

DANIELA IRLANDA CASTRO TARDÓN

**INTEGRATION BETWEEN HEALTHY AND MICROBIOME IN
HOLSTEIN COWS IMMEDIATELY AFTER CALVING**

São Paulo

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DANIELA IRLANDA CASTRO TARDÓN

**INTEGRATION BETWEEN HEALTHY AND MICROBIOME IN HOLSTEIN COWS
IMMEDIATELY AFTER CALVING**

Dissertation submitted to the Postgraduate Program in Veterinary Clinic of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Master's degree in Sciences.

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A Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, no cumprimento das suas atribuições, analisou e **APROVOU** a Alteração do cadastro (versão de 20/janeiro/2020) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Este subprojeto é parte integrante do projeto FAPESP 2016/16748-2, previamente aprovado pela CEUA n 3481060817. O subprojeto representa o mestrado da aluna Daniela Irlanda Castro Tardón, que inicialmente avaliaria apenas parâmetros de saúde da glândula mamária e microbioma do colostro e leite de transição. Após a análise global dos dados do projeto FAPESP 2016/16748-2, verificou-se que a apresentação dos dados na literatura seria mais adequada considerando-se todas as amostras obtidas a partir da vaca: colostro, fezes, secreção vaginal, já que a microbiota parece ser modulada pelo perfil metabólico e saúde das vacas no momento imediato após do parto. Sendo assim, solicitamos alteração da proposta inicial referente ao projeto de mestrado da aluna Daniela para inclusão das amostras de fezes e secreção vaginal, assim como dados clínicos obtidos no projeto FAPESP 2016/16748-2. ".

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DEDICATION

Dedico este trabajo a:

Mis padres, Irlanda Tardón and José Castro, por entregarme la educación y nunca medir esfuerzo para eso.

Mi marido y amigo, Danny Fuentes por creer en mí, por su compañerismo y por el amor que me entrega día a día.

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“Let’s not pretend that things will change if we keep doing the same things. A crisis can be a real blessing to any person, to any nation. For all crises bring progress. Creativity is born from anguish, just like the day is born from the dark night. It’s in crisis that inventive is born, as well as discoveries, and big strategies.”

Albert Einstein

RESUMO

CASTRO-TARDÓN, D.I. INTEGRAÇÃO ENTRE SAÚDE E MICROBIOMA EM VACAS HOLANDESAS IMEDIATAMENTE APÓS DO PARTO. 2020. 106 p. Dissertação (Mestrado em Ciências)- Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

O período de transição inclui eventos fisiológicos como o parto, colostrogênese e lactogênese, o que demanda o acionamento de mecanismos homeorréticos para atender as demandas energéticas impostas nesta fase do ciclo de produção. A inadequada adaptação da vaca no período pode evoluir para balanço energético negativo, doenças metabólicas, imunossupressão e alto risco para doenças infecciosas. Neste cenário, é factível acreditar que a microbiota da vaca sofre importantes alterações em diferentes sítios de colonização, e como causa ou efeito interfere na saúde do hospedeiro. O objetivo geral desta pesquisa foi avaliar as correlações entre parâmetros metabólicos e o estado de saúde da glândula mamária com a microbiota de vacas leiteiras imediatamente após o parto. Foram obtidas amostras sanguíneas de 20 vacas Holandesas múltíparas para a determinação de biomarcadores metabólicos, além de colostro, fezes e swabs vaginais para a realização do metagenoma. Reino Archaea foram apenas encontrados em amostras fecais e vaginais. As amostras fecais apresentaram maior abundância de Firmicutes, seguidas por Bacteroidetes e Actinobacteria. Amostras vaginais mostraram domínio dos Firmicutes seguidos pelos Bacteroidetes e Proteobacteria. Já a microbiota do colostro apresentou predominância de p_Proteobacteria, seguido do Firmicutes e Bacteroidetes. No nível taxonômico família, a *Ruminococcaceae* foi detectada como a mais abundante, seguida por *Bacteroidaceae*, *Lachnospiraceae* e *Clostridiceae* nas amostras fecais e vaginais. Por outro lado, as famílias predominantes detectadas nas amostras de colostro foram *Pseudomonadaceae*, *Staphylococcaceae* e, com menor abundância, *Enterobacteriaceae*. Foi observada correlação de Spearman positiva entre BHB e NEFA com as bactérias *Oscillospira*, *Treponema*, *Bacteroides* e *Methanocorpusculum* (Archaea). Ao contrário, o gênero *Lactobacillus* e a *Lachnospiraceae* apresentaram associação negativa com a condição de escore corporal, BHB e NEFA. Nas amostras de colostro, a bactéria do gênero *L7A-E11* apresentou correlação de Spearman positiva com a contagem de células somáticas (CCS) e correlação negativa com biomarcadores do metabolismo energético. O

Coprococcus e o *Treponema* apresentaram correlações negativas com a albumina, proteína total, triglicerídeos e colesterol. Por fim, o *Clostridiaceae* teve correlação positiva com a produção de leite das vacas. A análise das amostras vaginais mostrou que *Methanocorpusculum* teve uma correlação positiva com os biomarcadores do metabolismo energético, enquanto *Prevotella* e *L7A.E11* foram negativamente associados a esses marcadores. A análise de todo o ecossistema demonstrou que os parâmetros associados ao metabolismo, principalmente NEFA e BHB, estão associados a alguns gêneros bacterianos, principalmente em amostras fecais e vaginais de vacas holandesas imediatamente após o parto.

