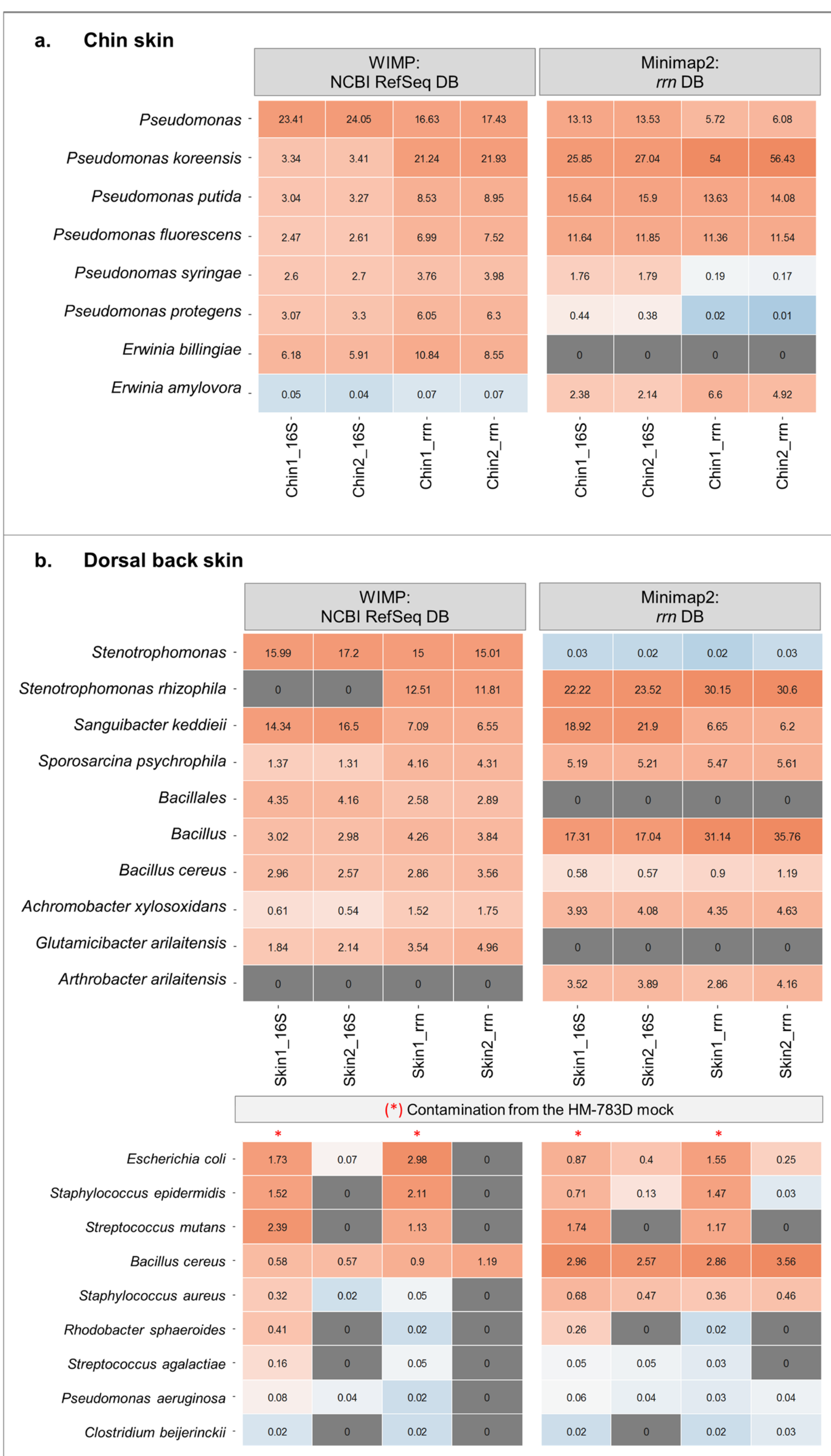


Figure 5. Microbiota composition of complex communities: skin samples of healthy dogs. (**A**) Chin samples: heat map representing the relative abundance of the main bacterial species in chin samples using WIMP and Minimap2. (**B**) Dorsal skin samples: heat map representing the relative abundance of the main bacterial species using WIMP and Minimap2. The lower heat map represents the remaining contamination from the previous run using HM-783D mock community within the same flowcell. Samples marked with a red line shared barcode with the mock community.

epidermidis, *Streptococcus mutans* and *Clostridium beijerinnkii*). For the Zymobiomics mock community, we found a consensus sequence for all the bacterial species with >99% of identity. The only two exceptions were *Salmonella enterica* that presented a consensus sequence with an identity of 98.8% and *Escherichia coli* that presented no consensus sequence (Table 2 and Supplementary Table 4).

For the complex microbial communities, the *de novo* consensus sequences usually presented lower identity than those of the mock community. For the dorsal skin, the ones with higher consensus accuracy were *Stenotrophomonas rhizophila* (99.3 %), *Streptococcus mutans* (99.1 %) and *Arthrobacter arilaitensis* (98.6 %), thus confirming the results retrieved using directly the raw reads. For the chin, the three contigs with higher consensus accuracy hit *Pseudomonas fluorescens* (>98.5%). We detected also other contigs with lower consensus accuracy values, with previously not seen taxonomic assignments (Supplementary Table 4).

Discussion

The full-length 16S rRNA and the 16S-ITS-23S of the *rrn* operon identified the bacterial isolate and revealed the microbiota composition of the mock communities and the complex skin samples, even at the genus and the species level. So, we present this long-amplicon approach as a method to profile the microbiota of low-biomass samples at deeper taxonomic levels.

The long amplicons were analyzed as raw reads (uncorrected), using both a mapping-based approach through Minimap2 and the "What's in My Pot" workflow by Oxford Nanopore to confirm results using a double approach. Although Nanopore sequencing has a high error rate (average accuracy for the *S. pseudintermedius* isolate: 89%), we compensated this low accuracy with longer fragments to assess the taxonomy of several bacterial communities. In general, the longer the marker, the higher the taxonomical resolution with both analyses performed. In this case, the longer 16S-ITS-23S marker remarkably improved the taxonomy assignment at the species level.

Moreover, we also performed de novo assembly of the 16S-ITS-23S amplicons obtaining consensus sequences that allowed us to validate some of the taxonomy retrieved with the long-amplicon raw reads. A de novo approach allowed retrieving consensus sequences with high accuracy (>99% of identity) for simple microbial communities. However, when working with more complex communities this consensus accuracy was generally lower. These lower accuracies found in complex microbial communities could be due to the lower sequencing depth, an uneven distribution of the bacterial species, and a mix of some closely similar species within the same contig.

Mock communities' analyses allowed us assessing the performance and the biases of the methodology, from the lab bench to the bioinformatics analyses and final results. In general, we found that the full-length 16S rRNA gene represents better the abundances of a microbial community; whereas, 16S-ITS-23S obtains better resolution at the species level.

So, do the long-amplicon approaches represent the actual bacterial composition? On one hand, we detected biases of our primer sets for both genetic markers, since some of the species of the mock communities were over- and under-represented. For example, *Actinomyces odontolyticus* and *Rhodobacter sphaeroides* seem to not amplify properly, neither with 16S rRNA gene, nor the 16S-ITS-23S of the *rrn* operon. Previous studies also detected the same pattern for these specific bacteria even when using or comparing different primer sets[16,21]. Overall, the 16S rRNA primer set seemed less biased than the 16S-ITS-23S of the *rrn* operon, which over- and underrepresented most of the bacterial species, suggesting that the 16S-ITS-23S primers should be improved for unbiased representation of the community.

On the other hand, with the HM-783D staggered mock community –with some low-abundant species– we aimed to assess the sensitivity of both approaches. With the 16S rRNA marker gene, we detected all bacterial members of both mock communities. However, when using the 16S-ITS-23S of the

Table 2. Zymobiomics contigs for the 16S-ITS-23S genetic marker and their taxonomic assignment through obtained through blasting.

Contig	Length	n° of reads	covStat	NCBI name	NCBI Accession	% Query coverage	e-value	% identity
tig00000001	4,346	10,900	13,568.84	*Salmonella enterica*	CP012344.2	99%	0.0	98.8%
tig00000003	3,972	2,237	15,554.25	*Bacillus subtilis*	CP002183.1	99%	0.0	99.4%
tig00000004	4,253	7,519	17,430.61	*Staphylococcus aureus*	CP029663.1	99%	0.0	99.1%
tig00000007	4,188	1,588	19,724.96	*Pseudomonas aeruginosa*	CP032257.1	100%	0.0	99.5%
tig00000009	4,061	645	18,811.23	*Enterococcus faecalis*	CP025021.1	100%	0.0	99.1%
tig00000010	4,064	1,107	17,360.31	*Listeria monocytogenes*	CP035187.1	99%	0.0	99.2%
tig00000012	4,019	1,347	16,386.90	*Lactobacillus fermentum*	CP034099.1	100%	0.0	99.4%

rrn operon, some of the low-abundant species in the HM-783D mock community were not detected. This was probably due to the fact that we obtained a lower number of reads –up to one magnitude less than with the 16S rRNA gene. Since we combined 16S rRNA and 16S-ITS-23S amplicons in the same run, this led to an underrepresentation of the 16S-ITS-23S amplicons and consequently a lower sequencing depth. This was probably due to the combination of various issues: i) not enough DNA mass to begin with the indicated number of molecules; ii) reads with shorter size than expected (~1,500 bp); iii) shorter fragments tend to be sequenced preferentially with Nanopore sequencing. Thus, for future studies our recommendation would be multiplexing samples with the same amplicon size to avoid underrepresentation of the longest one and improving PCR parameters or adding more PCR cycles to the longer amplicons to get more input DNA mass.

In the bioinformatics analyses, our aim was to confirm the results with two independent workflows and different databases rather than comparing them. We saw that the most abundant species were usually concordant with both strategies at a qualitative level. Some exceptions were due to the lack of that species in the *rrn* database, such as that seen for *S. pseudintermedius*. With the WIMP workflow and the 16S rRNA gene, many sequences did not reach species level. Previous studies analyzing the microbiota obtained with Nanopore reads have compared the performance of several databases using the 16S-ITS-23S[26] and software for the 16S rRNA gene[22]. They also found similar results as reported here: some false positives associated to specific software[22], as well as a high impact on the unclassified reads depending on the size of the database used[26].

When using EPI2ME (WIMP with NCBI Ref database), the amplicons from the *S. pseudintermedius* isolate were assigned to the correct bacterial species in ~98% and ~68% of the cases, using the 16S-ITS-23S of the *rrn* operon and 16S rRNA gene, respectively. In a previous study, Moon and collaborators used the full-length 16S rRNA gene for characterizing an isolate of *Campylobacter fetus* and the marker correctly assigned the species for ~89% of the sequences using EPI2ME[23]. The ratio of success on the correct assignment at the species level depends on the species itself and its degree of sequence similarity in the selected genetic marker. Within the *Staphylococcus* genus, the 16S rRNA gene presents the highest similarity (around ~97%) when compared to other genetic markers[37].

On the other hand, we observed that the mapping strategy (through Minimap2) could lead to a wrong assigned species if the interrogated bacterium is not represented on the chosen database. Minimap2 provides faster results than EPI2ME, but it needs an accurate comprehensive and representative database. Extra filtering steps using the alignment block length or Smith-Waterman alignment score could potentially be used to discard a wrong taxonomic assignment.

Switching to complex microbial communities, we found that dog chin was colonized by different *Pseudomonas* species. Recently, Meason-Smith and collaborators found *Pseudomonas* species associated with malodor in bloodhound dogs[38]. However, these were not the main bacteria found within the skin site tested, but were in low abundance, differing from what we have found here. On the other hand, Riggio and collaborators detected *Pseudomonas* as one of the main genera in canine oral microbiota in the normal, gingivitis and periodontitis groups[39]. However, the *Pseudomonas* species were not the same that we have detected here. It should be noted that we had characterized these chin samples with 16S V1-V2 amplicons in a previous study[27], where we found some mutual exclusion patterns for *Pseudomonadaceae* family. This taxon showed an apparent "invasive pattern", which could be mainly explained for the recent contact of the dog with an environmental source that contained larger bacterial loads before sampling[27]. Thus, our main hypothesis is that the *Pseudomonas* species detected on dog chin came from the environment, since they have been previously isolated from environments such as soil or water sources[40,41].

None of the most abundant species in dog dorsal skin had previously been associated with healthy skin microbiota either in human or in dogs. Some of them have an environmental origin, such as *Stenotrophomonas rhizophila*, which is mainly associated with plants[42]; or *Sporosarcina psychrophila,* which is widely distributed in terrestrial and aquatic environments[43]. The *Bacillus cereus* main reservoir is also the soil, although it can be a commensal of root plants and guts of insects, and can also be a pathogen for insects and mammals[44]. Overall, environmental-associated bacteria have already been associated with dog skin microbiota and are to be expected, since dogs constantly interact with the environment[27].

Regarding *Stenotrophomonas* in human microbiota studies, Flores *et al*. found that this genus was enriched in atopic dermatitis patients that were responders to emollient treatment[45]. However, previous studies on this skin disease found *Stenotrophomonas maltophila* associated to the disease rather than *Stenotrophomonas rhizophila*[46]. *Achromobacter xylosoxidans* has been mainly associated with different kind of infections, as well as skin and soft tissue infections in humans[47]. However, both dogs included in this pool were healthy and with representatives of both genus/species, a fact that reinforces the need to study the healthy skin microbiome at the species level before considering some species pathogenic. The other abundant bacteria detected on dog skin have been isolated in very different scenarios: *Sanguibacter keddieii* from cow milk and blood[48,49]; and *Glutamicibacter arilaitensis* (formerly *Arthrobacter arilaitensis*) is commonly isolated in cheese surfaces[50,51].

In general, we obtained taxonomy assignment down to species level with both the full-length 16S rRNA gene and the 16S-ITS-23S of the *rrn* operon, although it was not always feasible due to: i) high similarity of the marker chosen within some genera, especially for the 16S rRNA gene; ii) an incomplete database; and iii) sequencing errors. In the light of these results, for an increased resolution at the species level, the 16S-ITS-23S of the *rrn* operon would be the best choice. At the expenses of an increased taxonomic resolution, we could have missed few bacterial species due to unlinked *rrn* genes. While in host-associated environments (e.g. gut) bacterial species with unlinked

rrn genes are rare, if profiling natural environments (e.g. soil) this approach may be missing a significant proportion of the diversity[52]. So far, this genetic marker does not have as many complete and curated databases as 16S rRNA gene. If choosing to use 16S-ITS-23S genetic marker, we could add some filtering steps to filter out the "wrongly assigned taxonomy" and have more reliable taxonomic results, both using the alignment block length and the AS score using Minimap2.

Other gene-marker strategies have been further described to profile the microbiota with Nanopore sequencing. For example, sequencing 16S rRNA genes by Intramolecular-ligated Nanopore Consensus (INC-seq)[15,53] that allowed retrieving corrected full-length 16S rRNA genes. Another approach would be sequencing the cDNA from size selected small subunit rRNAs that allows retrieving many 16S rRNA genes using a primer-free approach[54]. However, these alternative strategies have been applied to the 16S rRNA gene that has a limited taxonomic resolution within some genera. Recently an approach using unique molecular identifiers (UMIs) for obtaining corrected full-length *rrn* operon has been applied to characterize a mock community[55]. The characterization of full-length ribosomal operons by the UMI approach has the potential to expand databases to make them more comprehensive with higher taxonomic resolution.

Further studies should be aiming to obtain reads with higher accuracy, either using consensus methods or applying new developments (new techniques, new basecallers or new R10 pores, etc.). Studies comparing marker-based strategies with metagenomics will determine the most accurate marker for microbiota studies in low-biomass samples.

Data availability

Underlying data

The datasets analyzed during the current study are available in the NCBI Sequence Read Archive, under the Bioproject accession number PRJNA495486.

Extended data

All the supplementary data has been added in an OSF repository (doi: http://doi.org/10.17605/OSF.IO/8MYKV)[22]. We provide here a complete list:

- Supplementary Table 1. Primer sequences for amplifying the full-length 16S rRNA gene and 16S-ITS-23S of the *rrn* operon.

- Supplementary Table 2. Samples included in the study, run summary and quality control results. *For mock communities, the mock DB was used. For complex communities, the *rrn* DB.

- Supplementary Table 3. Taxonomic assignments table of each sample with the different approaches.

- Supplementary Table 4. De novo results obtained with canu and their taxonomic assignment using BLAST.

- Supplementary Figure 1. Photo of the agarose gel electrophoresis of some of the samples.

- Supplementary Figure 2. Main workflow overview. Detailed bioinformatics workflow can be found in Supplementary File 1.

- Supplementary File 1. Bioinformatics workflow used for the mapping approach.

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Reviewer Report 21 February 2019

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Lee J. Kerkhof
Department of Marine and Coastal Sciences, Rutgers University, New Brunswick, NJ, USA

F1000 Research Cuscó et al. (https://doi.org/10.12688/f1000research.16817.1)

Comments to the authors:

The manuscript describes a study assaying 2 mock bacterial communities or 2 complex skin microbiome samples from dogs (chin or dorsal back) using both near-full length 16S rRNA genes and near-full length rRNA operons with the Oxford Nanopore MinION. The authors employ a library preparation method generating either 16S amplicons (1400 bp) or rRNA operons (4500 bp) including barcoding with a 1D ligation/sequencing kit and FLO-MIN 106 cells. The data analysis pipeline utilized Albacore basecalling, near-full length amplicon size selection, and screening by What's in my Pot (WIMP) and Minimap2 against both NCBI and rrn databases. The authors demonstrate increased resolution at the species level with longer reads, that there can be large losses of raw sequence reads by size selection for rrn amplicons in their hands, and that the data analysis software and database can influence the results of MinION bacterial community analysis.

It would have been very helpful for the authors to put these findings into context with other papers in the literature using MinION and rRNA genes. For example, their results support what others directly sequencing near-full length16S amplicons (e.g. Shin *et al.* (2016[1]), Mitsuhashi *et al.* (2017[2]), and Benitez-Paez *et al.* (2016[3])) or rRNA operons (e.g. Benitez-Paez *et al.* (2017[4]), Kerkhof *et al.* (2017[5])) have shown in mock communities or complex samples with respect to species-level resolution. Additionally, the screening of MinION reads with different 16S rRNA databases has also been described in the supplementary figures of Kerkhof *et al.* (2017[5]). Likewise, an acknowledgement of the various software packages that has been employed to analyze the MinION reads in the scientific literature would benefit the readership. It appears that QIIME, BLASTN, Centrifuge, LAST aligner, Discontinuous MegaBLAST, WIMP, and MiniMap2 have all been used to identify OTUs for the MinION platform for 16S rRNA genes or rrn operons. As the authors have shown, the software/database being used can be very influential in the results of MinION screens and a synopsis of what they have found in context with other investigators (% bacterial assignment vs. % error) may point to a best practice for future studies.

Other Specific Comments:

1. **Page 3:** I find it awkward/confusing to indicate the number of operons per microorganism per microliter here for the mock communities. Bacteria generally have 1-15 ribosomal operons in their genomes. I think it is clearer to just indicate the number of target rRNA operons is 10^3-10^6 for this particular DNA mixture.

2. **Page 3:** The barcoding expansion pack (EXP-PBC001) requires that the primers contain overhangs attached to the rRNA primers. This is not mentioned by the authors. Did they put overhangs on 27F/1492R/2241R? If so, the first round of target amplification may be affected by the presence of these overhangs. This should be indicated.

3. **Page 4:** The authors clearly show the danger of performing PCR and only characterizing the amplification product by Qubit fluorescence. If they had done agarose gels on the PCR reactions, they may have detected the short amplification products in their initial rrn operon reactions. Furthermore, these short reads are preferentially ligated using the SQK-LSK108 sequencing kit since there are more picomole ends. This best practice of visualizing PCR amplifications for size determinations before sequencing should be explicitly stated.

4. **Page 4:** I am a little confused by the 0.5 nM notation for PCR product in the barcoding reaction. If the authors used 50 microliter reactions, did they put 25 ng of 1st round PCR product in their barcoding reactions for a 15 cycle amplification? Can the authors just state the mass of DNA used to barcode? Secondly, Table 2 indicates BC1, BC2, and BC3 were not used. Was there a reason these barcodes were not utilized?

5. **Page 6:** Stating that the rrn operon profiling was more biased probably because of the lower sequencing depth does not recognize that others have not reported comparable bias or that it is probably a reflection of their potentially compromised amplification efficiencies. This conclusion should be viewed with caution, given the amplification issues noted above.

6. **Page 11:** The running of shorter (1500 bp) and longer (4500 bp) libraries on the same flow cell at the same time should enrich for the shorter reads. The MinION uses electrophoresis to move DNA molecules through the pores and smaller fragments should mobilize easier.

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular ecology of microbial systems

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 18 February 2019

https://doi.org/10.5256/f1000research.18384.r43564

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Kon Chu
Department of Neurology, Seoul National University, Seoul, South Korea

The study compared the results of microbiota profiling using two different markers (16S rRNA and the rrn operon) and different classification methods. Because other reviewers have already made comprehensive reviews and comments including several critical points, I would like to add only a few minor points to the manuscript:

1. Figure 2: according to the text, *Actinomyces odontolyticus* was detected using the 16S rRNA gene, however, '0' in the figure can create confusion. It would be better to represent the number of copies of *Actinomyces odontolyticus* using more decimal places or adding a caption for this species.

2. Figure 3a:

   - It would be better to change the figure (e.g. heatmap) to make it easier for readers to recognize under-represented and over-represented bacteria. *Listeria monocytogenes* also seems under-represented in the analyses using the mock database and rrn database, and the corresponding sentence in Page 7 may be changed.

   - Include the classification method (WIMP, minimap2) along with the name of the database, as in figure 4, to allow general readers to more easily match the methods and the database.

3. In the last paragraph of page 7, it seems that the criteria of the percentage of wrongly assigned species for the rrn operon are different from that for the 16S rRNA gene.