Palavras-chave: Microbiota. Periparto. Colostro. Perfil metabólico.

ABSTRACT

CASTRO-TARDÓN, D.I. INTEGRATION BETWEEN HEALTHY AND MICROBIOME IN HOLSTEIN COWS IMMEDIATELY AFTER CALVING. 2020. 106 p. Dissertação (Mestrado em Ciências)- Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

The transition period includes physiological events such as parturition, colostrumogenesis and lactogenesis, which requires the activation of homeostatic mechanisms to meet the energy demands imposed at this stage of the production cycle. The inadequate adaptation of the cow in the period can evolve to negative energetic balance, metabolic diseases, immunosuppression and high risk for infectious diseases. In this scenario, it is feasible to believe that the cow's microbiota undergoes important changes in different colonization sites, and as a cause or effect it interferes with the health of the host. The general objective of this research was to evaluate the correlations between metabolic parameters and the health status of the mammary gland with the microbiota of dairy cows immediately after calving. Blood samples were obtained from 20 multiparous Holstein cows for the determination of metabolic biomarkers, in addition to colostrum, feces and vaginal swabs to perform the metagenome. Archaea kingdom were only found in fecal and vaginal samples. Fecal samples showed a greater abundance of Firmicutes, followed by Bacteroidetes and Actinobacteria. Vaginal samples showed dominance of Firmicutes followed by Bacteroidetes and Proteobacteria. The colostrum microbiota, on the other hand, showed a predominance of Proteobacteria, followed by Firmicutes and Bacteroidetes. At the family taxonomic level, *Ruminococcaceae* was detected as the most abundant, followed by *Bacteroidaceae*, *Lachnospiraceae* and *Clostridiaceae* in fecal and vaginal samples. On the other hand, the predominant families detected in the colostrum samples were *Pseudomonadaceae*, *Staphylococcaceae* and, to a lesser extent, *Enterobacteriaceae*. A positive Spearman correlation was observed between BHB and NEFA with *Oscillospira*, *Treponema*, *Bacteroides* and *Methanococcus* (Archaea) bacteria. On the contrary, the genus *Lactobacillus* and *Lachnospiraceae* showed a negative association with the score of body condition, BHB and NEFA. In colostrum samples, the bacterium of the genus *L7A-E11* showed a positive Spearman correlation with somatic cell count (SCC) and a negative correlation with biomarkers of energy metabolism. The *Coprococcus* and *Treponema* showed negative correlations with

albumin, total protein, triglycerides and cholesterol. Finally, the Clostridiaceae had a positive correlation with the milk production of the cows. The analysis of vaginal samples showed that *Methanocorpusculum* had a positive correlation with biomarkers of energy metabolism, while *Prevotella* and *L7A.E11* were negatively associated with these markers. The analysis of the entire ecosystem showed that the parameters associated with metabolism, mainly NEFA and BHB, are associated with some bacterial genera, mainly in fecal and vaginal samples from Holstein cows immediately after calving.

Key words: Microbiota. Peripartum. Colostrum. Metabolic profile

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1 INTRODUCTION

The transition period of a dairy cow is defined as the period between 3 weeks before calving until 3 weeks after calving. The inevitable negative energy balance (NEB), the period of a few days before calving and at the beginning of lactation, reflects the most difficult phase for dairy cows. Dairy cows require a series of adaptations on their metabolic and endocrine systems to meet the energy requirements for the maintenance of pregnancy, calving and lactation. An inadequate physiological adaptation results in immunosuppression and the risk of infectious diseases (BAUMAN; CURRIE, 1980. GRUMMER, 1995; MULLIGAN; DOHERTY, 2008). Hence, this is a critical period associated with a peak in disease incidence, including those of metabolic, nutritional, or infectious nature. Therefore, this period has a strong impact on the production and profitability of dairy cattle.

In ruminants, nutritional challenges during the periparturient period can modulate composition and functionality of gastrointestinal tract (GIT) microbiota, resulting in altered microbial-driven metabolites along the GIT (WANG et al, 2012; LIMA et al, 2015). An altered GIT microbiome can affect systemic metabolic profiles (SHABAT et al, 2016). As such, changes in the GIT and circulating metabolite profiles may extend far beyond the GIT and affect overall physiology and immune homeostasis, which may modify the microbiota at several different body areas.

The microbiome and its metabolites have a crucial role in the maintenance of host homeostasis, and thus have become a booming research in both human and animal studies (LEE; HASE, 2014). The microbiota composition is of critical importance to the nutritive processes, health, and development of all known mammals, including cattle and other ruminants (ROUND; MAZMANIAN, 2009). Diverse microbial profiles exist in adult animals, and these differences are due to the varying niches imparted by localized variations in nutrient profiles, pH, transit rates, host physiology, immune cell populations, and the differing interactions between host-epithelia and symbiotic bacteria (YEOMAN et al, 2018). The suppression and over colonization of certain bacterial species in a specific niche results in disease pathogenicity and highlights the importance of understanding the interaction between host environment and its inhabiting microbes (RODRIGUEZ et al, 2015).

This research's hypothesis is that a correlation between the microbiome and the host has an influences in the health of the cow in the immediate period after calving.

2 OBJETIVES

2.1 General objective

The general aim of this study was to evaluate the correlation among metabolic parameters, mammary gland health status and the microbiota profile of dairy cows immediately after calving.

2.1 Specific objectives

1. Determine the microbial profile in fecal samples as a marker of the gastro intestinal tract microbiome, in colostrum and in vaginal secretion of multiparous cows immediately after calving, by sequencing the V3-V4 region of the 16S rDNA gene;
2. Correlate the metabolic status and the cow's microbiome immediately after calving;
3. Correlate the health of the mammary gland with the microbiome of the cow immediately postpartum.

3 LITERATURE REVIEW

This section will initially present the scenario to which the dairy cow is inserted in the transition period and will address information related to the profile of the microbiome in the peripartum.

3.1 Transition period scenario

The inevitable negative energy balance (NEB) in the last weeks of pregnancy and at the onset of lactation reflects the most challenging period for dairy cows. To meet the energy requirements for the maintenance of pregnancy, calving and lactation, dairy cows require a series of adaptations of the metabolic and endocrine systems (Bauman and Currie, 1980). This topic will address the main research conducted with a focus on metabolic adaptations in the transition period in dairy cows.

3.1.1 Metabolic adaptations

The transition period in dairy cows is comprised between three weeks prepartum to three weeks postpartum. It is an important and critical period for the health, productivity and profitability of dairy cattle (DRACKLEY, 1999). The transition from late gestation to early lactation is associated with extensive changes in metabolic, endocrine, and immune functions. During this stage, the cow goes through different adaptations that are necessary to maintain pregnancy and start lactation (GRUMMER, 1995; HERDT, 2000; SADRI et al, 2020). Calving is the critical moment of this period, because the dairy cow is subjected to several challenges that can affect her future lactations and fertility. Failure or inadequate adaptation to these changes can be a risk factor for different infectious diseases (DRACKLEY, 1999; VERNON, 2005).

The classical metabolic changes of the transition period start with the decrease of voluntary feed intake during the last weeks of pregnancy until parturition. Moreover, the increase of feed intake is slower than milk yield during the postpartum period (HAYIRLI et al, 2002). As consequence, a postpartum negative energy balance (NEB) is developed, since their dry matter intake does not increase sufficiently to meet the high energy requirement that follows the onset of lactation, especially in the last week before calving (BAUMAN and CURRIE, 1980; BAIRD, 1982).

The increase in nutrient requirements following parturition have been quantified in terms of mammary demands for synthesis of milk lactose, fat and protein versus the lesser prepartum needs of the conceptus for glucose, fatty acids (FA) and amino acids (BELL, 1995). It was estimated that in a Holstein cow producing 30 kg milk at 4 days postpartum, the mammary requirements for glucose, FA and amino acids are, respectively, 2.7, 4.5 and 2.0 times those of the gravid uterus during late pregnancy, and the estimated mammary requirement for energy is 3.0 times that of the uterus (BELL, 1995). On other hand, Goff and Horst (1997) estimated that the mammary requirement for calcium to produce 10 kg of colostrum on the day of parturition is more than double that for fetal growth in late gestation.

In order to supply the energetic demand, cows need to trigger homeorhetic mechanisms to attend a new physiological state and minimize the NEB. The concept of homeorhesis was applied to metabolic regulation by Bauman and Currie (1980) to account for the initiation and coordination of chronic metabolic changes in multiple body tissues that are necessary to support a dominant physiological state. Then, a key feature of homeorhesis is its mediation through altered responses to homeostatic effectors, such as insulin and adrenergic agents (BAUMAN & CURRIE, 1980).

Therefore, the cow needs a series of maternal adaptations to meet these metabolic requirements, along with the concept of directed partitioning of nutrients, the needs of the conceptus are given high priority by the homeorhetic controls it transmits to the dam. This imposition of pregnancy includes not only development of the fetus but, in addition, the growth of fetal membranes, the gravid uterus, and the mammary gland (BAUMAN & CURRIE, 1980). In Figure 1, a partial listing of metabolic changes which occur in transition period is presented. One of the major changes, which will be further discussed, occurs in adipose.