4. Table 3: I suggest making a caption for the difference between 'Staphylococcus' and 'Other Staphylococcus'.

5. If the authors would like to insist on better resolution by using the rrn operon, they need to demonstrate the data of the analysis using multiple species including species that tend to be under-represented or over-represented.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: neuroinfection, encephalitis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 08 February 2019

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Amanda Warr
Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, UK

Cusco *et al.* evaluate methods for long read sequencing and classification of marker genes from microbial communities, both for mock communities of known microbial composition and complex communities from dog skin from two anatomical locations, chin and back. They find that long read sequencing of 16S and the *rrn* operon is sufficiently accurate to classify microbes and that *rrn* is more sensitive at the species level.

This work demonstrates a valuable option for species identification from microbial samples using long reads, overcoming the current high error rate through covering a larger region. The work also highlights some of the issues that can arise from multiplexing amplicons of differing lengths when using Nanopore sequencing.

Overall the paper is well written and detailed, however there are a few details I feel could be addressed:

1. **16S length reads in *rrn* barcodes:** Do you expect this to be entirely from barcode misassignment or were these shorter fragments produced during PCR? You state that the loss of *rrn* amplicons during the length trimming step was probably due to over-representation of 16S amplicon on the flow cell, and most of the reads lost were roughly 16S amplicon sized - are you suggesting that there are large numbers of 16S reads that are assigned to *rrn* barcodes after 2 rounds of demultiplexing? Are these shorter reads actually whole 16S amplicons or fragments of *rrn*?

2. **Expected sensitivity given read count:** The authors state that failure to detect the less abundant species from the mock community in the *rrn* dataset was "probably" due to their being fewer reads. As the proportions of the species in the mock samples are known, theoretically what total number of reads would be necessary to detect the less abundant species? Given the number of rrn reads obtained, did the authors detect as many species as they would expect to detect and what is the minimum total number of reads they would need to be likely to detect the lowest abundance species?

3. **Differences in classification methods:** Differences in classifications between the mock community database/rrn database and the NCBI database may be attributable to differences in the tools, with minimap2 being used for the mock and *rrn* databases and WIMP (based on centrifuge) being used for the NCBI database. My understanding is that the authors are mainly interested in

classifications from different databases rather than differences between methods. While the authors do not directly compare the classification results between these different methods in text, some of the figures appear to imply that these results are directly comparable (e.g. Figure 3a). It would be useful if either all three databases were used with a single method (for example, using centrifuge with all three databases) or if these were at least more obviously separated or marked as coming from different classification methods in the figures.

4. **Classification rates against NCBI:** The authors should further discuss ways to improve the classification rates, will the biggest improvements come from reduced error rate, better classification tools, improving species representation in databases? The authors conclude that in the future we should aim to improve accuracy, but one of the main results here is that sequencing the full 16S/*rrn* overcomes the problem of the current error rate - perhaps highlight benefits such as improved barcode assignment and emphasise that while this works well classification against a large database would likely improve with increased accuracy. The authors also conclude that *rrn* offers higher resolution at species level, however I suspect that currently more species have 16S sequences in databases than *rrn*.

Additionally, I have a few minor corrections mainly around small grammatical errors and figure/table modifications:

- Page 5: Paragraph beginning "To assign taxonomy...", change "to strategies" to "two strategies". Also I would change the last sentence on the page to say "some of the reads excluded were the expected length of the 16S rRNA gene rather than the *rrn* operon". Figure 1 should also be labelling Albacore as the basecaller.
- Page 6: change "would allow us determining" to "would allow us to determine".
- Page 11, column 2, line 2: change "associated to" to "associated with".
- Figure 3a would benefit from separating the reference bar from the other bars or adding this bar to the other two plots (currently it is grouped with Mock database, but it is also relevant to the *rrn* database and the NCBI database).
- Figure 4 text is quite difficult to read.
- Table 2: the title of the final column isn't clear. Is this the % of reads that pass the quality filters before chimera detection? Could another column be added showing number of reads that pass this filter?
- Figure 5: there are several different colours of 0 in this heat map?

In the conclusion the authors have suggested ways to improve accuracy of this method in the future, I would add the R2C2 method (Volden *et al.*, 2018[1]) as an option to improve consensus accuracy here also, while designed for cDNA it could be applied to fragments of genomic DNA.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Genomics, long read sequencing, microbiome assembly

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 04 February 2019

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Rasmus H. Kirkegaard
Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University (AAU), Aalborg, Denmark

Title:
"Microbiota profiling with long amplicons using Nanopore sequencing: full-length 16S rRNA gene and whole rrn operon".

Summary of the key results:
The study demonstrates the use of nanopore sequencing for characterising low biomass samples with high levels of host DNA using a primer-based approach targeting the entire 16S rRNA gene or the 16S rRNA gene and the 23S rRNA gene.

Furthermore, it evaluates the ability of these methods in the context of known references using mock communities and a pure culture using both the WIMP software and a custom mapping-based approach.

The study demonstrates that nanopore sequencing can give accurate classifications even at the current level of error rate if the reference database contains the right sequences. The study also shows how sequencing the longer fragment spanning both the 16S rRNA and 23S rRNA genes improves the taxonomic classification when the database contains a matching sequence.

Is the work clearly and accurately presented and does it cite the current literature?
The study mentions that the classification methods rely heavily on reference databases so it would be relevant to include citations for papers with methods for producing new reference sequences for both 16S rRNA and the longer fragment in the discussion (metagenomics, artificial long reads, primer free methods). Methods for improving read accuracy are also mentioned as important but the only methods mentioned are future upgrades from the company, relevant existing literature is not included (INC-seq, UMIs etc.). The study concludes that sequencing the entire “rrn operon” would be the best choice but it would be relevant to compare the size of current databases for the 16S rRNA gene versus the rrn operon. The presence of conserved sites for designing better primers is also extremely important but not discussed. Furthermore, there is evidence that quite a few organisms have unlinked rRNA genes, which will thus be missed by a full operon approach.

Citations are also needed for bioinformatics tools for both processing and visualisation of the data.

Is the study design appropriate and is the work technically sound?
The study uses mapping to a reference database to point out that the sequences can get genus- and species-level classification. However, the method will always report a genus and a species even in the absence of the correct sequence in the reference database as indicated from the sequencing of the *S. pseudintermedius* pure culture with the “rrn” method. It will be important to simulate the impact on the results when there is no closely related sequences in the database. This could be done by removing all reference sequences within the Gammaproteobacteria and mapping the HM-783D to the modified database and monitor where the reads end up. It would also be helpful if there was a way to distinguish between reads that have the “correct” match and reads that just happen to map because the 16S rRNA gene is extremely conserved. Something similar would be relevant for the EPI2ME workflow but as the authors cannot control the reference database, it is probably not feasible. One of the advantages of the mock communities should be information about the copy numbers for the rRNA genes but there is no information on this included in the study and how it affects the results.

Are sufficient details of methods and analysis provided to allow replication by others?
The methods section lacks information about what happens after mapping the reads. How are the figures generated, what software is used, etc.? It would also be helpful if the specific scripts/commands used to run the bioinformatics analysis were available.

Figures:

Figure 1: bioinformatic workflow:
The figure gives a decent overview of the bioinformatics processing but seems to miss the visualisation tools used. The main role of Albacore is basecalling the raw data not just demultiplexing. The figure could be improved further if you include the wet lab part of the work, so it becomes clear why the demultiplexing step is included and where the raw data comes from. A mapping step is integrated in the chimera detection (removal?) workflow but it might be better to omit mentioning mapping in that step as it can be confusing that the figure has two mapping steps in a row.

Figure 2: heatmap mock community:
The caption needs to explain what the numbers represent e.g. percentage of sequenced reads/mapped reads. It would be great if the heatmap included the "true" composition of the mock community for comparison. Copy number for each organism in the mock would also be relevant to include in the figure. Since there are only two columns, it would be better to have the sample labels at the top and with horizontal text preferably with a name that makes it easier to interpret the figure.

Figure 3a: stacked bar chart:
Even though stacked bar charts are very common it is not easy to read as they lack a common baseline for most of the values (See https://solomonmg.github.io/blog/2014/when-to-use-stacked-barcharts/ and https://peltiertech.com/stacked-bar-chart-alternatives/). I suggest that you use more of the heatmaps instead of introducing bar charts.

Figure 3b: rarefaction curves:
It would be great if you could add a dashed line for the expected "true" value for the mock community.

Figure 3c: WIMP tree:
This figure is quite complex to read. If the point with running both WIMP and a mapping-based approach with the two different amplicon types is to compare the methods, I suggest that you try to integrate the information better into one combined figure. This way you can help the reader to understand your message.

Figure 4a: stacked bar chart+heatmap dog samples:
Remove the stacked bar chart.

Figure 4b: stacked bar chart+heatmap dog samples:
Remove the stacked bar chart.

Getting rid of the bar charts would allow for making a big heatmap with the data from Figure 4A and 4B combined. This way the reader can also compare the results from the two different sample sites. A naming system that makes it clearer that "_1" and "_2" are replicates would also help the reader interpret the figure. Presenting results aggregated at different levels, which could be included in one another is a bit confusing e.g., "*Bacillus cereus*" could be included in "*Bacillus*" which again could be included in "*Bacillales*".

Figure 5: heatmap mock community contamination:
It is confusing that several cells in the heatmap have a value of "0" but with very different colours. Adding some meaningful labels with the contamination vs no contamination on the top could help the reader understand the figure without reading the caption.

Tables:

Table 1: Primer sequences:
Fine but could be moved to supplementary.

Table 2: Samples and QC:
Make headers easier to understand e.g. "% seq 1st QC" could be "% of reads passing QC", "Albacore pass" could just be "# reads after basecalling" etc. Where is the number after chimera detection?

Add a column with data accession ID and move the table to supplementary then the sample names can also be expanded so the reader does not have to look to the bottom for an explanation of abbreviations. I suggest adding a column at the end with the number of reads mapping/classified for each sample so the reader know what fraction is included.

Table 3: Pure culture comparison WIMP vs. mapping:
You need to make it clear in the table that *Staphylococcus pseudintermedius* is missing from the "rrn" database. As the paper mentions genus- and species-level classification as the target you may benefit from aggregating the values for *S. pseudintermedius* and *S. pseudintermedius* HKU10-03 as splitting this into strains makes it more confusing as your numbers in the text do not match the ones in the table.

Supplementary Table 1:
It would be great to include the mock communities in this table as well.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: I am a co-owner of DNASense ApS (www.dnasense.com)

Reviewer Expertise: microbial biotechnology, nanopore sequencing

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 November 2018

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Alfonso Benítez-Páez

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Cusco and co-workers present an evaluation of both a mock community and the dog skin associated microbiota. The authors made use of the single-molecule Nanopore DNA sequencing technology and compared two different technical approaches by studying the nearly-full 16S rRNA bacterial gene and the nearly-full bacterial rRNA operon.

In my opinion, this work represents an important advance regarding the application of nanopore technology in the field of microbiome research.

The main strength of the work is its detailed technical description regarding the protocols for library preparation, sequencing and basecalling, that altogether facilitate the reproducibility. Moreover, the genetic data generated was properly deposited in a specialized database for public accession to whomever may want to replicate the analysis of long reads by similar approaches or new ones.

The figure quality is good and the information disclosed by them is well accompanied with appropriate captions.

Notwithstanding, I have some minor concerns about the work that should be clarified, at least for me:

1. The last paragraph of page 7 describes the level of reads correctly assigned to species level for the microbial isolate *Staphylococcus pseudintermedius*. However, some of the values cited in the text do not match, at least, explicitly in Table 3. So, the authors should revise this issue or better describe the information obtained.

2. The authors found that the study of a nearly-full 16S rRNA gene reflects in a better way the expected abundances of microbial species present in the mock community tested. This comparative analysis with regard to the results obtained by using the rrn operon should be accompanied by a linear regression analysis, declaring respective Pearson's "r" coefficients, that can measure more accurately the efficiency of both methods and better support the authors' observations and conclusions.

3. Additionally to the observed richness (observed species) and Shannon diversity, the authors could also include a microbial community evenness evaluation of reference and observed microbiome data from the different approaches evaluated in the study, so that additional conclusions could be addressed.

4. Given the issues with underrepresentation of "rrn" data as a consequence of mixing this type of synthetic DNA with nearly-full 16S rRNA amplicons, the authors should highlight this observation as a major issue of this approach and state a clear recommendation to avoid this type of multiplexing for future studies.

5. It is necessary to better describe the contamination issues described in the last paragraph of the results (page 9). I'm not sure if this cross-contamination came from re-utilization of a flowcell or if this came from contamination of the barcoded-primer, used during nested PCR, with

amplicons/DNA from the mock community. In a similar manner, the estimation of 6% of contamination has to be explained in detail (species/proportions discarded or having been taken into account to calculate this percentage).

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Human microbiome, Microbial genomics, Nanopore sequencing, Computational biology, Metagenomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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5 Manuscript draft 5

Short-communication: Milk microbiota profiling on water buffalo with full-length 16S rRNA using Nanopore sequencing

Short-communication: Milk microbiota profiling on water buffalo with full-length 16S rRNA using Nanopore sequencing

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Keywords: Milk microbiota, Water buffalo, Nanopore sequencing

Abstract

The identification of milk microbial communities in ruminants is relevant for understanding the association between milk microbiota and health status. The most common approach for studying the microbiota is amplifying and sequencing specific hypervariable regions of the 16S rRNA gene using massive sequencing techniques. However, the taxonomic resolution is limited to family and, in some cases, genus level. We aimed to improve taxonomic classification of the water buffalo milk microbiota by amplifying and sequencing the full-length 16S rRNA gene (1,500 bp) using Nanopore sequencing (single-molecule sequencing). When comparing with short-read results, we improved the taxonomic classification, reaching species level. We identified the main microbial agents of subclinical mastitis at the species level that were in accordance with the microbiological culture results. These results confirm the potential of single-molecule sequencing for in depth analysis of microbial population in dairy animals.

38 Unravelling microbiota population is of pivotal importance in veterinary and animal
39 sciences. The most common method used is targeted sequencing of a specific region
40 of the 16S rRNA gene. Nevertheless, no consensus about the "best practices" for 16S
41 microbiome studies has been agreed so far (Pollock et al., 2018). For low-biomass
42 samples, such as milk, targeted 16S rRNA gene sequencing approach is one of the
43 best approaches. However, the short length of the targeted 16S regions represents a
44 limitation for identifying taxa below genus level (Gao et al., 2017).
45 Milk microbiota in ruminants has already been investigated through massive
46 sequencing methods in cows and water buffaloes (McInnis et al., 2015; Oikonomou et
47 al., 2012; Quigley et al., 2013, Catozzi et al., 2017). The association between milk
48 microbiota and health status is particularly relevant for understanding mastitis disease,
49 by unravelling previously unreported microorganisms potentially related to mammary
50 gland pathogenesis (Hoque et al., 2019) and assessing the milk microbiota recovery
51 after treatment (Catozzi et al., 2019). Microbiological culture technique is considered
52 the gold standard for identifying pathogens in mastitis; however, bacteria are also
53 present in culture-negative samples collected from animals with clinical mastitis
54 (Kuehn et al., 2013). The change in milk microbiota during mastitis and lactation
55 stage has already been demonstrated in cow (Doyle et al., 2017; Lima et al., 2018),
56 goat (McInnis et al., 2015) and water buffalo (Catozzi et al., 2017). Given the limited
57 taxonomic resolution of sequencing a short fragment of the 16S rRNA gene, a full-
58 length 16S rRNA gene sequencing by single-molecule sequencers may represent the
59 strategy to reach species level identification and improve the identification of milk
60 microbiota from healthy and subclinical or clinical mastitis affected quarters. The
61 Oxford Nanopore Technologies MinION^{TM} is a portable sequencer, able to generate
62 long reads in real-time, allowing the direct characterization of the sample. The full

63 length 16S sequencing using MinION^{TM} has already been tested on mock
64 communities (Cusco et al., 2018; Li et al. 2016; Benítez-Páez et al., 2016), pure
65 microbiological culture (Cusco et al., 2018) and complex communities such as dog
66 skin (Cusco et al., 2018, 2017), mouse (Shin et al., 2016) and infant gut (Leggett et
67 al., 2017). The aim of this experiment was to explore the use of the full-length 16S
68 rRNA gene sequencing by MinION^{TM} from Oxford Nanopore Technology (ONT) as a
69 potential approach to improve the taxonomic classification of the bacteria present in
70 water buffalo milk quarters as compared to short-read sequencing analysis.
71 Twelve milk samples were included in this study (see Supplementary file 1 for
72 associated metadata). Six samples were collected from healthy quarters defined by
73 absence of clinical symptoms, negative microbiological culture and Somatic Cell
74 Count (SCC) < 200,000 cells/ml (milk samples 1, 2, 3, 9, 10, 12) and six from
75 subclinical mastitis quarters characterized by the absence of clinical symptoms and
76 positive microbiological culture (milk samples 4, 5, 6, 7, 8, 11). Milk collection,
77 microbiological culture and SCC analysis were performed as previously described
78 (Catozzi et al., 2017). Briefly, teat ends have been with a 2% povidone-iodine
79 (Betadine Solution) and the first three strains of milk were discarded in order to avoid
80 contamination. Milk samples were collected in sterile containers immediately
81 refrigerated and delivered to the laboratory where they were aliquoted. SCC was
82 measured in milk samples using Fossomatic (Foss) apparatus by means of the UNI
83 EN ISO 13366±2: 2007 technique for electronic optical fluorometric counters (Hoque
84 et al., 2019). The microbiological culture was applied for each samples: 10 ul of milk
85 were cultured using different media, including the incubation at 37˚ for 24h in aerobic
86 conditions on Trypticase soy (with 5% sheep blood), MacConkey and Baird Parker
87 agar, at 37˚C for 72h in aerobic conditions on Prototheca isolation medium and at 37˚

88 in microaerobic conditions on Mycoplasma agar. Gram staining, coagulase and
89 oxidase tests were performed on cultures with mastitis pathogens.
90 DNA extraction was carried out and quality assessed as previously reported starting
91 from 1 ml of milk (Catozzi et al., 2017). On one hand, V4-16S amplification (about
92 250 bp) was performed using 515F and R806 primers, as already mentioned (Catozzi
93 et al., 2019). Briefly, The forward and reverse primer were 5'–
94 CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNNNNNNNGAT
95 GTGYCAGCMGCCGCGGTAA– 3' (composed of the adapter linker, the key, the
96 barcode, different for each sample, and the forward primer 515F) and 5'–
97 CCTCTCTATGGGCAGTCGGTGATGGACTACNVGGGTWTCTAAT– 3'
98 (composed of the adapter linker and the R806 reverse primer), respectively. PCR was
99 carried out using the Thermo Scientific Phusion Hot Start II High-Fidelity DNA
100 polymerase kit; each PCR reaction contained RNAse and DNAse free water, 5x
101 Phusion Buffer HF (5 µl), dNTPs 2mM (2.5 µl), Primer Fw 10µM (1.25 µl), Primer
102 Rv 10µM (1.75 µl), Phusion High Fidelity Taq Polymerase 2 U/µl (0.25 µl) and 5 ng
103 of DNA (or 5 µl when the concentration was too low). The thermal profile consisted
104 of an initial denaturation of 30 sec at 98˚C, followed by 32 cycles of 15 sec at ˚98 C,
105 15 sec at 50˚C, 20 sec at 72˚C, followed by a final extension of 7 min at 72˚C. Each
106 PCR plate included a negative template control. The amplicons were sequenced using
107 massive sequencing (Ion Torrent Personal Genome Machine) as previously reported
108 (Catozzi et al., 2019). In addition to the V4-16S approach using massive sequencing,
109 the full-length 16S rRNA gene (about 1,500 bp) approach was performed using
110 single-molecule sequencing. A two-steps PCR was carried out as previously described
111 (Cusco et al., 2018). Briefly, the forward primer 5' –
112 TTTCTGTTGGTGCTGATATTGC**AGRGTTTGATYHTGGCTCAG** – 3',

113 composed of the ONT Universal Tag and the 16S-27F primer and the reverse primer
114 5' – ACTTGCCTGTCGCTCTATCTTC**TACCTTGTTAYGACTT** – 3', composed
115 of the ONT Universal Tag and the 16S-1492R primer - covered the region from V1
116 to V9, as already tested (Cusco et al., 2017). The ONT Universal Tag is needed for
117 the second PCR, where the barcode is added. PCR was carried out using the Thermo
118 Scientific Phusion Hot Start II High-Fidelity DNA polymerase kit. Each PCR reaction
119 contained RNAse and DNAse free water, 5x Phusion Buffer HF (5 µl), dNTPs 2mM
120 (2.5 µl), Primer Fw 10µM (1 µl), Primer Rv 10µM (2 µl), Phusion High Fidelity Taq
121 Polymerase 2 U/µl (0.25 µl) and 5 µl of DNA milk samples. One PCR negative
122 control was added, to test contamination of the water or kit reagents. The thermal
123 profile consisted of an initial denaturation of 30 sec at 98 °C, followed by 25 cycles of
124 15 sec at °98 C, 15 sec at 51 °C, 45 sec at 72 °C, and a final extension of 7 min at 72
125 °C. DNA purification performed by means of Agencourt AMPure XP beads
126 (Beckman Coulter) with a 0.5X concentration; after that, DNA was eluted in 21 µl of
127 RNAse and DNAse free water; then, quantity of PCR products were determined using
128 Agilent Bioanalyser 2100 and Qubit™ fluorometer. A molarity of 0.5nM of the first
129 PCR product was required for the second PCR, namely barcoding PCR. The
130 quantification of the first PCR was too low for 5 out of 12 samples and also for the
131 PCR negative control; for these samples, all volume was used for the second PCR,
132 while the corresponding dilution was assessed for the other samples in a total volume
133 of 20 µl. The barcoding PCR was carried out using the same kit used before; the PCR
134 mixture, with a total of 78 µl, was composed of RNAse and DNAse free water, 5x
135 Phusion Buffer HF (20 µl), dNTPs 2mM (10 µl), Phusion High Fidelity Taq
136 Polymerase 2 U/µl (1 µl), then the specific barcode (2 µl) (from PCR Barcoding kit –
137 EXP-PBC001) and in 20 µl of DNA from the first PCR were added. The thermal

138 profile consisted of an initial denaturation of 30 sec at 98 °C, followed by 15 cycles of
139 7 sec at °98 C, 15 sec at 62 °C, 45 sec at 72 °C, and a final extension of 7 min at 72
140 °C. The library preparation was carried out following the 1D PCR barcoding
141 amplicon protocol using the Ligation Sequencing Kit 1D (LQK-LSK108 kit). We
142 sequenced the full-length 16S rRNA using MinION™ in a flow cell Mk I (R9.4.1)
143 (FLO-MIN106).The bioinformatic workflow already reported by Cuscó and
144 colleagues was used to analyze data with minor modifications (Cusco et al., 2018).
145 Briefly, MinKNOWN software (version 1.14.1 – GUI version 2.1.14) was used to run
146 samples. Then, fast5 files were basecalled and demultiplexed using Albacore v2.3.1.
147 Barcode and adapter removal, in addition to another demultiplexing step, was
148 performed using Porechop (https://github.com/rrwick/Porechop); an extra trimming of
149 45 bases from each end was carried out, which corresponded to the length of the
150 universal tag and the custom primer, was carried out. Sequences were selected by
151 size, keeping those from 1,200 and 1,800 bases long. Sequence alignment and
152 mapping to the Greengenes database (DeSantis et al., 2006), previously trimmed for
153 the full-length 16S gene, was carried out using Minimap2 after they were checked for
154 chimeras by means of Yacrd (https://github.com/natir/yacrd) (MinION-Minimap2
155 worflow). After chimera removing, What's in my pot (WIMP) (Juul et al., 2015)
156 workflow on EPI2ME Oxford Nanopore Technologies, which uses NCBI RefSeq
157 database, was also performed. The data analysis applied after long read sequencing by
158 MinION™ included: i) MinION-Minimap2 workflow, as previously reported (Cusco
159 et al., 2018a) using a mapping-based approach; ii) MinION-EPI2ME workflow,
160 which was applied following WIMP workflow on EPI2ME (ONT) platform (based on
161 Centrifuge software) (Kim et al., 2016). Final results were compared with those from
162 short-read sequencing (Catozzi et al., 2019).