Since average values for dry matter intake (DMI) increase by only 30–50% between late pregnancy and Week 1 of lactation (ROCHE et al, 2005; DOUGLAS et al, 2006), much of the abruptly increased requirements for specific nutrients and energy must be met by increased hepatic gluconeogenesis and the mobilization of body stores of fat, protein and calcium. Indeed, was confirmed a major increase in hepatic gluconeogenesis in cows between 1-week prepartum and 1 week postpartum was confirmed (REYNOLDS et al, 2003; WHITE et al, 2012).

Figure 1 - A partial list of the metabolic changes associated with transition period in ruminants.

Physiological Function	Metabolic Changes	Tissue involved
Milk Synthesis	Increased use of nutrients	Mammary
Lipid Metabolism	Increased lipolysis Decreased lipogenesis	Adipose Tissue
Glucose Metabolism	Increased Gluconeogenesis Increased glygenolysis Decreased use of glucose Increased use of lipid as energy source	Liver Body tissue in general
Protein Metabolism	Mobilization of protein reserve	Muscle and other body tissue

Source: Adapted BAUMAN & CURRIE, 1980

As a compensatory mechanism to the high energy demand that occurs in the transition period, it triggers the massive mobilization of body fat which is achieved by a combination of increased lipolysis and decreased rates of lipogenesis and FA re-esterification in adipose tissue, leading to the net release of non-esterified FA (NEFA) and glycerol into the blood (BELL, 1995; DRACKLEY et al. 2001). These two components reach the liver through the bloodstream, then glycerol can be used in the production of glucoses, in the gluconeogenesis process, or combined with the NEFA for the recomposition of triglycerides (BRUSS, 2008).

During the transition period, the NEFA metabolized in the liver plays an important role as a source of energy. It is estimated that the liver is responsible for capturing 26% of the NEFA from the bloodstream (DRACKLEY et al, 2001). In this organ, the released NEFA can be metabolized in three ways: completely oxidized producing CO₂, water and ATP; oxidized to ketone bodies or to be re-esterified (DRACKLEY, 1999).

NEFA beta-oxidation process is carried out in the mitochondria of liver cells, there it is reduced to two carbons and converted to acetyl-Coa (acetylcoenzyme A), and in the presence of oxaloacetate the citric acid cycle enter, and it undergoes a series of reactions, generating energy and CO₂ (DRACKLEY, 1999; BEITZ, 1996). In the absence of sufficient amounts of oxaloacetate, acetyl-Coa is diverted to other metabolic pathways, becoming acetate, a precursor to ketone bodies such as beta-hydroxybutyrate (BHB) and acetone. Ketone bodies can be used as an energy source

in other extrahepatic tissues, especially in the heart and kidneys (DRACKLEY, 1999; BEITZ, 1996; HERDT et al, 2000).

In the peripartum, serum levels of NEFA and BHB are useful indicators of the ability of cows to face metabolic challenges in this period, measure fat mobilization and oxidation, respectively, and reflect the cow's success in adapting to the balance negative energy. At the time of calving, there is an increase in plasma concentrations of these indicators (HERDT, 2000; ARAUJO et al., 2009; MOREIRA, 2013). This was verified by a study by LeBlanc et al. (2005), they found that high concentrations of NEFA (≥ 0.4 mmol/L) 10 to 7 days before calving are associated with an increased risk of abomasum displacement, placental retention and decreased milk production. On the other hand, Duffield et al. (2009) reported that higher BHB values (> 1200 mmol/L) in the first or second postpartum week are associated with an increased risk of abomasum displacement and metritis.

High concentrations of NEFA suggest an accentuated BEN, with high rates of post-partum lipolysis (Li et al, 2012). Ospina et al (2010b) indicated that herds with more than 15% of the cows above 0.3 mmol/L of NEFA in the pre-delivery and 0.7 mmol/L, or 1,2 mmol/L of BHB in the post-partum present lower reproductive performance and greater chance of disease. Oetzel, (2004) suggested as a cohort point of 0.4 mmol/L of NEFA in prepartum and 1.4 mmol/L of BHB in postpartum, at least 10% of cattle being above those values in both parameters.

The stress of cows in the peripartum triggers an important endocrine mechanism that controls glucose levels, which are mediated by the activation of the somatotrophic axis that produces the release of catecholamines (epinephrine and norepinephrine) and cortisol, stimulate the sympathetic nervous system, resulting in a lipolysis that increases NEFA mobilization of adipose tissue and stimulates gluconeogenesis (GRUMMER, 1995; DRACKLEY, 1999). The activation of the somatotrophic axis favors lipolysis, this occurs due to an increase in the concentration of growth hormone (GH) and a decrease in IGF-1 levels. The GH increases lipolytic stimuli, attenuating the lipogenic response of insulin and inhibiting glucose capture by adipocytes (ROCHE et al, 2009).