163 The subclinical mastitis raw short-reads have been submitted to NCBI under
164 Bioproject accession number SUB4205063—Bioproject number: PRJNA477950. The
165 healthy raw short-reads and the raw long reads have been submitted to NCBI under
166 Bioproject accession number SUB5522471 —Bioproject number PRJNA534197.
167 The gel electrophoresis performed before sequencing showed that the expected band
168 at 1,500 bp was present in all samples, except for PCR negative control (Figure S1).
169 Unspecific bands around 700 bp were also present in some samples. Quality filtering
170 results are presented in Table S1. After the first quality procedure using Porechop
171 more than the 90% of the sequences was kept. The greatest loss of reads was reported
172 after the sequence length trimming between 1,200 and 1,800 bp, resulting, in some
173 cases, in a decrease of more than half of the sequences. The decrease is related to the
174 unspecific amplicons around 700 bp belonging to water buffalo genome,
175 demonstrating that the primers anneal to the host genome and should be improved to
176 avoid host DNA amplification. After applying the MinION-Minimap2 workflow, two
177 samples from healthy quarters were discarded. Sample 2 was removed for the limited
178 number of sequences and sample 3 was excluded from the analysis because the 80%
179 of the sequences belonged to the chloroplast taxonomic class. In subclinical mastitis
180 quarters, we found that long-amplicon results were consistent with those obtained by
181 microbiological culture of subclinical mastitis samples. MinION-EPI2ME procedure
182 allowed the detection of *Streptococcus agalactiae*, in addition to *Staphylococcus*
183 *aureus*. MinION-Minimap2 method was able to detect *S. aureus* species, but not *S.*
184 *agalactiae*, even though this species is present in the Greengenes database;
185 *Staphylococcus alactolyticus* was misrepresented instead of *S. agalactiae*.
186 Greenegenes is regarded as one of the best option for V3-V4 short-reads data analysis,
187 given the background of its rate of recall (sensitivity) of 93.2% at family level and

188 69.2% at genus level, which is the highest value obtained among the other software-
189 database comparisons (Almeida et al., 2018). However, the subclinical mastitis
190 samples that were positive to *S. agalactiae*, presented divergent results dependent on
191 the method used to analyzing it; EPI2ME found *S. agalactiae* whereas with Minimap2
192 with Greeengenes database found *S. alactolyticus*. We further checked some of these
193 sequences using BLAST and were *S. agalactiae*, indeed. This wrong result could be
194 linked to the poor representation of *S. agalactiae* in Greengenes database (2
195 references) when compared to *S. alactolyticus* (223 references) and their high
196 similarity within the 16S rRNA gene (94%). For what concerns *S. aureus*, samples
197 positive to this bacterium are all in accordance to the microbiological culture result,
198 except for sample 4, where the presence of coagulase-negative staphylococci was
199 mistaken for *Staphylococcus aureus*, which is a coagulase-positive bacterium (Table
200 1).
201 All taxa detected after short-reads, MinION-Minimap2 and MinION-EPI2ME
202 workflow are presented in Supplementary file 1. The 10 most abundant taxa obtained
203 by long reads sequencing are shown in Figure 1. In healthy quarters, most of the
204 species are in low-abundance, as detected by both workflow, with the exception of the
205 37% of *Enterococcus* spp in sample 12 (*Enterococcus casselifavus* and *gallinarum*
206 species detected by EPI2ME at 11% and 12%, respectively), as shown in Figure 1;
207 only the 10% of this genus was reported using V4-16S sequencing method. This taxon
208 is generally associated to feces contamination (Gelsomino et al., 2003; Klein, 2003).
209 However, *Enterococcus faecalis* was already found in bovine healthy milk quarters
210 with a SCC until 50,000 cells/ml (Bicalho, 2014). Moreover, the presence of fecal
211 contaminants in quarters milk samples may be linked to farming practice, that
212 includes the use of bathing pools. If this practice on one side decreases the animal

213 thermal stress, on the other, it might increase the risk of fecal contamination, as
214 already reported in our previous study of water buffalo microbiota (Catozzi et al.,
215 2017).

216 *Corynebacterium* genus is typically involved in flavor and aroma in cheese and is
217 often present in healthy samples from water buffalo (Catozzi et al., 2017) and cow
218 milk (Kuehn et al., 2013; Quigley et al., 2013; Addis et al., 2016). The present
219 findings demonstrated that *Corynebacterium* was present in a range from 2.8% to
220 7.5% and from 9.5% to 15.4% after applying MinION-Minimap2 and short reads
221 workflows, respectively. The main representative species, detected by MinION-
222 EPI2ME method, was *Corynebacterium efficiens* (Figure 1).

223 *Jeotgalicoccus psychrophilus* was detected by both MinION-Minimap2 and MinION-
224 EPI2ME workflow (relative abundance mean of 4.7% and 2.7%, respectively) in
225 healthy samples (Supplementary file 1); short-reads were able to detect this taxon
226 only until genus level, as already demonstrated in water buffalo milk (Catozzi et al.,
227 2017); the presence of this bacteria was also confirmed in goat milk (Callon et al.,
228 2007) and bovine teat canal (Gill et al., 2006) at the species level.

229 We also investigated whether the alpha diversity, emphasizing both the evenness
230 (Simpson) and the richness components (Shannon) of diversity, could be influenced
231 by the type of platform (Ion Torrent vs MinION^{TM} machines). Only the subclinical
232 mastitis samples were found as significantly different between the two technologies
233 using the Shannon index (P=0.01), which focuses on the diversity of the taxa.
234 Conversely, this difference was not observed when using the Simpson index, that
235 focuses on the major taxa present in the dataset, confirming the substantial
236 equivalency of the two platforms, when it comes to the detection of the major

237 components of the microbiome. However, in alpha diversity, only Ion Torrent was
238 able to distinguish samples according to their patho-physiological status (Figure S2).
239 The clustering of the samples was performed using PCoA (Principal Coordinates
240 Analysis), NMDS (Non-metric Multidimensional Scaling) and CAP
241 (Constrained Canonical Analysis of Principal Coordinates) using the Bray-Curtis
242 distance matrix (Figure 2). The different methods allow the visualization of the
243 differences between individuals, with the PCoA being the classical Multi-dimensional
244 scaling, the NMDS defining the positions using the non-parametric relationships with
245 the dissimilarities and their positions in a euclidean space and the CAP, which
246 decomposes the matrix analyzed before applying the PCoA. All methods show a
247 differentiation between both platforms and status of samples. This differentiation is
248 particularly outstanding in the CAP, with 15.2% of the variance linked to the type of
249 platform, while 6.6% connected to the distinction between healthy and subclinical
250 mastitis samples, suggesting that, despite of the methodology impact, the
251 discrimination based on status is still confirmed.
252 Due to the real-time, portable and fast method, Nanopore MinION^{TM} could provide a
253 supportive role for microbiological culture, especially in the field, where on-farm
254 culture systems have already been tested for mastitis pathogens in bovine milk (Ganda
255 et al., 2016). Furthermore, milk microbiota characterization up to species level is
256 relevant for identifying possible alterations and dysbiosis, which are not necessarily
257 detected by culture-dependent methods and short-reads sequencing. In conclusion, we
258 reported here the results of pilot study, which should be improved by: i) increasing the
259 number of samples; ii) reassessing the V1-V9 primer choice, as unspecific host
260 amplicons around 700 bp were detected in addition to the expected ones about 1,500
261 bp level. Furthermore, iii) The full-length 16S sequencing could not be enough to

262 reach species level identification, within some genera. iv) Test other databases or even
263 build up a specific database would be worth for milk microbiota analysis. The present
264 study should be regarded as a proof of concept of the application of the full-length
265 16S rRNA gene sequencing to milk microbiota. Even though further investigation is
266 needed to improve the accuracy, MinION™ sequencer has demonstrated to
267 successfully achieve long-reads output, portability and real-time analysis, in
268 comparison with short-read sequencing. These results are preliminary but promising
269 for the clinical point of view. The validation of sequencing results with
270 microbiological culture technique is essential to establish a standardized protocol to
271 identify the presence of pathogens in the field. The identification of potential
272 pathogens and resistance genes in milk through metagenomics represents one of the
273 future applications of nanopore technology.
274

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278

279 **Conflict of interest**
280 The authors declared no conflict of interest.
281

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284 ME 10/13 RC
285

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386 **Tables:**

387

388 **Table 1**: comparison between microbiological culture results and full-length 16S

389 sequencing using MinION-Minimap2 and MinION-EPI2ME workflows.

Sample	**Microbiological culture**	**MinION-Minimap2**	**MinION-EPI2ME**
4	Coagulase-negative staphylococci	*S. aureus* (77.3%) + Other *Staphylococcus spp*. (1.7%)	*S. aureus* (60.4%) + *S. aures subsp anaerobius* (3.8%) + Other *Staphylococcus spp*. (2.6%)
5	*S.agalactiae*	*Streptococcus spp*. (24.3%) + *Streptococcus alactolyticus* (35%)	*S. agalactiae ATTC 13813* (53.2%) + Other *Streptococcus spp*. (13.3%)
6	*S.agalactiae* + Coagulase-negative staphylococci	*Streptococcus spp*. (53.4%) + *Streptococcus alactolyticus* (30.4%)	*S. agalactiae ATTC 13813* (45.9%) + Other *Streptococci* (13.8%) + Other *Staphylococcus spp.* (9.1%)
7	*S. aureus*	*S. aureus* (93.1%) + Other *Staphylococcus spp*. (4.2%)	*S. aureus* (74.9%) + *S. aures subsp anaerobius* (3.3%) + Other *Staphylococcus spp*. (7.9%)
8	*S. aureus*	*S. aureus* (98.4%) + Other *Staphylococcus spp*. (0.63%)	*S. aureus* (79.6%) + *S. aures subsp anaerobius* (3.4%) + Other *Staphylococcus spp.* (6%)
11	*S. aureus*	*S. aureus* (12.3%) + Other *Staphylococcus spp.* (1.6%)	*S. aureus* (8.8%) + *S. aures subsp anaerobius* (1.3%)

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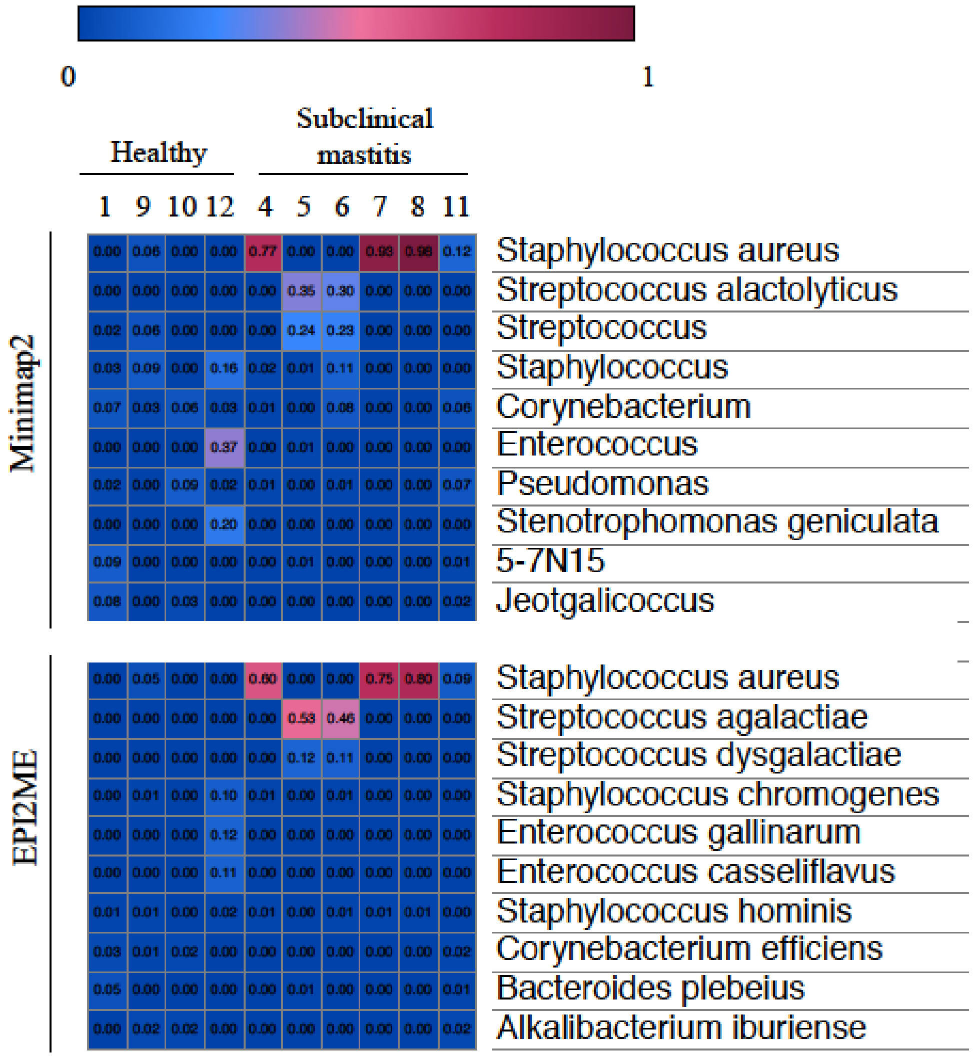
393 **Figures:**



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395 **Figure 1**: heatmap with the relative abundance of the top 10 taxa for healthy and
396 subclinical mastitis samples after using MinION-Minimap2 and MinION-EPI2ME
397 workflows. Values of relative abundance ranges from 0 to 1.

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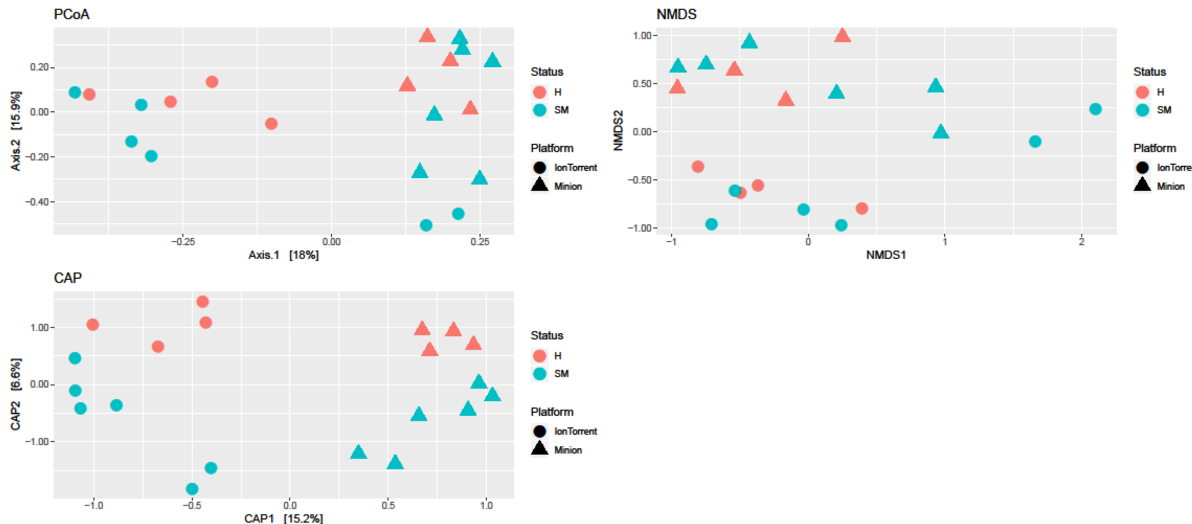

401 **Figure 2**: clustering of healthy (H) and subclinical mastitis (SM) samples by means of
402 PCoA, NMDS and CAP using the Bray-Curtis distance matrix. PCoA: Principal
403 Coordinates Analysis); NMDS: Non-metric Multidimensional Scaling; CAP:
404 Constrained Canonical Analysis of Principal Coordinates.

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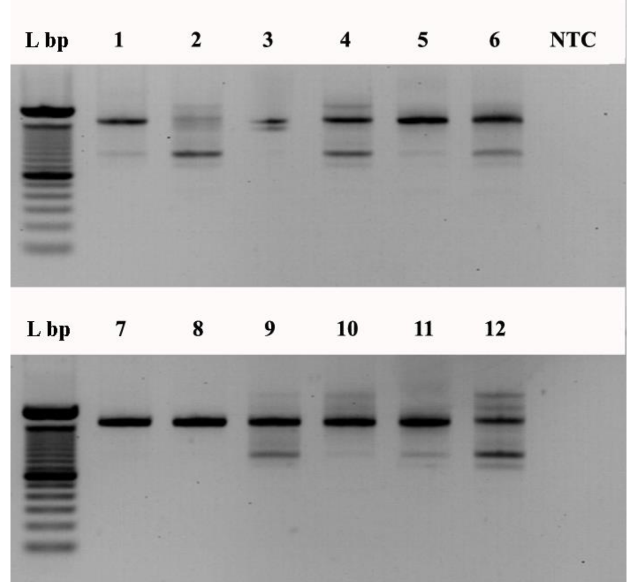
408 **Supplementary materials:**



409

410 **Figure S1**: gel electrophoresis after the full-length 16S gene amplification. L=

411 Ladder 100pb (the three main bands corresponds to 2000, 1500 and 600 bp from top

412 to bottom). Samples 1, 2, 3, 9, 10, 12 belong to heathy quarters; samples 4, 5, 6, 7, 8,

413 11 belong to subclinical mastitis quarters. NTC = PCR negative control.

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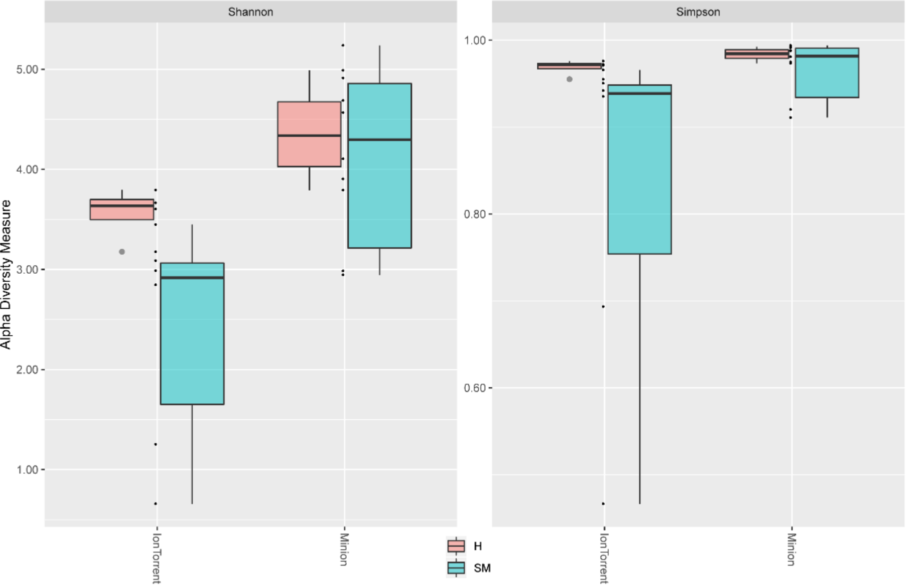

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416 **Figure S2**: alpha diversity measure in healthy (H) and subclinical (SM) affected

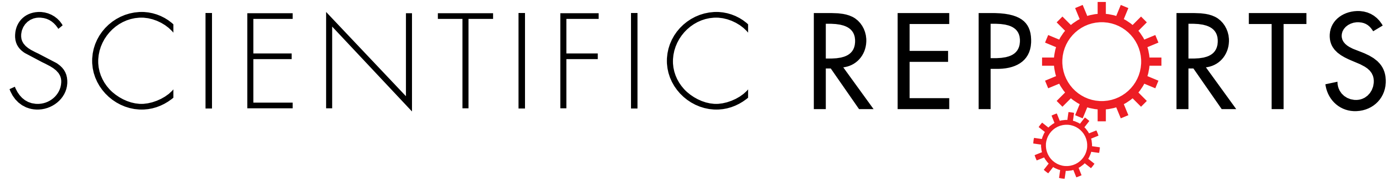
417 samples using Ion Torrent and MinION (using Minimap2 workflow) techniques.
418 Statistical significance differences are present i) between platforms in subclinical
419 mastitis samples in Shannon index ii) between status only when using Ion Torrent
420 machine.
421
422 **Supplementary file 1**: metadata and raw data for each sample are indicated after
423 analysis by MinION-Minimap2 and MinION-EPI2ME (at least order level) and short-
424 reads workflows (Data not shown).
425
426

Table S1: Sample reads after quality filtering procedures. Samples 1, 2, 3, 9, 10, 12 belong to heathy quarters; samples 4, 5, 6, 7, 8, 11 belong to subclinical mastitis quarters.

Samples	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Sequences raw fastq	16,209	34,527	11,292	28,333	17,875	29,177	18,878	23,493	19,919	14,810	17,045	19,221
Sequences after porechop + extra-trim of 45 bases	15,233 (93.9%)	33,269 (96.3%)	10,922 (96.7%)	27,704 (97.7%)	17,347 (97%)	28,458 (97.5%)	18,109 (95.9%)	22,949 (97.6%)	18,106 (90.8%)	14,021 (94.6%)	16,436 (96.4%)	18,646 (97%)
Sequences after trimming (1200-1800 bp)	9,785 (60.3%)	6,975 (20.2%)	6,192 (54.8%)	11,190 (39.4%)	13,205 (73.8%)	13,630 (46.7%)	16,390 (86.8%)	21,166 (90%)	8,993 (45.1%)	10,126 (68.3%)	11,081 (65%)	5,382 (28%)
Sequences after chimera removing	9,771 (60.2%)	6,974 (20.1%)	6,164 (54.5%)	11,180 (39.4%)	13,168 (73.6%)	13,607 (46.6%)	16,382 (86.7%)	21,159 (90%)	8,987 (45.1%)	10,103 (68.2%)	11,037 (64.7%)	5,375 (27.9%)
Final reads after Minimap protocol in R	8,309 (51.2%)	600 (1.7%)	5,682 (50.3%)	6,464 (22.8%)	9,396 (52.5%)	9,396 (32.2%)	16,109 (85.3%)	20,904 (88.9%)	7,298 (36.6%)	8,402 (56.7%)	9,436 (55.3%)	2,048 (10.6%)

6 Paper 6

Characterization of circulating miRNA signature in water buffaloes (*Bubalus bubalis*) during *Brucella abortus* infection and evaluation as potential biomarkers for noninvasive diagnosis in vaginal fluid



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Characterization of circulating miRNA signature in water buffaloes (*Bubalus bubalis*) during *Brucella abortus* infection and evaluation as potential biomarkers for non-invasive diagnosis in vaginal fluid

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Brucellosis is an infectious disease caused by bacteria from the *Brucella* genus that can be transmitted to humans through contact with infected animals or contaminated animal products. Brucellosis also causes financial losses in animal production. Ruminants are highly susceptible to brucellosis, and the causative agent water buffaloes (*Bubalus bubalis*) is *Brucella abortus*. Circulating microRNAs (miRNAs) are cropping up as promising biomarkers for several infectious diseases. The goals of this study were to characterize the serum miRNA signature associated with brucellosis in water buffaloes and investigate the miRNAs' potential use as biomarkers in vaginal fluids. Next Generation Sequencing was used to assess miRNA expression profiles in *Brucella*-positive and *Brucella*-negative blood sera; dysregulated miRNAs in blood serum and vaginal fluids were validated using RT-qPCR. ROC curves were generated to evaluate the diagnostic value of miRNAs for *Brucella*. GO and KEGG pathway enrichment analyses were exploited to investigate the biological functions of dysregulated miRNAs. The results showed that 20 miRNAs were modulated, of which, 12 were upregulated and 8 were downregulated. These findings were corroborated by RT-qPCR, and ROC curves indicated that the miRNAs can serve as potential biomarkers for *Brucella*. GO and KEGG pathway analyses pointed out that some of these miRNAs are related to immune response and apoptosis. These results provided an overview of miRNA expression profiles and highlighted potential biomarkers for *Brucella* infection in water buffaloes. We also demonstrated the potential of vaginal fluids in studies involving microRNA detection. Further functional and mechanistic studies of these miRNAs may improve our understanding of the biological processes involved in *Brucella* infection and host immune response.

Brucellosis is one of the most important zoonotic diseases in ruminants. The aetiological agent is *Brucella*, a gram-negative, facultative, intracellular pathogen[1,2]. The favoured reproductive niche of *Brucella* is the intracellular milieu of macrophages, dendritic cells and placental trophoblasts[3]. Brucellosis is a zoonosis of considerable importance to public health with more than 500,000 new human infections being estimated annually. Brucellosis also causes financial losses in animal production[4,5]. Routine screening and animal vaccinations have led to Brucellosis disappearing in western regions, although it remains endemic in developing regions such as the

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	MiRNA	Fold Change	*P* Val
UP	let-7f	2.6	0.003
UP	miR-126-5p	2.1	0.044
UP	let-7i	1.8	0.07
UP	miR-138	5.7	0.022
UP	miR-143	2.4	0.045
UP	miR-146b	3.7	0.033
UP	miR-151-3p	1.9	0.01
UP	miR-191	2.1	0.014
UP	miR-215	2.5	0.007
UP	miR-381	5.3	0.037
UP	miR-92a	1.9	0.021
UP	miR-92b	2.1	0.019
DOWN	miR-133a	−6.7	0.003
DOWN	miR-127	−3.2	0.044
DOWN	miR-150	−6.7	0.001
DOWN	miR-221	−2.7	0.017
DOWN	miR-30e-5p	−1.8	0.034
DOWN	miR-30d	−2.1	0.012
DOWN	miR-320a	−2.5	0.045
DOWN	mir-339b	−2.5	0.045

Table 1. Differentially expressed miRNAs in the serum of seropositive buffaloes compared with seronegative buffaloes by sequencing.

Middle East, Asia, Africa and South America and in some areas of Italy[6]. *Brucella* is transmitted to humans by consuming raw milk or after direct contact with infected animals. The infectious course of brucellosis is divided into three phases, each marked by distinct bacteriological, clinical and pathological profiles: (i) onset of infection; (ii) the acute phase during which clinical, haematological and pathological symptoms are first observed; and (iii) the chronic phase, characterized by intermittent clinical symptoms and evident pathological signs[2]. Ruminants are highly susceptible to brucellosis; small and large ruminants are preferentially infected by *B. melitensis* and *B. abortus*, respectively. In most areas examined thus far, namely, South America[7,8], Pakistan[9], Italy[10] and Africa[11], the main causative agent in water buffaloes is *B. abortus* biovar 1. In pregnant females, the bacterium invades the placenta, and subsequently the foetus, prompting abortion mainly during the last third of the pregnancy[12,13]. Nonpregnant animals, still shedding the bacteria through secretions, may be asymptomatic with no evident clinical or pathological signs[14]. *Brucella* infections must be diagnosed early to control disease spreading. *B. abortus* and *B. melitensis* ruminant brucellosis are diagnosed based on bacteriological and immunological tests, the latter being routinely used in control, eradication and surveillance programmes[15,16]. Serological tests are used to initially diagnose brucellosis, but the results can be negative, even when the bacterium is present, particularly during the early disease phases. Thoroughly understanding *Brucella* biology and identifying novel biomarkers are essential for diagnosis and prophylaxis protocols. MicroRNAs (miRNAs) are small noncoding RNA that regulate gene expression posttranscriptionally. They play pivotal roles in cellular homeostasis, and their expression is dysregulated during stress conditions, disorders and diseases[17]. MicroRNA are involved in pathogen-host interactions[18] and are stable in body fluids, from which they can be easily extracted[19]. Consequently, miRNAs are promising biomarkers for diagnosing several diseases and stress disorders in both humans[20,21] and animals[22–24]. Changes in miRNA expression patterns have been observed in association with infectious diseases[25–27] and as reactions to specific stresses such as thermal stress[28]. *Brucella* has also been shown to modulate *in vitro* expression of miRNAs involved in host immune responses[29–31].

Brucella infection reduces fertility by inducing abortion as well as suppurative placentitis[32]. Since no information has been reported on circulating miRNAs during *Brucella* infection in water buffaloes (*Bubalus bubalis*), the present study aimed to (a) assess miRNA expression profiles in the blood sera of water buffaloes infected by *B. abortus*; (b) extract and measure miRNA expressions in vaginal fluid during *B. abortus* infection; (c) determine whether miRNAs can be used as biomarkers to assess brucellosis; and (d) integrate miRNAs to their target genes and relative biological processes.

Results

Identifying differentially expressed serum microRNAs during *B. abortus* infection by miRNA sequencing.

Serum miRNAs were sequenced to determine the differential miRNA profiles of *B. abortus*-infected and healthy buffaloes. A total of 469 miRNAs were identified, of which, 20 showed significantly altered expression in seropositive animals compared with seronegative animals. In seropositive animals, the expressions of 12 miRNAs were upregulated 1.8- to 3.7-fold, while 8 miRNAs were downregulated 1.8- to 6.7-fold ($P \leq 0.05$) (Table 1).

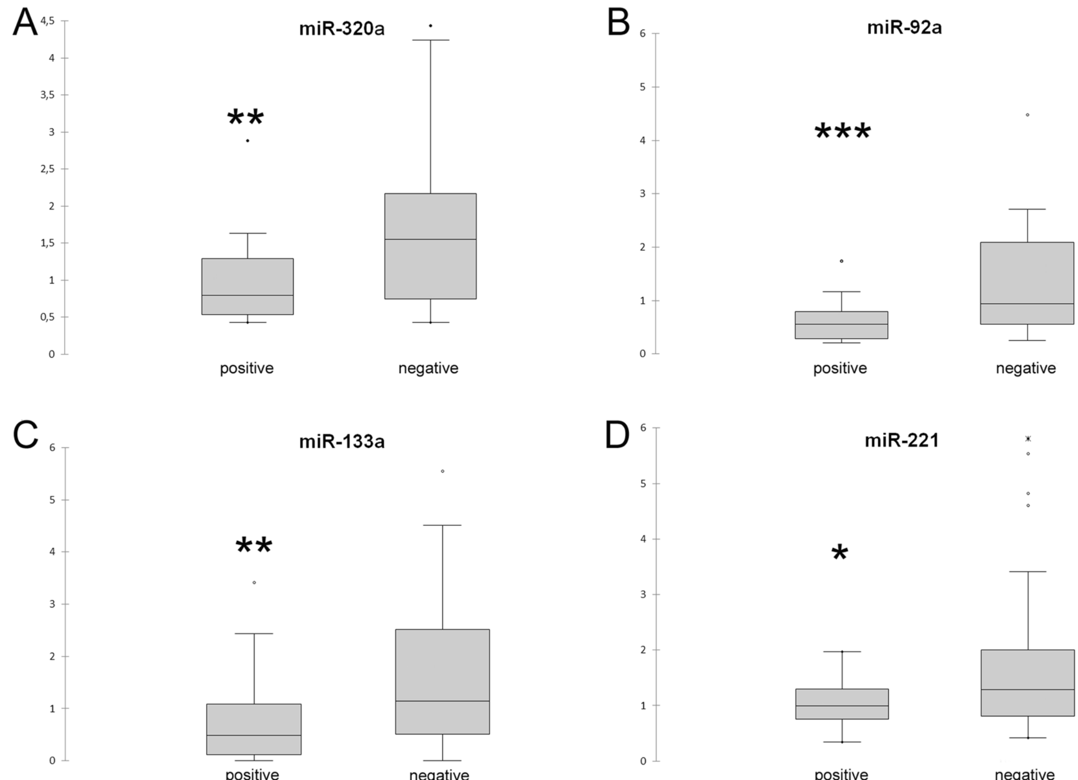

Figure 1. Box plots of DE-miRNAs in the blood serum. Significance was declared at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Black lines inside the boxes mark the medians. Whiskers indicate variability outside the upper and lower quartiles.

Validating microRNA sequencing results and quantifying differentially expressed miRNAs in blood serum and vaginal fluid samples. qPCR validation was performed on the 30 sequenced samples and a separate independent cohort of 30 buffaloes. To validate the sequencing results, 12 differentially expressed (DE)-miRNAs were selected, and their relative abundance was quantified using RT-qPCR. MiRNA levels were normalized to that of cel-miR-39, an artificial spike-in which was used as an internal control. The selected miRNA targets were detected in all blood serum and vaginal fluid samples. RT-qPCR results for the blood serum demonstrated that the levels of four miRNAs (miR-320a, miR-133a, miR-92a, and miR-221) were significantly downregulated in seropositive buffaloes (Fig. 1). An analysis of 12 selected miRNAs from the vaginal fluid demonstrated that 10 miRNAs (miR-let-7i, miR-150, miR-320a, miR-191, miR-let-7f, miR-339b, miR-30e, miR-151, miR-126-5p, and miR-92a) were significantly differentially expressed between *Brucella*-positive and negative buffaloes. Figure 2 presents an overview of these results. DE-miRNA levels were unaffected by the animals' oestrus phases (linear regression, $P > 0.05$).

Assessing the diagnostic value of DE-miRNAs during *B. abortus* infection. To analyse the diagnostic value of DE-miRNAs in the blood serum and vaginal fluid, ROC curves were analysed, and the associated area under the curve (AUC) was used to confirm the diagnostic potency of each miRNA. The ROC was analysed as previously reported[22]. Table 2 summarizes the diagnostic performance of each DE-miRNA and shows combinations of some DE-miRNAs. The AUC was fair for blood serum miR-320a and miR-92a and poor for blood serum miR-133a and miR-221 (Supplemental Material 1). The AUC was excellent for vaginal fluid miR-151 and miR-30e, with calculations of 0.957 and 0.931, respectively; good for miR-let-7f, miR-339b, miR-150 and miR-191 ($AUC \geq 0{,}799$); fair for miR-let-7i, miR-92a and miR-320a; and poor for miR-126-5p (Fig. 3). To test potential collinearity, a Spearman correlational analysis was performed on the vaginal fluid miRNAs with excellent and good AUC values, suggesting that relative concentrations of miR-151, miR-339b, miR-150, miR-191, and miR-30e are positively correlated with each other (data not shown).

Discriminant analysis was applied to further investigate the potential for improving diagnostic performance by analysing multiple DE-miRNAs. Statistical analysis was performed examining the weighted average relative quantification (RQ) values of the miRNAs with an $AUC > 0.7$ for serum blood (miR-320a and miR-92a) and $AUC > 0.8$ for vaginal fluid (miR-let-7f, miR-151, miR-30e, miR-191, miR-150 and miR-339b) (Fig. 4). Median expression levels in the blood sera were 0.649 (range, 0.229 to 1.639) and 0.542 (range, 0.188 to 1.889) and in the vaginal fluid 11.62 (range, −6.6 to 40.36) and −6.16 (range, −67.6 to 73.8) in *Brucella*-positive and *Brucella*-negative buffaloes, respectively (Fig. 4A,C). The predicted probability of being discriminated as positive from the logit model based on the two blood sera [$\text{logit} = (0.6045 \times \text{expression level of miR-320a}) + (0.4045 \times \text{expression level of miR-92a})$] or the six vaginal fluid miRNAs [$\text{logit} = (0.936 \times \text{expression level of let-7f}) + (4.183 \times \text{expression}$

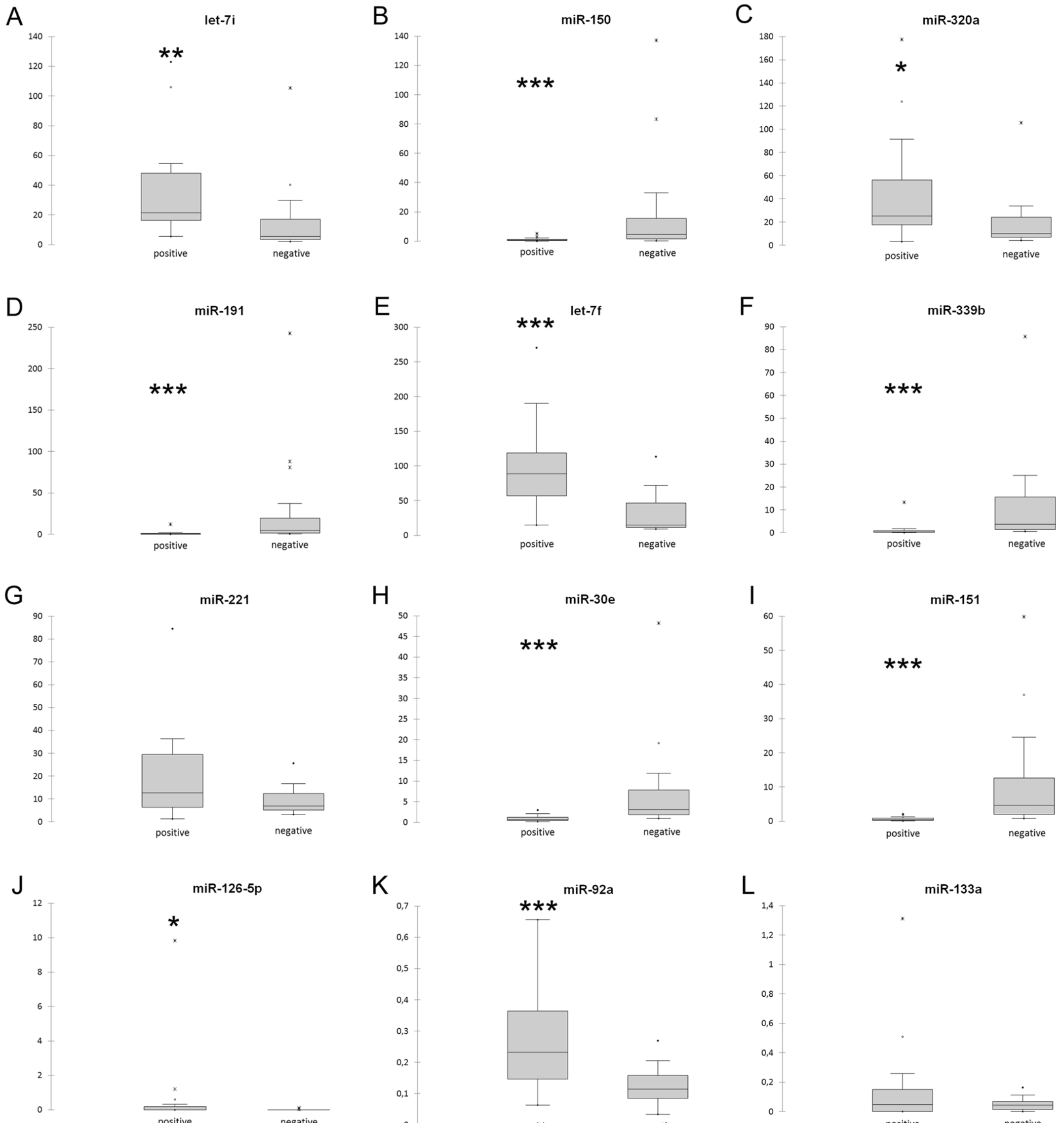

Figure 2. Box plots of the DE-miRNAs in the vaginal fluid. Significance was declared at **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. Black lines inside the boxes mark the medians. Whiskers indicate variability outside the upper and lower quartiles.

level of miR-151) + (−13.777 × expression level of miR-339b) + (−21.946 × expression level of miR-30e) + (5.372 × expression level of miR-150) + (3.784 × expression level of miR-191)] was used to construct the ROC curves (Fig. 4C,D). The AUC for the combined blood serum miRNAs was 0.753 (95% CI 0.620–0.857) with a cut-off value of 0.90 and 80% sensitivity and 56.25% specificity. The AUC for the combined vaginal fluid miRNAs was 0.88 (95% CI 0.742–0.959) with a cut-off value of 0.023 and 95.45% sensitivity and 85% specificity. Clustering patterns were further visualized using multidimensional scaling plots (MDS) (Fig. 5). These plots generate distances between samples corresponding to the biological coefficient of variation between miRNAs in each sample. The MDS plots comparing seropositive and seronegative buffaloes showed distinct groupings of infected and control animals in both the blood serum and vaginal fluid samples, highlighting a clear miRNA expression distinction by infection type.

miRNA target prediction and pathway enrichment. Predicted significant DE-miRNA targets were computationally fetched from the TargetScan database, and genes with cumulative weighted context++ scores (CWCS) < −0.4 were further analysed. mRNA was enriched using DAVID bioinformatics to explore the function and pathogenesis of these candidate biomarkers. Gene Ontology (GO) analysis was performed using DAVID at three levels: Molecular Function (MF), Cellular Component (CC) and Biological Process (BP). Figure 6A illustrates the top 10 items that were significantly enriched by target genes for each of the above GO levels. The enriched GO terms in MF mainly included protein binding and kinase activities. The CC items in which the predicted targets were involved were related to proteins involved in structure (nucleus and cytoplasm) and function

	MiRNA	AUC	95% CI	*P Value*	Cut-Off	Sensitivity	Specificity
BLOOD SERUM	miR-320a	0.736	0.603–0-844	0.0005	1.33	84	65.62
	miR-92a	0.749	0.616–0.854	0.0001	0.82	80	65.62
	miR-133a	0.693	0.556–0.808	0.0069	1.08	76	56.25
	miR-221	0.661	0.524–0.781	0.026	1.25	72	56.25
	miR-92a, miR-320a	0.753	0.620–0.857	0.0001	0.90	80	56.25
VAGINAL FLUID	miR-126-5p	0.67	0.491–0.819	0.0142	0.04	42.86	92.86
	miR-92a	0.76	0.589–0.886	0.0011	0.099	86.36	50
	miR-320	0.727	0.554–0.862	0.014	11.12	86.36	64.29
	miR-let7f	0.88	0.728–0.964	<0.0001	25.13	95.45	64.29
	miR-let7i	0.799	0.632–0.913	0.0007	7.57	95.45	64.29
	miR-151	0.957	0.843–0.996	<0.0001	2.0936	100	75
	miR-30e	0.931	0.806–0.986	<0.0001	2.133	95.24	70
	miR-339	0.89	0.753–0.966	<0.0001	1.85617	95.25	70
	miR-150	0.826	0.676–0.926	<0.0001	3.31	95.24	55
	miR-191	0.898	0.763–0.970	<0.0001	2.09	95.24	70
	miRNAs combination	0.88	0.742–0.959	<0.0001	0.023	94.45	85

Table 2. Sensitivity, specificity, and area under the curve (AUC) for DE-miRNAs in the blood serum and vaginal fluid. MiRNAs combined in the vaginal fluid include miR-let-7f, miR-151, miR-30e, miR-191, miR-150 and miR-339b.

(signal transduction and replication, such as G-protein complexes and transcription factors, respectively). Most GO BP items converged on regulating proliferation-apoptosis processes. KEGG pathway analysis was carried out on the whole targets of miRNA biomarkers using DAVID. Since altered immune responses are believed to contribute to the bacteria's ability to hide and survive in the host, mRNA targets encoding for genes involved in immune pathways were enriched. The list of immune-related targets included 78 genes that were employed in further analyses. The top 15 significantly enriched KEGG pathways are outlined in Fig. 6B, with chemokine signalling, transcriptional misregulation, Chagas disease and FoxO signalling being the top pathways.