Hypoinsulinemia resulting from a decrease in insulin production by the pancreas, and a decrease in insulin response are homeoretic mechanisms present in the peripartum (BELL; BAUMAN, 1997; DRACKLEY et al, 2001). This allows a greater availability of glucose for the fetus and mammary gland, independent insulin tissues,

while the other tissues begin to use ketone bodies to meet their energy needs (BELL, 1995).

Glucose is an important monosaccharide as a source of energy in all mammals, and during lactation it helps in lactose synthesis. Its concentration in ruminants depends on hepatic gluconeogenesis, lactate and amino acids (KERR, 2002). Its blood concentration it is regulated in such a way that allows small fluctuations, it is mainly controlled by insulin and glucagon hormones, however, several other factors can interfere with its concentrations, such as the action of catecholamines, glucocorticoid hormones and somatotropin (KANEKO, 2008).

In ruminants the main sources of glucose are cellulose, hemicellulose and pectins and, to a lesser extent, starch and disaccharides. They practically do not absorb glucose from the gastrointestinal tract, as it is fully fermented into volatile fatty acids in the rumen. In the ruminal epithelium, short-chain volatile fatty acids are absorbed and metabolized: 80% of the butyrate is converted to acetoacetate and BHB; and 50% of propionate can be metabolized to lactate or pyruvate (GONZALEZ; SILVA, 2006). The rest of the absorbed propionic acid is transformed into glucose in the liver through gluconeogenesis (THRALL et al, 2012).

Hepatic gluconeogenesis can be carried out by various biochemical pathways, depending on the glucose precursors. In ruminants, the main source of glucose is propionate, but there are other precursors of gluconeogenesis, such as glycerol obtained from lipolysis; lactate of anaerobic glycolysis in skeletal muscle; and amino acids from proteolysis (GONZALEZ; SILVA, 2006).

The normal concentration of glucose in adult ruminants is between 45 to 75 mg/dL (RADOSTITS et al, 2007), it is usually lower in the first weeks of lactation in relation to the concentration before calving. The metabolic changes that occur in the peripartum period require major adjustments in glucose production and utilization in maternal liver, adipose tissue, skeletal muscle, and other tissues. The hepatic glucose synthesis during late pregnancy and early lactation is increased to accommodate uterine or mammary demands. The balance of this monosaccharide is altered; Increased glucagon and insulin resistance increase gluconeogenesis and reduce glucose utilization by peripheral tissues. However, this recover as the animal progresses through mid-lactation (BELL, 1997).

Another metabolic marker to consider are triglycerides, they are formed by three long chain fatty acids attached to a glycerol-3-phosphate molecule. They can form in

intestinal cells, in adipocytes, hepatocytes, mammary glands and kidneys. Its synthesis is stimulated under conditions of high concentrations of insulin and low concentrations of glucagon and is inhibited in the opposite situation. Triglycerides produced in the liver bind phospholipids, cholesterol and apoproteins to form very low-density lipoproteins (VLDL). This lipoprotein transports triglycerides through plasma to other tissues. When triglycerides are removed from lipoproteins by the enzyme lipoprotein lipase present in various tissues, it becomes a low-density lipoprotein (LDL), which is then transformed into high density lipoprotein (HDL) (BASOGLU et al., 1998; THRALL et al., 2012).

Normal triglyceride levels for bovine species vary between 0 and 14 mg/dL (KANEKO, 2008). However, a study conducted in Brazil showed a reference value of 10.95 to 22.12 mg/dL in adult cows (POGLIANI; BIRGEL, 2007). The physiological states of pregnancy, calving and lactation are the ones that most represent changes in triglyceride levels. Normally, triglyceride concentrations in dry cows are lower than in lactating cows, and in the puerperium these values are especially low (POGLIANI; BIRGEL, 2007). Due to this great physiological change, the reference values must be specific for these phases of the production cycle. This study suggests the adoption of levels of 19.68 to 36.23 mg / dL in cows with 6 to 9 months of gestation and 6.5 to 15.8 mg / dL in cows with less than 30 days in lactation.

Ruminants have to virtually produce all of their cholesterol, the liver being the main organ responsible. Its synthesis is made of acetyl-CoA, beginning in the cytoplasm and ending in the endoplasmic reticulum (KANEKO, 2008). Cholesterol is a precursor to steroid hormones and vitamin D. It can be exported by the liver as a component of lipoproteins (VLDL) or in bile acids (THRALL et al, 2012). The cholesterol synthesized in the liver is exported as part of the VLDL for circulation and is used in other tissues. In bovine blood, most cholesterol is related to HDL, which represents 80% of circulating lipoproteins (BASOGLU et al, 1998).