Discussion

Although serum circulating miRNAs are some of the most promising clinical diagnostic or therapeutic biomarkers for diagnosing various diseases and stress disorders in both humans[33] and animals[22,23], their diagnostic potential in veterinary medicine remains to be fully explored.

To the best of the authors' knowledge, the present study is the first to address the relationship between *Brucella* infection and miRNAs in dairy water buffaloes. We adopted a multistep approach, using miRNomics as a first step to identifying differentially expressed miRNAs in blood serum, then validated them in blood serum and vaginal fluid, which was shown to be a reliable source of microRNA for the first time. We selected vaginal fluid as a potential miRNA source because the reproductive system is the preferential target organ for *Brucella* infection. We found that (I) the levels of four miRNAs were significantly downregulated in the blood serum of seropositive buffaloes; (II) the levels of 10 miRNAs were significantly higher in the vaginal fluids of seropositive than in seronegative animals; (III) diagnostic accuracy for *B. abortus* was excellent ($0.90 < AUC < 1$) for two miRNAs (miR-151 and miR-30e) and good ($0.80 < AUC < 0.90$) for four miRNAs (miR-let-7f, miR-339b, miR-150 and miR-191); and (IV) the diagnostic accuracy of the combination of six differentially expressed miRNAs was good ($AUC = 0.88$) with 95.45% sensitivity and 85% specificity. We also demonstrated that miRNAs can be efficiently extracted from vaginal fluids.

Sequencing results provided a list of differentially abundant miRNAs but validation with RT-qPCR demonstrated that the differential expression rate in the serum between *Brucella*-seropositive and seronegative animals was not statistically significant. Therefore, the results suggested that miRNA expression levels were similar in the blood sera of seropositive and seronegative water buffaloes. Nevertheless, technical bias inherent in sequencing technologies may generate distorted results. A substantial distortion between miRNA levels in NGS data and true miRNA abundancy may occur using Illumina sequencing technology[34]. Because vaginal fluids may express the same miRNAs as in the serum during *B. abortus* infection[35], these miRNAs were quantified, and the results are promising.

In addition to well-established tests, such as serological and microbiological tests, a molecular approach was used to broadly assess *Brucella* spp. infections[36–38]. MiRNAs regulate gene expression in many pathophysiological pathways, including those involved in microbial infection. Changes in host miRNA expression occur following infection with exclusively extracellular (*Helicobacter pylori*) or intracellular (*Salmonella enterica*) gram-negative bacteria, as well as in response to gram-positive bacteria (*Listeria monocytogenes*) and other pathogens (*Mycobacterium* and *Francisella* species)[17,39]. The infected cells modulate miRNAs, suppressing host immune responses and promoting microbial pathogenesis. For example, let-7 family miRNAs, which were upregulated in the present study, are involved in modulating two major cytokines, IL6 and IL10, which have opposite effects on the immune response: specifically IL6 promotes, whereas IL10 dampens the proinflammatory programme. The miR-150 family (both miR150 and miR-151 were downregulated in the present study) is involved in lymphocyte

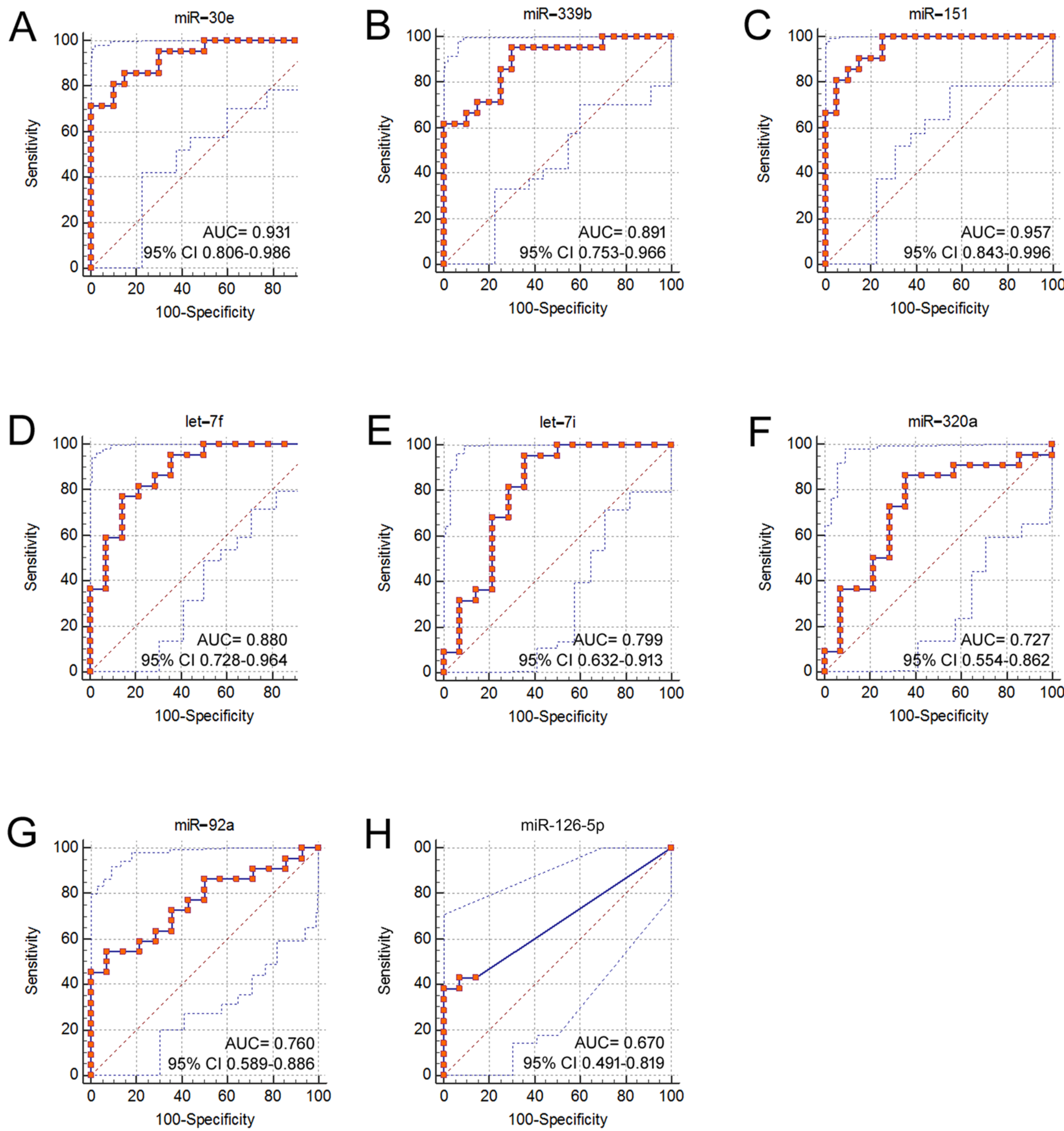

Figure 3. Receiver-operator characteristic (ROC) curve analysis of DE-miRNAs in the vaginal fluid. AUC, area under the curve; CI, confidence interval.

development and regulation; a recent study identified that serum circulating miR-150 is a general lymphocyte activation sensor and may serve as a biomarker for human lymphocyte activation in healthy and diseased conditions[40]. The miR-30 family, to which miR-30e belongs and which was downregulated in the present study, is involved in modulating host SUMOylation by downregulating UBC9, a protein involved in T cell development[41]. The results reported herein suggest that *B. abortus* may influence the immune responses of infected buffaloes by modulating the production and exocytosis of miRNAs that influence both innate and adaptive immune responses, potentially acting on phagocytic cells and lymphocytes as previously suggested for cattle[42]. Accurate screening tests are important for the success of brucellosis eradication programmes. This study suggests that vaginal DE-miRNA levels accurately differentiated *B. abortus*-infected buffaloes from controls. Many miRNAs aberrantly expressed mRNAs have been identified between *Brucella*-infected and noninfected buffaloes, and the relative expressions of DE-miRNAs, such as miR-151 and miR-30e, were used to discriminate seropositive *vs* seronegative animals. These miRNAs had high sensitivity (>94%) up to 100% for miR-151, but low specificity, which reached 85% when some of these were combined. These data showed that miRNAs collected from vaginal fluids may be suitable for use in screening because they can solve many problems that arise from other tests[14,15]. The sensitivity and specificity of the immunological tests used to routinely diagnose brucellosis in cattle have been summarized by several authors[16,43,44] who believe that more than one test should be applied to serologically diagnose brucellosis in the field. The main issue concerns test sensitivity, which normally does not exceed 90%, whereas specificity can reach 100%. Quantifying a panel of miRNAs in the vaginal fluid increases sensitivity to 95% with an adequate 85% specificity. The authors believe that applying this miRNA panel may help to identify infected animals, particularly in extensive breeding systems where brucellosis is endemic or transmission is relatively low. Combining immunological and molecular tests may enable an ideal balance between specificity and

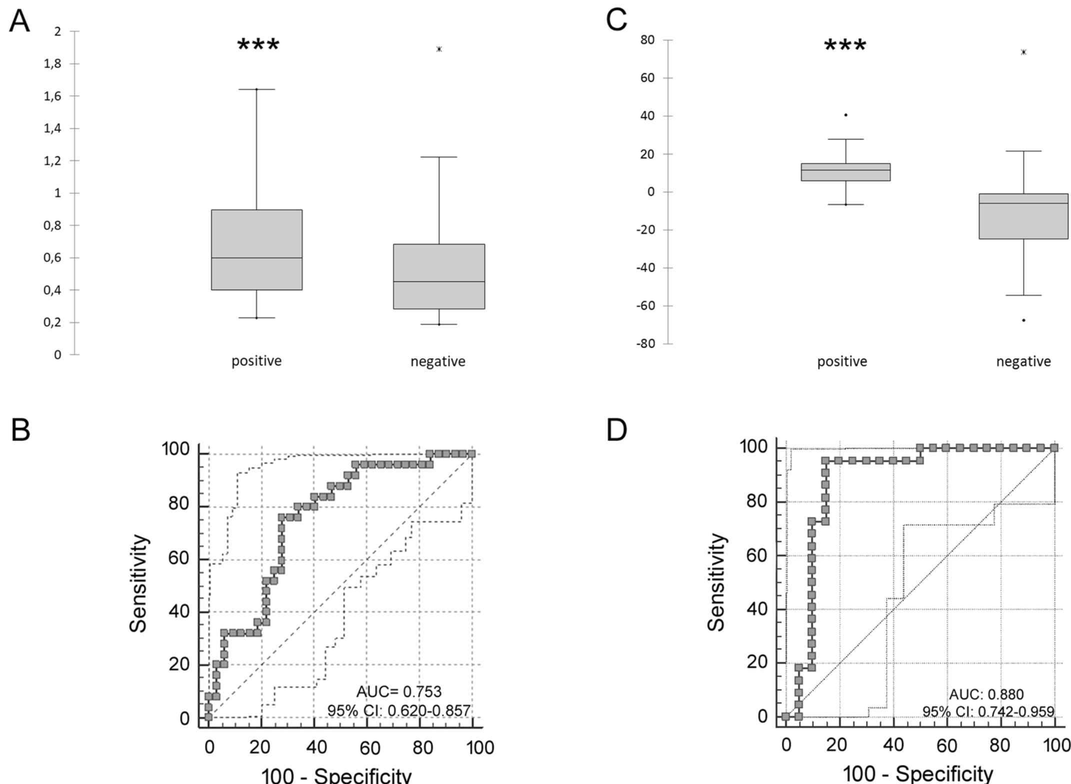

Figure 4. Average expression of the DE-miRNAs in the blood serum and vaginal fluid. Weighted average relative quantification (RQ) values of DE-miRNAs in the blood serum (**A**) and vaginal fluid (**C**). ROC curve analysis, constructed using the logit model, for DE-miRNAs in the blood serum (**B**) and vaginal fluid (**D**). AUC, area under the curve; CI, confidence interval. Black lines mark the medians. ***$P < 0.0001$.

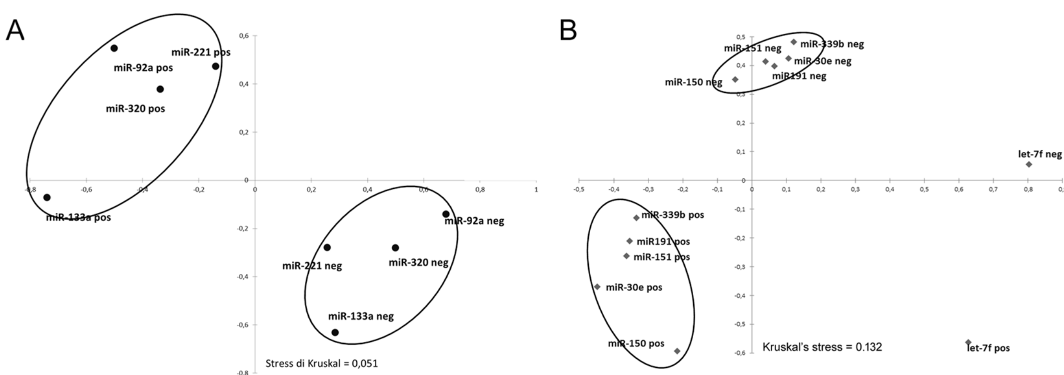

Figure 5. Multidimensional scaling plots comparing miRNA expression levels in the (**A**) blood serum and (**B**) vaginal fluids of *Brucella*-positive and negative buffaloes. For comparison to the seronegative buffaloes, seropositive buffaloes are grouped together.

sensitivity. Our results suggest that noninvasive molecular biomarkers may help to more accurately assess and monitor *Brucella* infections.

Gene Ontology analysis demonstrated that DE-miRNAs regulated mRNA coding for proteins involved in several molecular functions, cellular components and biological processes involved in *Brucella* pathogenesis. For example, these DE-miRNAs may target genes coding for G-proteins, plasma membranes and membrane rafts, which are essential for *Brucella* endocytosis, highlighted at the CC and MF levels. The pivotal role of these mechanisms was elucidated by Rossetti and colleagues[45], who demonstrated that pathogens attaching to the cell surface induce a zipper-like mechanism for internalization, and binding activates the GTPases, promoting cytoskeleton reorganization and thus host cell membrane rearrangement. Inside cells, these pathogens reside in the *Brucella*-containing vacuole (BCV) and influence two other processes potentially targeted by DE-miRNAs: the inflammatory reaction, which is modulated by T4SS (type IV secretion system)[46], and changes in host cell transcription processes[47]. PI3K (phosphatidylinositol 3-kinase) activities, highlighted by both GO and KEGG analysis,

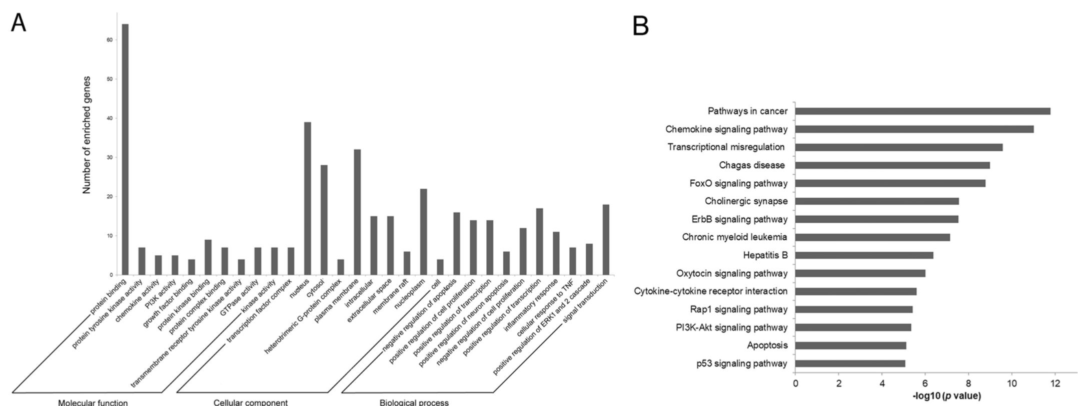

Figure 6. Target prediction and pathway enrichment. (**A**) GO annotation of genes regulated by identified DE-miRNAs. The target genes were annotated by DAVID at three levels: molecular function, cellular component and biological process. The top 10 significantly enriched items are shown. (**B**) Pathway enrichment analysis for genes regulated by DE-miRNAs. Genes regulated by DE-miRNAs were retrieved and enriched in KEGG using DAVID. The *P* value was negative 10-base log transformed. The top 15 enriched KEGG pathways are reported.

are involved in *Brucella* uptake[48] and modulating host cell proinflammatory responses. PI3K promotes binding in TIR-containing proteins and subsequent TLR signalling activation. Sengupta and colleagues[49] demonstrated that *Brucella* expressed the TIR-containing protein, Btp1, which competes with MyD88 and interferes with the TLR4/TLR2 cascade, dendritic cell maturation, and proinflammatory cytokine production. KEGG analysis identified genes and pathways related to cell apoptosis: proliferation and chemokine signalling. *B. abortus* infection does not lead to cell death[50], and *B. suis* inhibits apoptosis induced by chemical stimuli in human macrophages[51]. The mechanisms underlying apoptosis inhibition, include modulating genes from the BCL2 pathway[51], with both pro- and anti-apoptotic activities. The DE-miRNAs may modulate apoptosis-related genes, promoting cell survival by targeting prosurvival BCL2 proteins, such as BCL2 (B-cell lymphoma 2) and MCL1 (ML1 myeloid cell leukaemia 1), and prodeath proteins, such as BAK1 (Bcl-2 homologous antagonist/killed 1) and PUMA (p53-upregulated modulator of apoptosis). Apoptosis is an important innate immune mechanism that eliminates pathogen-infected cells; the possibility that *Brucella* may modulate host miRNAs regulating apoptotic signalling pathways might partially explain *Brucella's* ability to promote propagation and evade host defences.

In conclusion, the present study identified for the first time DE-miRNAs in the blood serum and vaginal fluid of water buffaloes affected by *Brucella abortus* compared with healthy animals. These miRNAs were involved in regulating the transcriptions of genes related to the molecular pathogenesis of brucellosis. Moreover, we found that miR-let-7f, miR-151, miR-30e, miR-191, miR-150 and miR-339b extracted from vaginal fluids are potentially useful biomarkers of *Brucella* infection. Although this study provided new and important insights into brucellosis pathogenesis, further experiments involving more animals are required to validate the miRNAs' potential use in diagnosing brucellosis. A better grasp of the mechanisms regulated by these miRNAs will be important for developing interventions such as designed therapy and vaccines.

Materials and Methods

Ethical statement. Samples were collected during routine disease testing for the national brucellosis eradication programme. The study design was approved by Campania Region DGR No. 352/2013 and DRD No. 603 dated 11/03/2014. All experiments were performed in accordance with the relevant guidelines and regulations.

Animal husbandry, sample collection and oestrus phase classification. Blood and vaginal fluid samples were collected from 60 buffaloes (*Bubalus bubalis*) raised on 3 farms in the Caserta area in June and July 2016 during routine national brucellosis prophylaxis programme screening. Animals were housed in an open yard breeding system. Blood was collected by tail venepuncture in serum and Monovette EDTA tubes (Sarstedt Company, Nümbrecht, Germany) and centrifuged at 800 × g for 15 min. Serum and plasma were stored at −80 °C. To collect the vestibulovaginal fluid swabs, the buffaloes were restrained and a sterile swab (sterile swab on a wooden stick with a cotton tip in a polypropylene test tube (12 × 150 mm), FL Medical s.r.l. Unipersonale Padova, Italy) was introduced into the cranial vagina. The swab was exposed to the vestibule-vaginal fluid, pulled inside the pipette and stored at −80 °C.

Oestrous cycle stage was determined by progesterone (P4)[52–54] and oestradiol (E2)[55] plasma concentrations using radioimmune assays (RIA).

Serological and bacteriological analysis. Serology for brucellosis was performed using the Rose-Bengal and complement fixation tests on all samples, as recommended by the World Organization for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017 (version adopted in May 2016)[56]. Microbiological analysis was performed as previously reported by O'Grady and colleagues[57].

MiRNA extraction, library preparation, and sequencing. Total RNA was extracted using the miRNeasy Serum/Plasma Kit (Qiagen, catalogue number 217184, Milan, Italy). Serum was thawed on ice and centrifuged at 3000 × g for 5 min at 4 °C. An aliquot of 200 μl per sample was transferred to a new tube, and RNA was

miRNA	Assay ID
cel-miR-39-3p	000200
hsa-let-7i	002221
hsa-miR-320a	002277
hsa-miR-92a	000431
hsa-miR-126-5p	000451
hsa-let-7f	000382
hsa-miR-151-3p	002254
hsa-miR-30e-5p	007791_mat
hsa-miR-150	006586_mat
hsa-miR-339b	241893_mat
hsa-miR-191	002299
mmu-miR-221	001134
hsa-miR-133a	002246

Table 3. List of TaqMan probes (ThermoFisher Scientific, Monza, Italy) and assay IDs.

extracted using miRNeasy Serum/Plasma Kits (Qiagen, catalogue number 217184, Milano, Italy) in accordance with the manufacturer's instructions. Animals were divided into six pools of 5 by the presence or absence of *Brucella* infection, and the pools were sequenced.

Libraries were prepared using TruSeq SmallRNA Sample Prep kits (Illumina, San Diego, CA, USA) following the manufacturer's instructions. Both RNA samples and final libraries were quantified using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and quality tested by the Agilent 2100 Bioanalyzer RNA Nano assay (Agilent Technologies, Santa Clara, CA, USA). Libraries were then processed with Illumina cBot for cluster generation on flowcells, following the manufacturer's instructions and sequenced on single-end mode at the multiplexing level requested on HiSeq. 2500 (Illumina, San Diego, CA). The CASAVA 1.8.2 version of the Illumina pipeline was used to process raw data for both format conversion and demultiplexing[58]. The reads obtained were mapped to the *Bos taurus* database because the complete *Bubalus bubalis* genome is lacking.