The cholesterol levels considered normal are between 80 and 120 mg/dL (KANEKO, 2008). Yet, cholesterol varies widely due to sex, race, age and the puerperium (POGLIANI; BIRGEL, 2007). Considering this, research indicated a reference value between 94.63 and 146.93 mg/dL to be used in cows at the end of pregnancy and between 32.2 and 103.3 mg/dL for cows at the beginning of lactation (POGLIANI; BIRGEL, 2007). The cholesterol concentration is low on the day of calving and gradually increases in the first weeks after calving (CAVESTANY et al, 2005). Low cholesterol concentrations have been associated with metabolic problems, liver

steatosis, placental retention and low voluntary consumption (GRUM et al, 1996; KANEENE et al, 1997; GURETZKY et al, 2006).

Skeletal muscle is a reservoir of amino acids during the transition period (Bell, 1995). Amino acids play an important role in increasing gluconeogenesis during the beginning of lactation. The obvious importance of glycolytic amino acids as precursors of glucose immediately postpartum demonstrate the need to quantify the protein supply and metabolizable use by these animals (BELL et al, 2000; DRACKLEY et al, 2001). Due to its great importance in the homeostasis of the organism, considerable information can be obtained by measuring the total protein and its fractions (albumin, globulin and fibrinogen) (SMITH, 2009).

In adult animals, the protein concentration generally remains stable and may change during pregnancy due to the additional protein necessary for fetal growth (SMITH, 2009). Normal levels of total protein in bovine serum are between 6.8 and 8.6 g/dL (Smith, 2009). In the transition period, there are some changes in these concentrations. In the physiological state of pregnancy and lactation, hormonal changes, nutritional status, hydrous balance, the process of colostrumogenesis and other factors interfere with the plasma concentration of total proteins (BUTLER, 1983; JAIN, 1993).

Albumin is the most abundant plasma protein (about 50%) and is responsible for 75% of the oncotic pressure of intravascular fluid. It acts in the transport processes of non-esterified fatty acids, calcium, hormones, bilirubin and bile acids. Its synthesis is related to hormonal patterns, nutritional status, general liver conditions and stress (JAIN, 1993; THRALL et al, 2012).

According to some authors, the decrease in albumin concentrations at the beginning of lactation can also occur due to the demand for amino acids for the production of milk proteins, which reduces the synthesis of albumin and other proteins (CONTRERAS, 2000). Cows with protein deficiency do not respond adequately in case of illness and are more likely to get sick due to the lack of amino acids available to the immune system (VAN SAUN, 2000). An important tool to help diagnose liver disease is the albumin: globulin. Chronic liver damage results in a drop in albumin levels and an increase in globulin, the critical value for liver health is at a rate below 0.5 (BIRGEL JUNIOR et al, 2003).

Lipid accumulation in liver may contribute to health disorders and decreased milk production (DRACKLEY, 1999). In addition, most infectious diseases and

metabolic disorders occur during this time. Milk fever, ketosis, retained fetal membranes, metritis, and displaced abomasum primarily impact cows during the periparturient period (MALLARD et al, 1998).

3.1.2 Physiology and health of mammary gland in the peripartum period

Because of the metabolic requirements of the gland, particularly in early lactation, it has been suggested that the cow should be considered an appendage to the mammary gland, rather than vice versa (Bauman et al. 2006).

In the last third of pregnancy there are alterations of the secretory capacity of the mammary epithelial cells, the decrease in parenchyma occurs gradually during lactation, this decrease may be reduced to the metabolic conflict between pregnancy and lactation. In this phase, the drying of the cows is carried out approximately 60 days before calving, to prioritize the pregnancy and start a new productive cycle. (CAPUCO et al, 1997).

Between lactations, a nonlactating period is necessary for optimal milk production in the succeeding lactation. With cessation of milking, alveolar structure is largely maintained and little or no loss of cells occurs. However, increased apoptosis and cell proliferation, relative to that in lactating glands during the same stage of gestation, suggest that a nonlactating period serves to promote cell turn over prior to the next lactation (CAPUCO et al, 2001). Therefore, the dry period is important for the replacement of senescent and damaged cells of the mammary epithelium, increasing the epithelial component of the mammary gland before the next lactation. Replacement cells may be responsible for the expansion and maintenance of the secretory cells of the mammary gland that influence milk production in subsequent lactation (SORENSEN; ENEVOLDSEN, 1991; CAPUCO et al, 1997).

Colostrogenesis begins weeks before calving and stops abruptly at the time of parturition under hormonal influences, such as prolactin that is necessary for the structural and functional differentiation of mammary cells (BRANDON; WATSON; LASCELLES, 1971; BARRINGTON et al., 2001).