Validation by qPCR. Total RNA was extracted using the miRNeasy Serum/Plasma Kit (Qiagen, catalogue number 217184, Milano, Italy) as reported above. One millilitre of Qiazol (Qiagen) was added to an aliquot of 200 µl per sample. After incubation at room temperature for 5 min, 3.75 µl (25 fmol final concentration) of the exogenous synthetic spike-in control *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen, catalogue number 219610) was spiked into samples at the beginning of the extraction procedure. Reverse transcription was performed in 15 µl volume reactions using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, catalogue number 4366596, Monza, Italy) using miRNA-specific stem-loop RT primers. The mix reactions contained 1.5 µl 10× miRNA RT buffer, 1 µl MultiScribe reverse transcriptase (50 U/µl), 0.30 µl 100 mM dNTP mix, 0.19 µl RNase Inhibitor (20 U/µl), 6 µl of custom RT primer pool and 3.01 µl of nuclease-free water. The custom RT primer pool was prepared by combining 10 µl of each 5× RT primer in a final volume of 1000 µl; the final concentration of each primer in the RT primer pool was 0.05× each. Three µl of serum RNA was added to each RT reaction. Each RT reaction mixture was incubated on ice for 5 min, 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min.

To validate the sequencing results, the qPCR experiments were designed following MIQE guidelines[59]. Small RNA TaqMan assays were performed per the manufacturer's instructions using the selected primer/probe assays (ThermoFisher Scientific, Monza, Italy) reported in Table 3. Quantitative reactions were performed in duplicate in scaled-down (12 µl) reaction volumes using 6 µl TaqMan 2X Universal Master Mix II (ThermoFisher Scientific, Monza, Italy, catalogue number 4440044), 0.6 µl miRNA specific TaqMan Assay 20× and 1µl of the RT product per reaction on the Eco Real-Time PCR detection system (Illumina, Milan, Italy). The standard cycling programme was 50 °C for 2 min, 95 °C for 10 min and 40 cycles at 95 °C for 15 sec and 60 °C for 60 sec. Data were normalized relative to the cel-miR-39 expression. MiRNA expression levels are presented as fold changes normalized to cel-miR-39 expression using the $2^{-\Delta\Delta Cq}$ formula. The significant miRNA targets were determined using the TargetScan database (http://www.targetscan.org/vert_71/), functional mRNA were enriched using DAVID bioinformatics resources (https://david.ncifcrf.gov/), and biological pathways in KEGG were examined for enrichment (http://www.genome.jp/kegg/).

Statistical analysis. Statistical analysis was performed using XLStat for Windows (Addinsoft, New York, U.S.A.) and MedCalc 14.0 (MedCalc Software bvba, Ostend, Belgium). Statistical significance was accepted at $P < 0.05$. Data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene tests, respectively. Because the data were not normally distributed, nonparametric statistical tests were applied. The Kruskal-Wallis test was used to assess differences in miRNA concentrations. *P* values were adjusted using the Bonferroni correction. Linear regression was used to investigate relationships between DE-miRNAs and oestrus phases. Receiver operating characteristic (ROC) analysis was performed to determine the diagnostic accuracy of targets that statistically differed between *Brucella*-positive and negative animals. The diagnostic values were calculated for miRNAs that showed significant differential expression in the buffalo blood. Distance matrices were processed by multidimensional scaling (MDS) to obtain a dimensionally reduced map of the miRNA coordinates. Linear regression was used to investigate relationships between miRNAs and the buffaloes' ages. Spearman's Rho test was performed to evaluate whether the expression levels of the various miRNAs were correlated.

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Author Contributions

G.G., G.B. and C.L. designed the study. E.D.C. and F.C. guided the experimental performance. A.M. and D.V. collected the samples. C.L., C.C., V.Z. and G.P. performed the laboratory experiments and bioinformatic data analysis. C.L. wrote the main manuscript. All authors critically read and approved the manuscript.

Additional Information

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7 Manuscript draft 7

Evaluation of lymphocyte-related mRNA and miRNA in wheal exudates of water buffaloes *Bubalus bubalis* positive to the intradermal *M. bovis* and *M. avium* PPD test

Evaluation of lymphocyte-related mRNA and miRNA in wheal exudates of water buffaloes (*Bubalus bubalis*) positive to the intradermal *M. bovis* and *M. avium* PPD test

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19 ## Abstract

20 Bovine tuberculosis (TB) is a zoonotic disease primarily caused by *Mycobacterium bovis*
21 (*M.bovis*). The immune system activity changes during the disease process, showing a pro-
22 inflammatory milieu in the first phase that shifts to an anti-inflammatory environment in
23 the chronic phase. Programs of control and eradication for TB include a screening relying
24 an single intradermal tuberculin tests (SIT) using *M.bovis* purified protein derivatives
25 (PPD-B) and PPD avium (PPD-A) in single intradermal comparative cervical tuberculin
26 (SICCT), followed by additional analysis that includes measurement of gamma-interferon
27 (IFNγ) and serological immune responses. The diagnosis is confirmed after culling by
28 finding tuberculosis lesions and the positive microbiological culture of *M. bovis*. Although
29 applied to water buffalo as well, SIT sensitivity and specificity is reduced in this species
30 as compared to cows. The test sensitivity may be reduced by the coinfection with non-
31 tuberculous mycobacterial species generally present in the environment such as
32 *Mycobacterium avium* (*M.avium*). This study aims to investigate for the first time the
33 different lymphocyte switching between animals positive for *M.bovis* and for *M.avium*
34 PPDs in terms of expression of transcription factors, cytokines and miRNA within the
35 water buffalo wheal exudate after comparative SIT. The investigation was carried out on
36 mRNA extracted from wheal exudate of 36 animals of which 24 were *M.bovis*
37 positive(*M.bovis*+) and 12 *M. avium* positive classified (*M.avium*+). The polarization
38 toward Th1, Th2, TReg and Th17 lineages was addressed by measuring the abundance of
39 the respective cytokines and transcription factors, namely *TBET*, *STAT4*, *IFNγ*, *IL1β* for
40 Th1, *STAT5B*, *IL4* for Th2, *FOXP3*, *IL10* for TReg and *RORC*, *STAT3*, *IL17A* for Th17.
41 MicroRNA involved in immune response against TB, namely miR-122-5p, miR-148a-3p,

miR30a, miR-455-5p, were equally measured. We found that IFNγ was upregulated in *M.bovis*+ as compared to *M.avium*+ samples (fold change=2.54; p = 0.037), and positively correlated with two Th1 transcriptional factors, namely *TBET* (R2=0.43; p=0.025) and *STAT4* (R2=0.42; p=0.021). Among miRNAs, only miR-148a-3p was found to be increased in TB+ animals (p=0.03). We demonstrated the presence of a pro-inflammatory Th1-associated profile in *M.bovis*+ animals. miR-148a has been known to positively regulate *TBET*, suggesting its role in maintaining the pro-inflammatory milieu mediated by Th1 subsets.

51 ## Introduction

52 Bovine tuberculosis (TB) is a zoonotic disease caused by *Mycobacterum tuberculosis*
53 complex (MTB), which includes genetically related species belonging to the genus
54 *Mycobacterium*. MTB has been well adapted to wild and domestic animals and humans
55 (Malone and Gordon, 2017). In 2016, 147,000 new cases of zoonotic TB were estimated
56 in people globally, with more than 12,000 deaths associated to the disease (www.fao.org).
57 *Mycobacterium bovis* represents the major infectious agent in ruminants (Pesciaroli et al.,
58 2014), including water buffalo (Araújo et al., 2014; Barbosa et al., 2014). *M. bovis* is an
59 obligate intracellular pathogen and the transmission between animals primarily occurs by
60 inhalation (Menzies and Neill, 2000; Smith, 2003). In a second phase, the microorganism
61 adheres to the alveolar surface of the lungs, where it is phagocyted by macrophages which
62 in turn activate T cells (Neill et al., 2001). During the first TB phase, the disease remains
63 localized at cellular level and the bacterium is generally eliminated by macrophages
64 together with cytotoxic T (CTL) and Type 1 helper T lymphocytes (Th1). The polarization
65 toward Th1 lineage is associated to the developing of a type IV hypersensitivity reaction
66 with the consequent production of cytokines like IFNγ, IL1β, IL12, TNFα, playing a
67 pivotal role in the cell-mediated anti-mycobacterial immunity in both humans (Winslow et
68 al., 2008; Lin and Flynn, 2015) and cattle (Wang et al., 2011). The *Mycobacterium* may
69 adopt strategies to escape from the immune system at macrophage level, by inhibiting the
70 maturation and acidification of the phagolysosomes and promoting apoptosis (Zhai et al.,
71 2019), and at CD8+ and CD4+ T lymphocyte level, by delaying their activation (Urdahl et
72 al., 2011). If these adaptive reactions are successful, *Mycobacterium* survives and the
73 disease evolves toward a chronic evolution (Palmer and Waters, 2006). During the chronic

74 phase, the CD4+ T polarization shifts from Th1 to Type 2 lymphocytes (Th2), which favor
75 humoral immunity and the production of anti-inflammatory cytokines (e.g. IL4, IL13). A
76 Th2 polarisation also occurs after *M. bovis* experimental infection in vaccinated cows as
77 compared to those not vaccinated (Widdison et al., 2006). Type 17 (Th17) and regulatory
78 (Treg) T cells are also involved in tuberculosis immune response (Agrawal et al., 2018),
79 the pro-inflammatory activity of Th17 being antagonized by TReg (Saini et al., 2018). In
80 cattle, IL17A has been evaluated as possible biomarker for *M. bovis* tuberculosis,
81 demonstrating a strong correlation with IFNγ level in TB positive animals (Waters et al.,
82 2016).

83 The spread of TB is screened by using the Single Intradermal tuberculin test (SIT). SIT
84 were performed by accredited veterinary services according to Italian legislation (DD n.
85 226 03/10/2016). After measuring the skin fold of the shoulder using a calliper, PPD-B
86 (0.1 ml) (Istuto Zooprofilattico Umbria e Marche, Italy) were inoculated and the reaction
87 in terms of skin thickness, which features an inflammatory wheal, is evaluated after 72
88 hours (Good et al., 2018). In addition to SIT, supplemental tests (e.g. IFNγ) are also
89 included to confirm TB diagnosis, which is finally confirmed by pathology and
90 microbiology after culling. SIT is also used for water buffalo TB diagnostics, although it
91 is not as sensitive and specific. Water buffalo skin is thicker, making the interpretation of
92 SIT more difficult. *Mycobacterium avium,* a non-tuberculous *Mycobacterium* generally
93 present in the environment, may also interfere with the *M. bovis* PPD test, on the
94 background that the water buffalo skin is often covered with mud due to their attitude for
95 frequent bathing. The aim of this study was to understand the molecular basis of the cross-
96 reactivity between *M.bovis* and *M.avium*. The intradermal immune reaction within the

97 wheal exudate was investigated during a single intradermal comparative tuberculin test
98 (SICTT), using PPD from *M. bovis* and *M.avium*, by measuring the abundance of cytokines
99 and transcription factors related to T cell switching and miRNA associated to tuberculosis
100 and immune response.

101

102 ## Materials and methods

103 ### Experimental design

104 A total of 36 dairy water buffaloes (*Bubalus bubalis*) from 17 farms were enrolled. A
105 number of 24 animals were positive to single intradermal tuberculin tests (SIT), single
106 intradermal comparative cervical tuberculin (SICCT), IFNγ assay and/or culture test for
107 *M.bovis and with presence of tubercolar lesions* The remaining 12 animals were positive
108 to single intradermal tuberculin tests (SIT) and were negative for *M.bovis* and positive for
109 *M.avium* in SICCT and IFNγ assay. The animals positive to *M. bovis* to SICCT were sent
110 to the slaughter house, where the diagnosis was confirmed for the presence of tubercular
111 lesion (*M.bovis+*). The others were classified as positive for *M. avium* only (*M.avium+*).
112 After the test reading, the exudate was collected by means of a fine-needle aspiration
113 biopsy taken from the wheal generated by the intra-dermal reaction. The experimental
114 design illustration is available in Figure 1. T cells switching targets were focused on
115 transcription factors and cytokines related to Th1 (Schulz et al., 2009), Th2 (Zhu et al.,
116 2003), Th17(Ivanov et al., 2007) and regulatory T cells (Treg) (Chen et al., 2011). Four
117 miRNAs - miR-122-5p, miR-148a-3p, miR-30a and miR-455-5p - associated to immune
118 response, infectious disease and tuberculosis (Chiodini and Rossiter, 1996; Cousins, 2001;
119 Smith, 2003; Ahluwalia et al., 2017; Wu et al., 2019),were selected.

120

121 ### Study population

122 The animals included in this study are part of the regional water buffalo TB eradication
123 program. As part of the State Prophylaxis operations, all buffaloes tested positive for SIT

124 were isolated and submitted to SICCT and blood collection after a minimum of 42 days'
125 post SIT. Buffaloes that tested positive for SICCT were slaughtered.
126

127 **Intra-dermal reaction test and exudate collection**

128 The SICCT was performed by intradermal inoculation of 0.1 ml (30,000 I.U./ml) of PPD-
129 B (Istituto Zooprofilattico Sperimentale Umbria e Marche, Italy) on the left shoulder and
130 0.2 ml (25,000 I.U./ml) of PPD-A (Istituto Zooprofilattico Sperimentale Umbria e Marche,
131 Italy) on the left shoulder of each animal used Inj-Light tubercolin syringes. Before the
132 inoculation of the tuberculins and 72 hours later, the skin-fold thickness was measured with
133 the use of a calliper. The animal was considered reactive to bovine tubercolin if a swelling
134 at the site of PPD-B injection was positive and >4mm compared the site of PPD-A
135 injection. Conversely, the animals were considered avian reactors if exceeded the avian
136 reaction by >4mm compared to the bovine reaction. In all other cases the animals were
137 considered negative. At the time of slaughter, the exudate generated by the inoculation of
138 tubercolin, was aspirated using a syringe with fine-needle. The samples were quickly
139 mixed with an RNA later and stored at a temperature of -80°C until processing.
140

141 **Collateral performed tests (Microbiological culture, IFNγ test)**

142 ***IFNγ assay***

143 Heparinised blood samples were taken from each animal before the SICCT. They were
144 then transported to the laboratory immediately and at room temperature. The samples were
145 submitted to the laboratory and stimulated with two couple of purified protein derivatives
146 (PPDs), avian (PPD-A) and bovine (PPD-B), from Lelystad (Thermo-Fisher Scientific,

147 Lelystadt, Netherlands) and from Italy (Italian PPDs, Istituto Zooprofilattico Umbria e
148 Marche, Italy); phosphate buffer saline (PBS) was used as a negative control. Also a
149 positive control (pokeweed mytogen: PWM) was included for to verify lymphocyte
150 function and the possible use of lymphocyte inhibiting substances. Plasma samples were
151 analyzed using Bovigam (Bovigam, Thermo-Fisher Scientific, Schlieren, Switzerland)
152 following the manufacturer's instructions. Animals were considered positive if the optical
153 density (OD) of the sample stimulated with the two pairs of PPD-B was greater than or
154 equal than OD of PBSx2 and the OD ratio PPD-B/PPD-A was greater than or equal than
155 1.1 (interpretation of the Eradication Program for the water buffalo species in Campania,
156 Italy).
157
158 ***Bacteriology***
159 Slaughtered animals were subjected to post-mortem examination to detect the presence of
160 tuberculosis compatible lesions. Therefore, tissue samples (retropharyngeal, mandibular,
161 tracheobronchial, mediastinal, mesenteric, hepatic, sub iliac, supra mammary, popliteal,
162 prescapular lymph nodes, spleen, tonsils) were collected for culture of *M.bovis*. The
163 samples were transported to the laboratory, frozen and then processed in accordance with
164 the OIE manual (OIE Manual for Terrestrial Animals Cap. 2.4.6 par B.1, 1.1, 1.2 - 2015).
165
166 **RNA and miRNA extraction**
167 The RNA and miRNA were simultaneously extracted using the miRNeasy Micro kit
168 (QIAGEN). Briefly, 1 ml of QIAzol lysis Reagent (QIAGEN) was added to the wheal
169 exudate (50-100µl), homogenized and incubated for 5 minutes. Then, 3.75 µl (final

170 concentration of 25 fmol) of the *Caenorhabditiselegans* miRNA cel-miR-39 (QIAGEN)
171 was introduced as exogenous synthetic spike-in control. The procedure was carried out
172 following the manufacturer's instructions, with a final elution of 20µl for both RNA and
173 miRNA.
174
175 **Quantification of immune-related transcription factors and cytokines mRNA by RT-**
176 **qPCR**
177 The quality and quantity of recovered RNA was assessed using a NanoDrop ND-1000 UV–
178 vis spectrophotometer. The DNase treatment (DNase I, RNase free kit - Fermentas) and
179 the reverse transcription (iSCRIPT cDNA Synthesis kit – BIORAD) were scaled up with a
180 final volume of 60µl per each sample. qPCRs were carried out in duplicate for all targets
181 listed in Table 1. Each reaction was composed of 7.5µl of SsoFast™EvaGreenSupermix
182 (BIORAD), forward and reverse primers (listed in Table 1), RNase and DNase free water
183 and 1 µl of cDNA with a final volume of 15 µl. The thermal profile consisted of 95 °C for
184 10 min, 40 cycles of 95 °C for 10 s and 60, 61 or 61.5 °C (Table 1) for 30 s; the melting
185 curve was assessed by 80 cycles starting from 55 °C with an increase of 0.5 °C each 5s up
186 to 95°C. The CFX Connect Real-Time PCR Detection System (BIORAD) was used to
187 perform the qPCR. Two reference genes (YWHAZ and H3F3A) were selected and the
188 mean of reference gene abundance was used for normalization purposes using the 2^{-}
189 $^{\Delta\Delta Cq}$method. qPCR efficiency and R^2 were determined by means of a relative standard
190 curve (Table 1). Negative controls of qPCR were included by adding nuclease free water.
191 The Minimum Information for Publication of Quantitative Real-Time PCR (MIQE)
192 guidelines were followed (Bustin et al., 2009).

193 Digital PCR (dPCR) was carried out to quantify the Th17-related targets, namely RORC
194 (Assay ID Bt03256306), STAT3 (Assay ID Bt01653077) and IL17A (Assay ID
195 Bt03210252). YWHAZ (Assay ID Bt01122444) was used for data normalization. All
196 probes were checked for identity with water buffalo genome. A total of 12 samples (6 from
197 TB+ and 6 from TB-) wereincludedbased on qPCR results. Each reaction was composed
198 of 1 µl of cDNA, 8µlQuantStudio 3D Digital PCR Master Mix v2 (Applied Biosystem),
199 0.8 µl of TaqMan Advance (Applied Biosystem) and RNase and DNase free water up to
200 16µl of final volume. Fifteen µl of each reaction were loaded into the chip and run using
201 the QuantStudio 3D Digital PCR System (Thermofisher Scientific). The thermal profile
202 consisted of 95 °C for 10 min, 45 cycles of 60 °C (for YWHAZ, RORC and STAT3) or
203 56°C (for IL17A) for 1 min and 98°C for 30 s, followed by 60°C for 2 min. One negative
204 template control was used for each PCR and then applied to establish the threshold for data
205 analysis performed by means of the QuantStudio 3D AnalysisSuiteCloud Software.
206
207 **Quantification of immune-related miRNA**
208 Two µl of miRNA were reverse transcribed to cDNA by using TaqMan Advanced miRNA
209 cDNA Synthesis Kit (Applied Biosystems), following the manufacturer's procedure. The
210 cel-miR-39 spike-in (Assay ID478326_mir) and four miRNA, namely miR-122-5p (Assay
211 ID 480899), miR-148a-3p (Assay ID 477814), miR-30a (Assay ID 478273) and miR-455-
212 5p (Assay ID 478113), were quantified by qPCR using the Maestro CFX thermocycler
213 (BIORAD). All probes were checked for identity with water buffalo genome. Each reaction
214 contained 7.5 µl of 2X TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific),
215 0.75 µl of miRNA specific TaqManAdvance assay (20X) (Thermo Fisher Scientific), 1 µl

216 of cDNA and DNase and RNase free water up to the final volume of 15 µl. The thermal
217 profile was composed of 50°C for 2 min, 95 °C for 3 min and 40 cycles of 95°C for 15s
218 and 60 °C for 40s. Data normalization was carried out through the spike-in and miRNA
219 quantification was performed on CFX Maestro™ Software (BIORAD) using the $2^{-\Delta\Delta Cq}$
220 method. Negative controls of qPCR were included by adding nuclease free water.

221

222 **Statistical analysis**

223 Statistical analysis was performed using SPSS 23 (SPSS Inc., Chicago, IL, USA) and
224 XLSTAT softwares. The Shapiro-wilk test was used to assess data distribution. Where data
225 showed a not-normal distribution, they were normalized calculating the Log10. After
226 assessing the equality of variances through Levene test, the parametric t-test for
227 independent samples was performed to analyse the data. Mann-whitney test was used when
228 data were not-normally distributed. Target correlation was verified using the Pearson test.
229 The threshold for statistical significance was considered at $p<0.05$.

230

231

232 ## Results

233 **Quantification of transcription factors and cytokines mRNA related to T cell**
234 **switching by RT-qPCR**

235 The expression level of 11 targets, including transcription factors and cytokines, related to
236 T cell switching, were measured. In details, Th1 (*TBET, STAT4, IFNγ, IL1β*), Th2
237 (*STAT5B, IL4*), TReg (*FOXP3, IL10*) and Th17 (*RORC, STAT3, IL17A*) mRNA targets
238 were quantified by qPCR on 36 samples. Since the abundance levels of Th17-related genes
239 were under the limit of detection using conventional RT-PCR, the analysis was repeated
240 using dPCR on 12 samples. Results are presented in Figure 2. For Th1 related targets,
241 *M.bovis*+ samples presented an upregulation of IFNγ mRNA (fold change=2.54; p = 0.037)
242 as compared to *M.avium*+, whereas no differences were found for of IL1β, STAT4 and
243 TBET. For Th2 related targets, the mRNA of IL4 was not detected and no difference
244 between the expression level of STAT5B between the two groups was observed. For Treg
245 related targets, IL10 and FOXP3 were detected in all samples, but there was no differential
246 expression between *M.bovis*+ and *M.avium*+ animals. For Th17 related targets, quantified
247 using dPCR and TaqMan probes, all targets were detected, but no differences were
248 significant, even if a trend of decreased expression of STAT3 and IL17A in *M.bovis*+
249 animals was appreciable. A positive correlation was observed between IFNγ and TBET
250 (R^2=0.43; p=0.025) and STAT4 (R^2=0.42; p=0.021).

251

252 **Quantification of immune-related miRNA**

253 MiRNAs were detected only in 9 out of 36 tested samples. Results are reported in Figure
254 3. Four TB-related miRNAs (miR-122-5p, miR-148a-3p, miR30a, miR-455-5p) were

255 measured. Although all miRNAs targets were over-expressed in *M.bovis+* animals, only

256 miR-148a-3p was different between two groups (p=0.03).

257

258 ## Discussion

259 To the best of the knowledge of the authors, this is the first study to investigate at molecular
260 level the differences between the wheals induced during inoculation of *M.bovis* PPD and
261 *M.avium* PPD. This study addresses one of the main issue related to *M.bovis* diagnosis in
262 water buffalo using the screen with comparative SIT. The study aimed at determining
263 whether the two PPD induced different switching toward Th1, Th2, TReg and Th17
264 lineages, and whether microRNA that were found to be involved in immune reaction
265 against MTB were equally differentially expressed. It was found that the main difference
266 within the two wheals was the upregulation of IFNγ and miR-148a-3p, that were
267 upregulated in *M.bovis*+ wheals. As the intradermal reaction result after *M. bovis* SIT is
268 difficult to read in water buffalo due to anatomical and behavioral differences as compared
269 with cow, we performed a SIT also using PPD from *M. avium,* which may interfere with
270 the normal test. The local immune response after SIT has not been investigated so far,
271 neither in water buffalo nor in other ruminants. It is known that this immune reaction,
272 which is regarded as a type IV hypersensitivity reaction, features a polarization toward Th1
273 lineage during the early infection phases, which thereafter shifts to a more Th2 oriented
274 response. A strong effect mediated by Th1 with an increase of IFNγ has been demonstrated
275 in *M.bovis* challenged animals as compared to the vaccinated ones (Widdison et al., 2006b).
276 The high expression of the Th1-associated IFNγ was also observed in polymorphonuclear
277 cells derived from *M. bovis* infected cows (Blanco et al., 2009). The findings presented in
278 this study demonstrated that one of the source of IFNγ is located in inflammatory wheal
279 during SIT test. Furthermore, the Th1 profile in *M.bovis*+ samples was also confirmed by
280 the positive correlation between IFNγ and two Th1-related transcriptional factors, namely

281 TBET and STAT4. Interestingly, *M.avium* PPD does not increase IFNγ production. IFN-γ
282 is an important cytokine in defenses against mycobacterial disease (Schroder et al., 2004)
283 and its seems involved in limiting infection by *M.avium* as well (Koets et al., 2015).The
284 switch toward Th2 lineage is limited to the chronic phase of *M.bovis* infection. The Th2-
285 related IL4 and IL13 cytokines were found to inhibit autophagy in both murine and human
286 infected macrophages with the consequent survival of the bacterium (Harris et al., 2009).
287 The Th2 pattern has also been linked to host protection as demonstrated after cattle
288 vaccination (Widdison et al., 2006). In our study, we did not observed any differential
289 expression in Th2 targets between *M.bovis*+ and *M.avium*+ groups. We did not find any
290 difference neither in Treg targets either. The role of TReg response has been poorly
291 investigated in cattle. In humans, it has been found that TRreg inhibits human memory γδ
292 T cells, reducing the production of IFNγ (Li and Wu, 2008) and depressing the T cell
293 mediated immune response (Hougardy et al., 2007). We did not find any differential
294 expression between *M. bovis* and *M. avium* positive samples, even if a decrease in *M. bovis*
295 positive animals was appreciable. Th17 cytokine subsets have been regarded to have pro-
296 inflammatory functions (Dong, 2009), with a particular activity against intracellular
297 pathogens (Khader and Gopal, 2010). They are also involved in immunity during
298 tuberculosis in humans (Torrado and Cooper, 2010) and in cattle as well (Waters et al.,
299 2016), although in humans controversial results have been observed regarding the Th17
300 role during the disease (Lyadova and Panteleev, 2015), showing both an increase (Wang
301 et al., 2011) or a decrease (Shu et al., 2017) in TB patients.
302 In the second part of the study, we also measured four immune-related miRNAs, namely
303 miR-122-5p, miR-148a-3p, miR-30a, miR-455-5p, that were demonstrated to be involved

304 in immune reaction during TB, within the wheal exudate. MiR-148a-3p was found to be
305 upregulated in a statistically significant way in water buffalos inoculated with *M.bovis* PPD
306 as compare to those inoculated with *M.aviu*m PPD. The upregulation of miR-122-5p has
307 been linked to infectious diseases (Wang et al., 2016), the increase of miR-148a-3p has
308 been found in TB patients (Ueberberg et al., 2014), as well as a higher expression of miR-
309 30a and miR-455-5p has been associated with tuberculosis in humans, with a reduction of
310 macrophage autophagy (Chen et al., 2015) and M1 pro-inflammatory activity (Ahluwalia
311 et al., 2017), respectively. Among the quantified miRNAs, all of them showed an
312 upregulated trend in *M.bovis*+ as compared to *M.avium*+ animal, but only the differential
313 expression of miR-148a-3p was statistically significant. The finding that miR-148a-3p was
314 upregulated is consistent with a polarization toward Th1 lineage, since this miRNA was
315 also been found to promote Th1 switching, activating TBET transcription factor (Haftmann
316 et al., 2015), and specific miRNA-148a antagomirs have also been demonstrated to
317 selectively target theTh1 cells (Maschmeyer et al., 2018), suggesting the role of miR-148a
318 in contributing to the pro-inflammatory environment mediated by Th1 cells. A study on
319 circulating miRNAs during *M.avium* infection did not detect any change in miRNA-148a
320 abundance (Farrell et al., 2015). We may therefore speculate that the production of miR-
321 148a-3p is local, and does not reflect the systemic asset of microRNAs.
322
323

324 ## Conclusion

325 The characterization of immune-related mRNA and miRNA within wheal exudate of water
326 buffalo after comparative SIT was carried out by inoculated with *M.bovis* and *M.avium*
327 PPD. As compared to *M.avium*, the major finding is that the wheal exudate in *M.bovis*
328 positive animals present an upregulation of the Th1-related IFNγ and miR-148a-3p,
329 suggesting the development of type IV hypersensitivity in *M.bovis* positive animal only.
330 Further studies are needed to improve miRNAs purification and better unravel the immune
331 response in *M. avium* PPD positive wheal exudate.

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501 **Tables:**

502 Table 1: Sequences of oligonucleotide primers used in the current study and design on the
503 basis of GenBank sequences, except YWHAZ from (Lecchi et al., 2012); H3F3A from
504 (Puech et al., 2015);IL4 from (Patra et al., 2013); IL10 from (Shah et al., 2012).

Gene	Accession number	Sequenza		Concentrazione primer (nM)	Efficienza (%) (R^2) Tm (°C)	Lunghezza amplicone
T-bet	XM_006074324.2	Fw 5'→3'	GCCGTCCCCAGCCTTTTCTGTC	250	94,4%; 0,998; 61,5°C	170
		Rv5'→3'	ACCCACAGCCAGAAGCAGCACC			
STAT4	XM_025277672.1	Fw 5'→3'	CGTTGGTCGTGGCCTGAACT	300	94,2%; 0,996; 61,5°C	95
		Rv 5'→3'	TGGCCCAGGTGAGATGACCA			
IL1β	NM_001290898.1	Fw 5'→3'	AGCTGCATCCAACACCTGGACC	300	99,1%; 0,996; 61,5°C	110
		Rv 5'→3'	ACAATGACCGACACCACCTGCC			
IFNγ	NM_001290905.1	Fw 5'→3'	GCTCTGCGTGCTTCTGGGTTT	300	109,1%; 0,994; 61,5°C	117
		Rv 5'→3'	GGGCCACCCTTAGCTACATCTG			
STAT5B	XM_025280120.1	Fw 5'→3'	TCTCCCCCGACCCCCATTTTCC	250	93,7%; 0,995; 61,5°C	81
		Rv 5'→3'	CCACGACTTCCCTTGCCCCAAC			
IL4	AY293620	Fw 5'→3'	GTACCAGTCACTTCGTCCAT	300	99,2%; 0,990; 52,0 °C 20sec (+est72°C 25sec)	197
		Rv 5'→3'	GCTCCTGTAGATACGCCTAA			
IL17A	XM_006056757.2	Fw 5'→3'	AACTCCAGAAGGCCCACCGA	300	100,8%; 0,995; 61,5°C	75
		Rv 5'→3'	CTCAGGGTCCTCATTGCGGT			
FOXP3	XM_006073647.2	Fw 5'→3'	ACCTGGAAGAATGCCATCCGCC	300	90%; 0,997; 61,5°C	147
		Rv 5'→3'	TGTGGGGTTGGAACACCTGCTG			
IL10	AB246351	Fw 5'→3'	TGCCACAGGCTGAGAACCA	300	97,7%; 0,991; 60°C	60
		Rv 5'→3'	TCTCCCCCAGCGAGTTCA			
H3F3A	NM_00101489	Fw 5'→3'	CGCAAACTTCCCTTCCAGCGTC	250	94,3%; 0,995; 61,5°C	102
		Rv 5'→3'	TCACTTGCCTCCTGCAAAGCAC			
YWHAZ	NM_174814	Fw 5'→3'	GCATCCCACAGACTATTTCC	250	97,3%; 0,998; 61,5°C	119
		Rv 5'→3'	GCAAAGACAATGACAGACCA			

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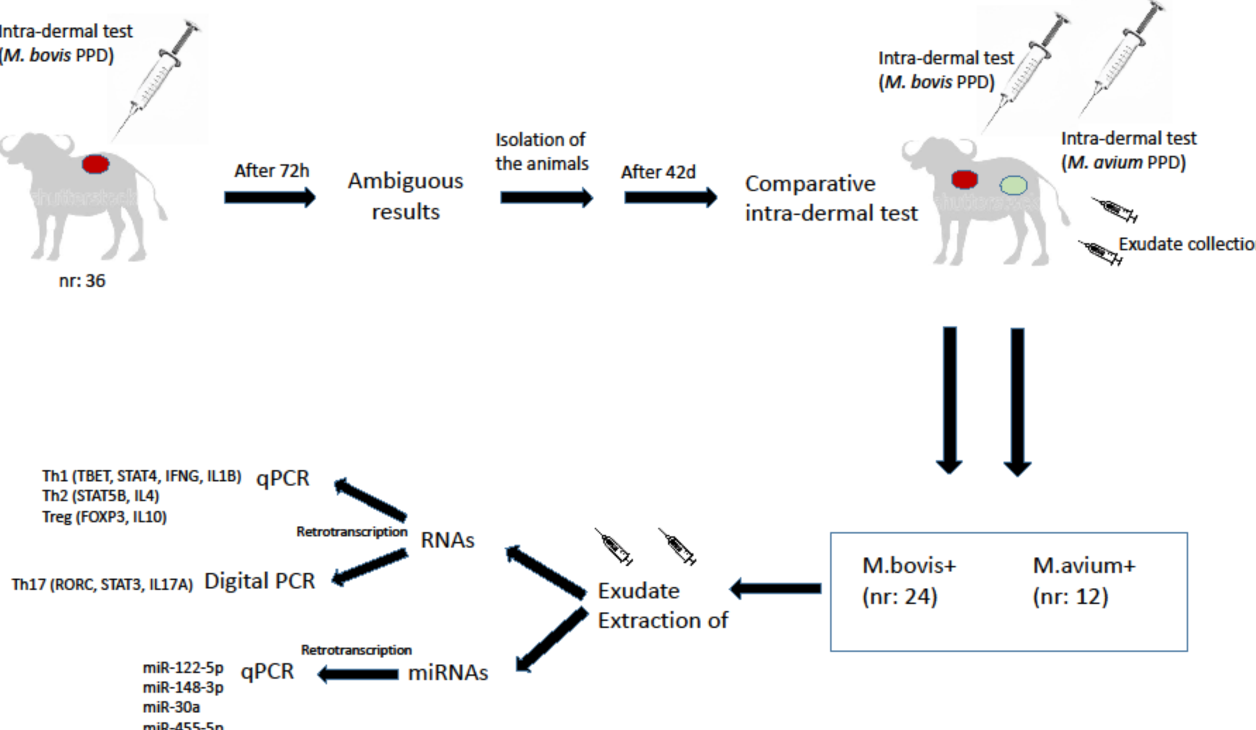
506

507 **Figures:**



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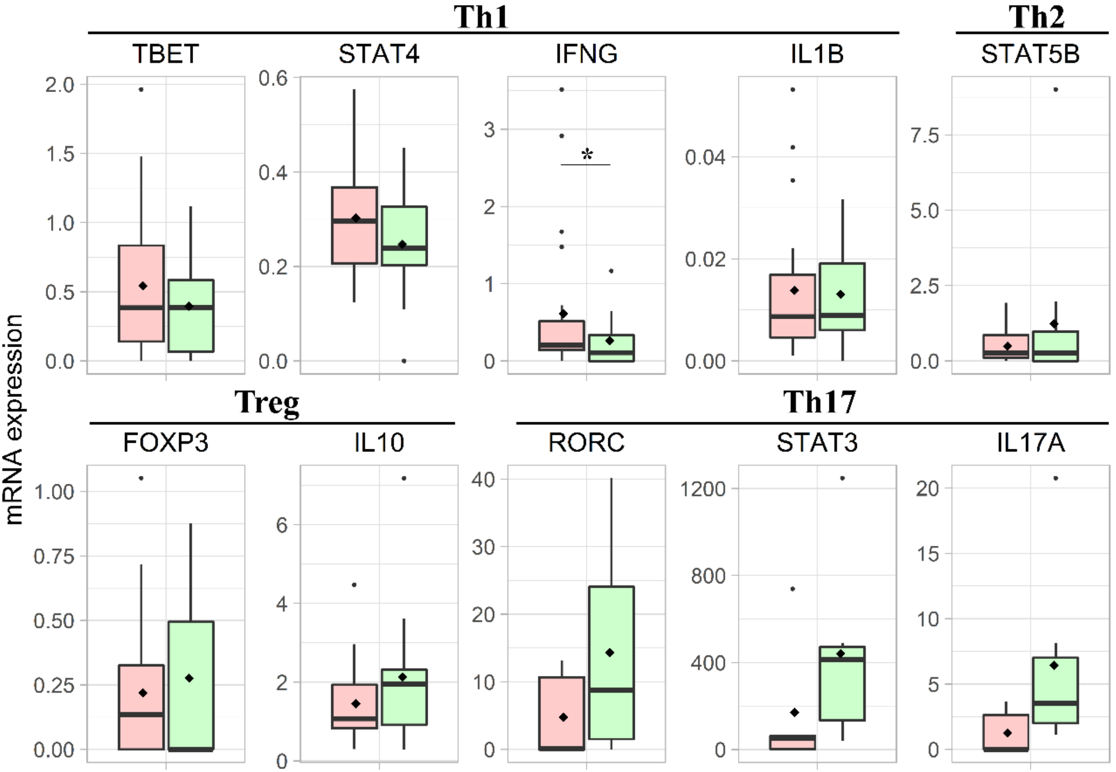
509 Figure 1: experimental design and sample collection of wheal exudate

510



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512 Figure 2: Relative expression of transcription factors and cytokines related to Th1, Th2,
513 Treg and Th17 polarization. Results for the target genes were normalised using the mean
514 of reference genes (YWHAZ and H3F3A). Data are shown as the mean ±SE of 36 animals
515 for Th1, Th2, Treg polarization (qPCR) and 12 animals for Th17 polarization (dPCR).
516 Significance was declared for * $p<0.05$. The black lines inside the boxes mark the medians.
517 Whiskers indicate variability outside the upper and lower quartiles. *M.bovis+* group is
518 shown in red; *M.avium+* is shown in green.

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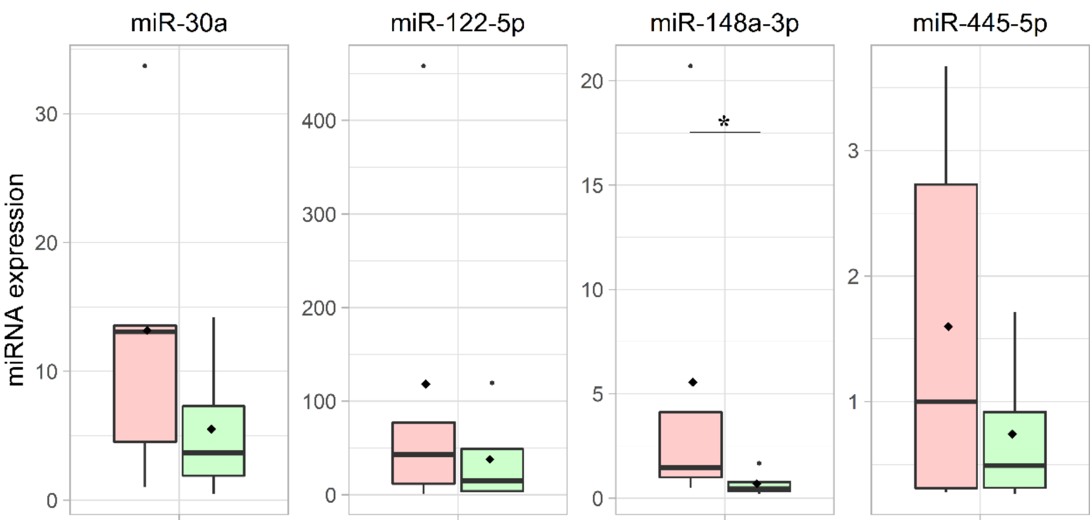

521 Figure 3: box plots of immune-related miRNAs. Significance was declared for * p<0.05.
522 The black lines inside the boxes mark the medians. Whiskers indicate variability outside
523 the upper and lower quartiles. *M.bovis+* group is shown in red; *M.avium+* is shown in
524 green.

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8 Manuscript draft 8

Acute phase proteins in water buffalos during the peripartum period

Acute phase proteins in water buffalos during the peripartum period

Introduction

Water buffaloes (*Bubalus bubalis)* are important dairy animals ranking second in milk production worldwide (FAOstat, 2019). Compared to Asia where about 98% of buffalo milk are produced, the share of Europe is only 0.2%; Italy is nevertheless the 6th biggest producer of buffalo milk worldwide (FAostat, 2019, data from 2017). In Italy, buffalo milk is mainly used for producing mozzarella, a fresh soft cheese. The number of water buffalos and the amounts of buffalo milk produced in Italy in 2017 are 8.6 and 7.5-fold greater than 50 years ago, respectively, i.e. compared to 1967 (FAOstat, 2019). During the same time the number of dairy cows (*Bos taurus*) has decreased to about 51% of the 1967 values but the amount of cows' milk produced was 16% greater than in 1967. Even though these are average values and individual variation is high (Ingvartsen and Moyes, 2013), these numbers indicate that the average milk yield per animal has more than doubled in case of cows (2.2-fold), whereas in case of buffalos the average individual milk yield was largely maintained or even slightly less than 50 years ago (0.87-fold). For dairy cows, concerns have been raised since more than 20 years that the increase in milk yield, mainly driven by genetic selection but also improved feeding and management strategies, is putting cows at risk for various metabolic but also infectious diseases (Gröhn et al., 1998; Ingvartsen and Moyes, 2013).

The most critical phase during the lactation cycle in dairy ruminants, in particular in dairy cows, is the transition from late pregnancy to early lactation, with a focus on the peripartum period. The negative nutrient balance during the first weeks of lactation, during which feed intake cannot meet the need for the rapidly increasing milk production, imposes metabolic stress which may result in several diseases and decrease fertility (Esposito et al., 2014). During the peripartum period, dairy cows have

26 compromised liver function together with increased inflammation and oxidative stress
27 (Bionaz et al., 2007; Trevisi et al., 2012). Some degree of inflammation during this
28 period has been demonstrated to be physiologically related to normal function of the
29 reproductive and the immune system, supporting the metabolic adaptive reactions as
30 well. For example, the increase of Acute Phase Proteins (APP), a family of proteins that
31 are overexpressed during systemic inflammation, is believed to be related to the control
32 of inflammation, given that the function of most of them is exquisitely anti-
33 inflammatory (Ceciliani et al., 2012). If not properly balanced, and if resolution of
34 inflammation is delayed, the inflammatory status during peripartum may result in an
35 impaired health and productivity of cows (Bradford et al., 2015). The increased
36 metabolic rate during the peripartum period also demands more oxygen which in turn
37 increases the production of oxygen-derived reactants, collectively termed reactive
38 oxygen species (ROS). Augmented ROS production is normally controlled by the
39 antioxidant defense systems. The failure of balancing pro-oxidative and anti-oxidative
40 reactions is termed oxidative stress, which in turn interferes with proper resolution of
41 inflammation (Bradford et al., 2015).
42 In periparturient water buffalos, the information about the metabolic status is limited. A
43 recent study carried out in Murray water buffaloes showed that the energy balance is
44 negative in the first 4 weeks of postpartum (Golla et al., 2019). A similar study on Italian
45 Mediterranean Buffaloes identified an increase of Non-Esterified Fatty Acid (NEFA),
46 but not of β-hydroxybutyrate (BHB) during the weeks postpartum (Fiore et al., 2018).
47 The physiological situation in terms of inflammation. Beside their anti-inflammatory
48 properties, APP are regarded as reliable biomarkers to monitor the inflammatory status
49 of dairy cows during the peripartum period (Ceciliani et al., 2012). Little information
50 about water buffalo acute phase proteins is available, both during peripartum and also
51 in general. In this species, studies on APP are limited to acute diseases (Horadagoda et

52 al., 2002, 2001), identifying Serum Amyloid A (SAA), Haptoglobin (Hp) and α_1-acid
53 glycoprotein as major acute reactants. A recent study (Gianesella et al., 2019),
54 monitored the modification of Hp around peripartum, using a turbidimetric assay, and
55 showing no differences between ap and pp period. To the best of the knowledge of the
56 authors, no information is available on oxidative stress during peripartum in water
57 buffaloes.
58 In view of the virtual absence of the typical production diseases known from dairy cows
59 during early lactation in water buffalos, as well as the lower metabolic challenge due to
60 lesser milk yields, the main hypothesis behind this study was that the pattern of changes
61 in APP and oxidative stress indicators during the peripartum period would be similar to
62 dairy cows but the deflection would be less pronounced. For characterizing
63 inflammation in water buffalos, we assessed their blood concentrations of three positive
64 APP, namely Hp, SAA and AGP by means of ELISA. The measurement of the energy
65 balance of the water buffalos in the present study to characterize their metabolic status
66 is ongoing. Therefore, for what concerns the relationship between APP content and
67 metabolic status, the results presented in this study should be considered as preliminary.
68

69 **Materials & Methods**

70 *Animals and sampling*

71 The samples included in the present study were collected from Italian Mediterranean
72 Buffaloes (*Bubalus bubalis*) during diagnostic routine sampling collection aimed at
73 monitoring the health status of the herd during the transition period (from -8 weeks to +
74 8 weeks around calving). The analyses were carried out on leftover serum: This practice
75 is regulated by the Ethical Committee of the University of Milan (Comitato Etico
76 15.02.16 Parere numero 2/16) "allowing the use, under informed consent of the owners,
77 of the residual volume of samples for studies on metabolic biomarkers". In order to have

78 the same feeding and management conditions for the animals studied, the present
79 investigation was limited to samples collected from one single private farm located in
80 the North of Italy (Lombardia region, Province of Cremona, Italy). The herd size in this
81 farm was about 140 buffalo cows in milk; the average milk yield per 270 days of
82 lactation was 2827 ± 654 kg with 8.47% fat and 4.74% of protein. All buffalo cows had
83 a dry period of 120 days and were fed with Total Mixed Rations (TMR) for dry and
84 early lactating buffalos, respectively. The composition of the diets used during the dry
85 and the subsequent early lactation period is reported in Table 1. Individual feed intake
86 could not be quantified and therefore the energy intake of the animals during the dry
87 period and during lactation was estimated based on the energy content of the respective
88 rations and the amount of feed assigned per animal and day (Table 1). The energy
89 content of the roughage and concentrate components as well as the amount of TMR fed
90 per animal was not measured. Water was available *ad libitum*. Health status was
91 assessed daily by visual inspection of gross appearance, behavior and apparent feed
92 intake by the herdsmen. Ten buffalo cows (lactation number 4.6 ± 1.6; means ± SD)
93 were randomly selected and blood samples were collected from the *V. subcutanea*
94 *abdominis* after the morning milking (or corresponding time for the dry cows) in
95 approximately weekly intervals (8.9 ± 3.3 days) aiming to span the time from 8 weeks
96 (wk) *ante partum* (ap) until 8 wk *post partum* (pp). The average daily milk yield of these
97 10 cows during the recorded lactation time was 9.0 ±1 .9 kg with somatic cell counts
98 (SCC) of 165,454 ± 129,278 cells/mL (means ± SD). The blood samples were
99 transferred to the laboratory at the University of Milan, serum was separated and stored
100 frozen at – 80 °C until used.