The colostrogenesis process produces the physiological edema in the mammary gland (MG), observed in 95% of the cows. This fact is the result of the increase in blood flow necessary for colostrum formation and nutrient demand for MG (DENTINE; MC DANIEL, 1983; HIBBIT et al, 2008). Other factors may also contribute

to it, such as hormonal influences, the pre-parturition inadequate diets, canionic diet, that contains high levels of sodium and potassium and damage to gland tissue caused by oxidative stress resulting from inflammatory processes (RANDALL et al, 1974; BLOCK, 1984). In addition, the authors report that MG edema is a risk factor for the appearance of mastitis within 30 days after calving (VAN DORP et al., 1999; WAAGE et al., 2001).

This process occurs prepartum and results in the formation of secretory colostrum that is typically collected at first milking postpartum. Colostrogenesis is thought to occur during the later stages of mammary development and is termed lactogenesis (NEVILLE et al, 2002). Mammary differentiation is called lactogenesis and consists of the set of processes that lead to the initiation of full lactation. Lactogenesis was shown long ago to occur in two stages: Lactogenesis I and II (HARTMAN, 1973).

The lactogenesis has been described as a period of proliferation and differentiation and the physiological differentiation includes the mammary epithelial cells capacity to conduct colostrogenesis (STARK et al, 2014). Near parturition, the gland undergoes a second set of developmental processes that lead to the secretion of colostrum, and then milk. This phase is referred to as lactogenesis II and is characterized by the closure of tight junctions between alveolar cells, and the movement of cytoplasmic lipid droplets and casein micelles into the alveolar lumina. A transient increase in the transfer of immunoglobulins and other protective substances characterizes colostrum formation (NEVILLE et al, 2002; STARK et al, 2014).

Colostrum is the first secretion after the dry period and is mainly composed of immunoglobulins, with IgG1 being the predominant isotype, which constitutes more than 90% of all proteins in this secretion. In addition, it contains viable cells that secrete a range of components related to the immune system, including cytokines, proteins and antimicrobial peptides. The main role of colostrum is to protect newborns and these immunological factors play an important role in the defense of the mammary gland against the invasion of pathogens (LARSON, 1992; OVIEDO-BOYSO et al, 2007; STELWAGEN et al., 2009).

The periparturient period is associated with rapid differentiation of secretory parenchyma, intense mammary growth, copious synthesis and secretion, and marked accumulation of colostrum and milk (OLIVER; SORDILLO, 1987). This series of alterations that occur during the dry period have characterized this stage as critical for

the appearance of mastitis, by observing the high rates of new mammary gland infections compared to the lactation phase (SMITH et al., 1985). Mammary infections that began during this period or that persisted from previous lactation in an apparent way, manifest clinically *in a subsequent* lactation (TODHUNTER et al, 1995; BRADLEY; GREEN, 2000). The first three weeks of the dry period and the pre and postpartum periods are critical phases for the appearance of new mammary infections (EBERHART, 1986).

Clinical mastitis occurs mostly in early lactation and is caused by environmental pathogens such as *Escherichia coli* and *Streptococcus* spp (OLIVER; SORDILLO, 1987). Environmental and contagious pathogens are relevant for the occurrence of breast infections in the dry period. The most important contagious pathogens are *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis*, while environmental pathogens include *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli*, *Klebsiella* and *Enterobacter*. As pathogens are considered minor, the coagulase negative *Staphylococcus* sp, which can be found in the skin of the teat, and finally *Corynebacterium bovis*, whose main reservoir are the mammary glands and the infected teat ducts. In addition to these, mastitis can be caused by *Prototheca* sp, *Pasteurella* sp, *Arcanobacterium pyogenes* and *Pseudomonas* sp (EBERHART, 1986; RADOSTITIS et al., 2002; CONSTABLE et al, 2017).

The entry path for these pathogens is ascending and can be facilitated by the presence of risk factors in the transition period. In addition to the anatomical changes that occur in the peripartum, such as inflammation of the mammary gland, the physiological edema and the proximity of the teat to the ground, there are immune factors linked to the function of the neutrophils that have been associated with the predisposition of the animals to infectious diseases during this period (SHELDON et al, 2006; RINALDI et al, 2008; CONSTABLE et al, 2017).

The clinical stage observed in this phase will depend on the clinical picture, level of infection and the pathogens involved, but, in general, mastitis manifests with a decrease in milk production, an increase in the volume and redness on the mammary gland or the affected rooms, pain, increased local or systemic temperature, which can even lead to the death of sick animals (RUEGG, 2011; RADOSTITIS et al, 2002).