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104 *Analysis of the Acute Phase Proteins Hp, SAA and AGP*

105 The concentrations of Hp in serum were measured by an in-house developed enzyme-
106 linked sorbent immunoassay (ELISA) for bovine Hp as described earlier by (Hiss et al.,
107 2009). In brief, the Hp ELISA is performed in a sandwich format, i.e. bovine serum is
108 coated to the wells of microtiter plates; after blocking free binding sites with casein, the
109 plates are washed, test serum or standard serum together with the rabbit polyclonal
110 antibody are incubated and after additional washing steps, the secondary anti-rabbit
111 antibody, labeled with horseradish peroxidase is added, and finally measuring the
112 optical density (OD) generated from a tetramethyl benzidine substrate is assessed. The
113 concentration of the standard serum was established using purified Hp and was also
114 calibrated against a standard obtained from an European Concerted Action on the
115 standardization of animal acute phase proteins (QLK5-CT-1999-0153). Crossreactivity
116 of the anti-bovine antibody with water buffalo samples was confirmed by Western blot
117 and dilutional linearity. Parallelism with the bovine standard curve were confirmed
118 when using serum from water buffalo for both the coating and as sample. A high degree
119 of accuracy and a broad analytical range of the assay at varying dilutions as prerequisites
120 for a valid relative quantification were thus substantiated. Given that one of the aims
121 was the absolute quantification, in order to improve the reliability of the water buffalo
122 Hp ELISA assay, water buffalo Hp was purified to homogeneity from serum following
123 the protocol of (Hiss et al., 2004) with minor modifications. In brief, affinity
124 chromatography on hemoglobin-sepharose followed by gel filtration as described
125 previously (Hiss et al., 2004) was used. The identity, purity and the concentration of the
126 bubaline Hp obtained was assessed by mass spectrometry, SDS-page and by assessing
127 the total protein content via the Bradford assay, respectively. After establishing a
128 bubaline serum standard curve, the modified assay was used for the samples collected

129 throughout the experiment. The pooled coefficient of intra and inter-plate variation was
130 10.2%.

131 The relative concentrations of AGP and of SAA were measured with commercial
132 sandwich ELISAs for bovine samples (Life Diagnostics Ltd., Stoke on Trend, UK -Cow
133 AGP-11 and Cow SAA-11). The application of the SAA assays specific for the bovine
134 species was previously validated in water buffalo (Glidden et al., 2018). Briefly, for
135 SAA assay, dilutions of 1:25, 1:50, 1:100 or 1:400 were used, depending on the protein
136 concentration. The ELISA was run following the manufacturer's instructions. For the
137 AGP assay, samples were diluted 1:20,000 or 1:5,000 when the concentration was too
138 low. Diluted samples were assayed according to the manufacturer's protocol. In both,
139 the SAA and the AGP assay, all samples were run in duplicate and the intra-assay
140 variability, measured by the coefficient of variation (CV%), was always less than 15%.
141 The optical density was measured at 450 nm on a Multiskan Plus MK II
142 Spectophotometer (Labsystem Oy, Helsinki, Finland) and the concentrations were
143 calculated using the four-parameter logistic regression of the XLStat software for
144 Windows (Addinsoft Inc, Long Island City, NY, USA).
145
146 *Statistical analyses*

147 Depending on the individual calving dates, the times of sample collection resulted in
148 different days relative to calving for the individual cows. The average number of
149 samples per cow was 9.45 ± 2.58 (minimum 6 and maximum 13). For the statistical
150 evaluation we classified the days relative to calving into weeks but limited the weeks
151 that were considered to those from which we had samples from at least 4 buffalo cows,
152 i.e. to the time between 6 weeks before to 8 weeks after calving. Linear mixed models
153 with time (wk) as repeated effect considering the nested periods ap and pp and cow as

154 random effect were used to evaluate the time courses of the different variables using
155 IBM SPSS25. Data are shown as weekly means ± SEM; the level of significance was
156 set at $P \leq 0.05$; P values > 0.05 and < 0.1 were considered as trends.
157 Calculations about energy supply and energy requirements were based on UFL values
158 (unité Fourragère Lait, i.e., forage unit for lactation, 1 UFL equals 7.11 MJ of net
159 energy). For dry cows, energy requirements of 6 to 7 UFL/day were taken as basis, as
160 suggested for pluriparous animals in their 10th month of gestation, assuming a body
161 weight of 600 kg. For lactating cows, the needs for maintenance (=1.4 UFL + 0.6
162 UFL/100 kg of BW, assuming a body weight of 650 kg, = 5.3 UFL) were added to the
163 requirements for milk production (0.74 UFL/kg of milk; milk was standardized to 8.3%
164 fat, 4.3% protein from the individual average milk composition data).
165

Results

Serum concentrations of the Acute Phase Proteins Hp, SAA, and AGP

In a first step, the study focused on unraveling whether the peripartum period in water buffaloes is associated with a systemic inflammation by measuring the concentration of APP. As a preliminary step, where required, the ELISA assays available for bovine species were validated in water buffalo species.

Measurement of serum Hp concentrations

In order to determine the serum concentration of Hp, the first step of this study was to validate the ELISA developed for bovine Hp in water buffalos. For improving the precision of the measurement, Hp was purified to homogeneity from water buffalo serum. The identity and purity of Hp purified from buffalo serum was confirmed by Western blotting, by mass spectrometry of the Hp band separated by SDS-PAGE and by silver staining of the SDS-PAGE gels in which the purified fraction were tested. Using a bubaline serum for coating in the sandwich ELISA and the bubaline standard, dilutional linearity was confirmed. The results of serum Hp concentration measurement are reported in Figure 1A, demonstrating that that the minimal and maximal concentrations found in water buffalos herein were 3 and 31 mg/mL, respectively. The Hp peak values were observed in the first week after parturition and was about 2-fold greater than the preceding concentrations ante partum and about 4 fold greater than the average of weeks 4 to 7 post partum, indicating also that the concentrations ante partum were greater than those observed from week 4 to 8 post partum (Figure 1A).

190 *Measurement of serum SAA and AGP concentration*

191 The time course of serum SAA and AGP concentrations is shown in Figure 1 B and C,
192 respectively. For SAA, peak values were observed in the first week after parturition and
193 a trend (P = 0.054) for time was found. The serum concentration of SAA ranged from a
194 minimum of 0,2 µg/mL to a maximum 7,9 µg/mL.

195 In case of AGP, minimum and maximum concentrations ranged from 50 to 120 µg/mL,
196 as presented in Figure 2C. The increase of the AGP concentrations from prepartum to
197 early lactation was later than for Hp and SAA and was also not limited to a peak value
198 but was continued until 6 weeks postpartum.

199

200 **Discussion**

201 The findings of this study yielded a picture of the peripartum situation in water buffaloes
202 combining the inflammatory status, as assessed by measuring the APP, The results
203 presented suggested that the metabolic adaptation of the water buffalos studied herein,
204 is not entirely corresponding to the changes commonly observed in dairy cows.

205 The acute phase proteins concentration, at least for Hp and SAA, was basically
206 correspondent to those reported for dairy cows, indicating a peak during the period after
207 the calving. The concentrations of Hp in buffalo serum assessed with the modified
208 ELISA using the bubaline standard were distinctly greater when compared to *Bos taurus*
209 cows: in previous studies it was observed that basal values < 1 mg/mL and peak values
210 around calving of up to 2 to 5 mg/mL (Hachenberg et al., 2007; Saremi et al., 2012) in
211 dairy cows. According to the standardization with the purified bubaline Hp together
212 with its purity, the greater values in buffalos as compared to cattle might be considered
213 as real. However, the Hp concentration baseline reported using an ELISA assay

214 (Glidden et al., 2018) were sensibly lower as compared to the present study. The Hp
215 concentrations reported for healthy versus parasite affected water buffalos were 0.1
216 versus 2.2 mg/mL from a hemoglobin-binding activity-based assay (El-Deeb and Iacob,
217 2012). When assessing Hp via its characteristic of inhibiting the peroxidase activity that
218 is proportional to the amount of hemoglobin, mean Hp concentrations of 0.6 mg/mL
219 were reported in healthy water buffalos (Tajik et al., 2012). Similarly, using a
220 biochemical assay, Hp concentrations between 0.2 and 1.1 mg/mL were reported in
221 water buffalo calves before and after injection with lipopolysaccharide, respectively
222 (Horadagoda et al., 2002). In all assays, the standard is the critical issue for the absolute
223 concentrations that are reported, but the information about the nature of standardization
224 is hardly reported. However, provided the common criteria of assay validity, such as
225 dilution linearity and reproducibility, as demonstrated herein for the modified ELISA,
226 relative comparisons are valid. For the purpose of the current study, i.e. characterizing
227 the time course of the Hp concentrations during the peripartum period in water buffalos,
228 the assays format we used was adequate. Moreover, the results of the present study were
229 in the same order of magnitude of a previous study on water buffalo peripartum
230 (Gianesella et al., 2019). The distinct peak that was observed in week 2 post partum
231 resembles the longitudinal profile reported in cows by (Zaworski et al., 2014). These
232 results were different from those previously reported, that measured Hp concentration
233 using a turbidometric assay (Gianesella et al., 2019) at day – 7, +7, +30, and +50. The
234 concentration of Hp did not change around peripartum, although it decreases at days
235 +30 and +50, corresponding to week 4 and week 7 of the present study. In dairy cows,
236 the common pattern of change described in the literature is that the basal values before
237 and after the peak at calving and early lactation are at about the same level, and peak
238 values are about 10 to 20-fold of basal (Hachenberg et al., 2007; Saremi et al., 2012;
239 Zaworski et al., 2014). However, when studying only from week 2 ante partum,

240 decreases from week -2 to -1 ante partum, followed by an increase towards week 1 post
241 partum were also reported (Deng et al., 2015).

242 To the best of the knowledge of the authors, this is the first report on SAA profile in
243 water buffalos during the transition period available until now. Values reported in
244 African buffalo (*Syncerus caffer*) are of in the same range than those report in the present
245 study (e.g. 0.4 µg/mL), increasing 12 times in the two weeks after calving. The
246 concentration after calving is also in the same range of concentration of SAA in water
247 buffaloes after experimental infection with FMDV (Glidden et al., 2018) and were in a
248 similar range as reported previously for water buffalos (3 – 4 µg/mL; (Tajik et al., 2012)
249 but lower than reported by (El-Deeb and Iacob, 2012) for healthy versus parasite-
250 affected water buffalos (24 versus 156 µg/mL). The distinct peak we observed in week
251 2 post partum resembles the longitudinal profile reported in cows by (Zaworski et al.,
252 2014). In the latter study using weekly sampling from 3 weeks before to 4 weeks after
253 calving, the peak values were reached 1 and 3 weeks post partum but were more than
254 10-fold greater than the ante partum values. In contrast, Razavi et al. (Razavi et al.,
255 2019) reported a decline in the circulating SAA concentrations from week -3 to -1 before
256 calving and constantly low levels until week 3. In the study of (Jafari et al., 2006), an
257 increase from day – 7 to the day of calving was observed, with declining concentrations
258 until day +21.

259 The concentrations we found were much lower than what was reported by (El-Deeb and
260 Iacob, 2012) in diseased versus healthy buffalos (370 versus 240 µg/mL) using a
261 commercial radial immune diffusion kit.

262 Also for AGP, this is the first report about its peripartum changes in water buffalos. In
263 cows, AGP is a moderate APP with a specific anti-inflammatory activity (Ceciliani et
264 al., 2012). The AGP concentrations during the transition period in dairy cows have been

265 reported to increase from day – 7 to +7 with a decline in the following 2 weeks (Jafari
266 et al., 2006). Other studies also demonstrated that AGP is increased in cows around
267 peripartum (Cairoli et al., 2006), although the concentration of protein was not
268 measured, and the increase refers only to 2D electrophoretic semi-quantitative maps.
269 On the contrary, the present study clearly demonstrates that AGP concentration is
270 increased starting from the 4th week after peripartum, with a peak at the 7th week. This
271 result is difficult to explain, on the background that no inflammatory status could be
272 identified during the seventh weak from calving.

273 The increase in concentration of the acute phase proteins around calving was largely
274 confirming previous data from both water buffalos and dairy cows. For Hp, the
275 concentrations ante partum were above the basal level observed post partum, which may
276 indicate some immunological stress but may also be a buffalo-specific pattern. For AGP,
277 there are too few studies, even in dairy cows, to allow for a solid evaluation of the
278 observed time courses being normal. The patterns obtained for SAA are well in
279 concordance with most of the previous reports. Taken together, the presence of an
280 inflammatory state around parturition, even in absence of disease, is in line with finding
281 in dairy cows but also many other species, including non-ruminants (Bradford et al.,
282 2015). Nevertheless, the magnitude of increase is greater in diseased animals as shown
283 for haptoglobin in dairy cows (Qu et al., 2014). The biological purpose of the
284 inflammatory reaction around parturition in healthy animals was suggested to support
285 the homeorhetic shifts in metabolism required for a successful transition period
286 (Bradford et al., 2015). In addition, this reaction may also be important in tissue
287 development and remodeling, e.g. for the mammary gland, the liver and adipose tissue
288 (Bradford et al., 2015).

289 Taken together, the buffalo cows in the present study were not or only slightly under
290 metabolic stress but exhibited an acute phase reaction around parturition which is likely
291 necessary for the adaptation to lactation.
292

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Table 1: Composition of the diets fed to dry and lactating water buffalo cows

	Dry period		**Lactation**	
	% of TMR	**Energy content (UFL/kg)**	**% of TMR**	**Energy content (UFL/kg)**
Ingredients				
Barley straw	21.1	0.3	1.65	0.3
Hay	21.1	0.5	-	-
Ryegrass hay	-	-	4.95	0.5
Concentrate for dry cows*	5.3	1.07	-	-
Concentrate for lactating cows**	-		20.8	1.07
Triticale silage	18.4	0.18	-	-
Lucerne hay	-		6.6	0.18
Corn silage	18.4	0.25	59.4	0.25
Water	15.7		6.6	
Calculated energy content# (UFL/kg)		2.05		12.5

* Vitamins and mineral additives per kg: 50,000 IU Vit. A, 49.25 mg niacin, 90 mg Vit. E, 6,000 IU Vit. D, 7.5 mg copper, 2.5 mg iodine, 150 mg iron, 108.5 mg manganese, 3 mg selenium, 200 mg zinc, 20,000 mg urea

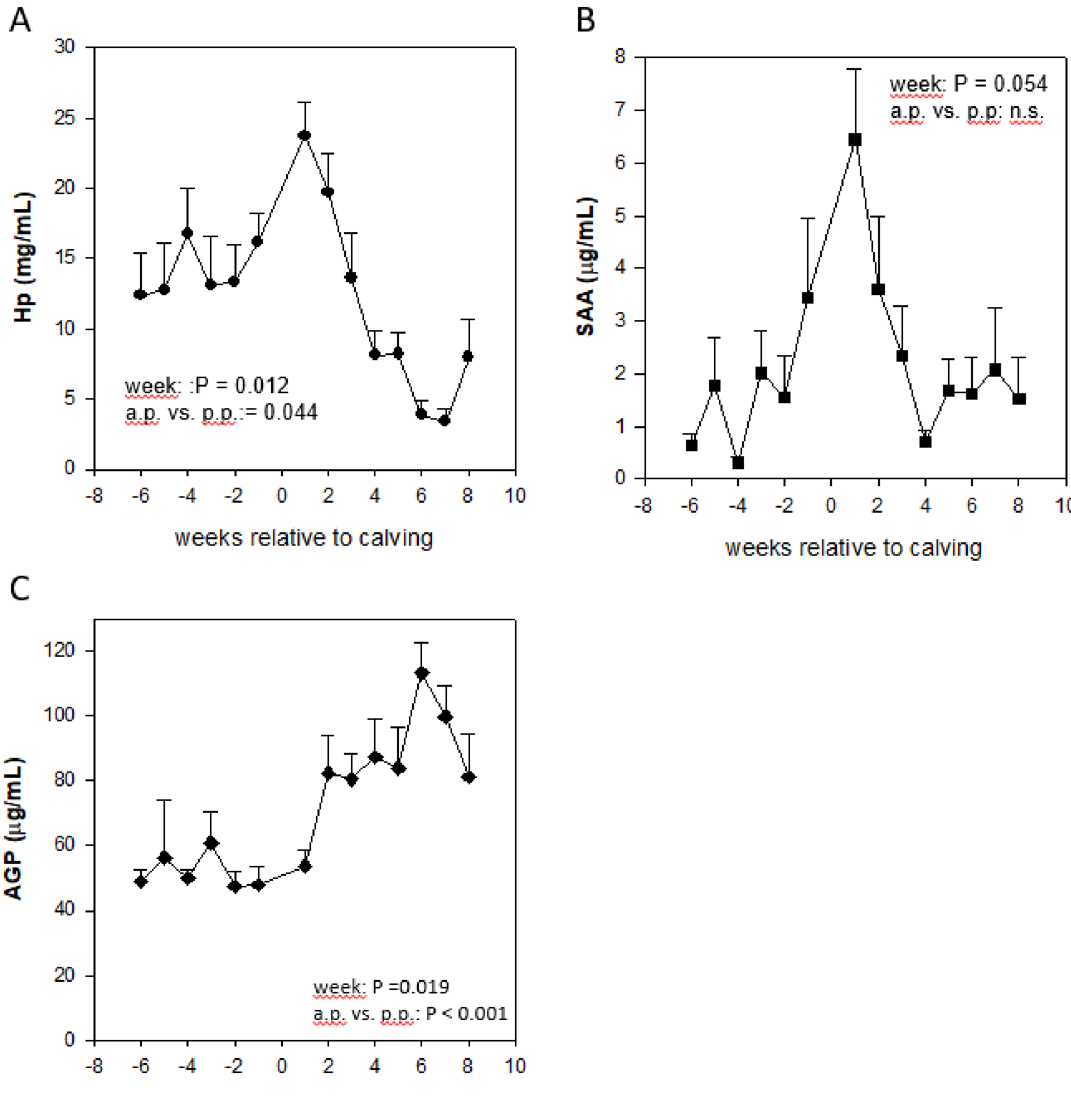
*Vitamins and mineral additives per kg: 18,625 IU Vit. D, 143,995 IU Vit. A, 312.8 mg choline chloride, 165.6 niacinamide, 10.9 mg calcium-D-pantothenate, 2.56 mg Vit. B1, 0.01 mg Vit. B12, 2.66 mg Vit. B2, 1.69 mg Vit. B6, 348 mg Vit. E, 0.94 mg Vit. K3, 7.5 mg copper, 8.35 mg iodine, 524 mg iron, 210 mg manganese, 3 mg selenium, 361 mg zinc oxide, 54.8 zinc sulfate, 125.1 DL-methionine, 29.8 L-lysine, 15.4 mg L-treonine, 2.5 mg tryptophane, 2 x 10^7 CFU *Saccharomyces cerevisiae* NCYC Sc 47.38 .

493

494 #The targeted feed and energy intakes for dry cows were 19.0 kg/day and 5.77 UFL/day,
495 respectively. For lactating cows 19.0 kg/day and 5.77 UFL/day, respectively, were
496 targeted.

497

498 **Figure Captions**



499

500 Figure 1: Time course of the serum concentrations (means + SEM) of three different
501 acute phase proteins. (**A**) Haptoglobin (Hp), (**B**) Serum Amyloid A (SAA), and (**C**)
502 Acidic Glycoprotein (AGP) in water buffalo cows from 6 weeks ante partum (a.p.) until
503 8 weeks post partum (p.p.).

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