3.2. Microbiome of the cow in the peripartum

3.2.1 General aspects of microbiome

The human body is inhabited by trillions of microbial cells whose coordinated actions are believed to be important for life (LEY *et al.*, 2006). Among the main benefits of the interaction between microorganisms and host are those related to energy metabolism through digestion of undigested carbohydrates and vitamin production (WANG *et al.*, 2012). These microorganisms can also exert antibacterial and immunomodulatory activities through different mechanisms: competition for binding sites; antimicrobial substances; intestinal barrier formation by inducing mucin production, epithelium permeability reduction; development of immune tolerance to commensal / symbiotic microorganisms, and induction of an immune response towards pathogens (GABORIAU-ROUTHIAU *et al.*, 2009; WANG *et al.*, 2012; FERNÁNDEZ *et al.*, 2013). These microbial populations can reach high densities, and collectively form a complex community known as **microbiota**. **The Microbiota**, develops over the course of the host's infancy to eventually reach its adult form (MILANI *et al.*, 2017; LOUZOPONE *et al.*, 2012).

Microorganisms that reside steadily in the host environment form the resident, endogenous or native microbiota (KOBOZIEV *et al.*, 2014). The bacteria present in a certain organism can be part of three populations; commensal, symbionts and pathobionts (HOOPER *et al.*, 1998). Commensal bacteria are permanent residents and offer no benefit or harm to the host; symbiotic microorganisms are known for their beneficial health functions; and pathobiont organisms are also permanent residents of the microbiota but have the potential to become pathogenic and produce diseases (HOOPER *et al.*, 1998).

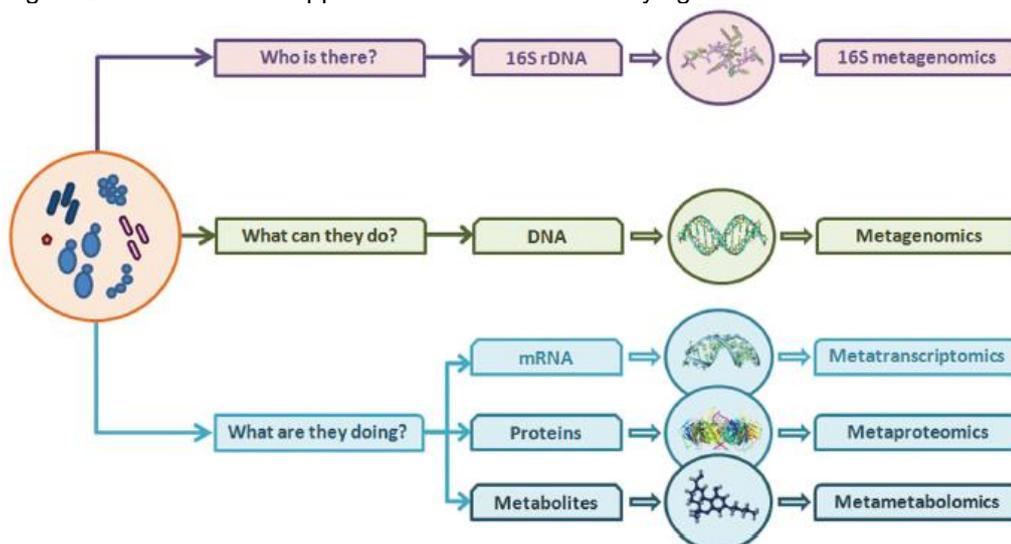
Microbiomics is an emerging investigative field which seeks to identify the constituents of the microbiome, analyze the microbial genome, characterize the interactions between the microbiome and host, and determine its influence on the pathobiology of disease. It is estimated that of the cultivability of the bacteria present in our organism varies between 10 - 50%, for example: higher in fecal samples, the cultivable fraction remains a minority (SHERMANA *et al.*, 2014). The reasons for this culture anomaly include the unknown bacterial growth requirements, the selectivity of the media used, the stress imposed by the culture procedures, the need for strictly

anoxic conditions and the difficulties to simulate the interactions of the bacteria with other microbes and host cells (CLEMENTE et al., 2012).

During the last decade there has been a dramatic increase in the application of approaches based on the diversity of sequences of the 16S ribosomal DNA gene (rDNA), a conserved bacterial gene region with hypervariable sequences that differ between species, to explore the diversity of bacterial communities in a variety of ecosystems (BARKO et al, 2018). Commonly, these methods involve amplification and sequencing of targeted microbial DNA regions followed by statistical data. In addition to DNA sequencing technologies, it has greatly increased advances in bioinformatics analysis of microbial identity and diversity based on sequence similarity and comparisons to reference microbial genomic databases (OUWEHAND et al, 2004; GREEN et al, 2006).

Along with these advances in technology for DNA sequencing, the term "metagenome" emerged, and it is defined as analysis of the collective genomes that are present and that is based on the objective sequencing of the 16S rDNA (ADDIS et al, 2016). As a result of rapid evolution of metagenomic sciences, a wide range of approaches is now available for detailed characterization, allowing gathering information that goes from its taxonomic composition to its functional potential and the molecules it produces as a result of its operation (Figure 2 and Figure 3) (ADDIS et al., 2016). The genomic content of a microbial community gives insights into its functional potential, but no information can be inferred about the functional activities in the microbiota. To reach this goal, additional -omics data shall be collected from the microbial community by means of metatranscriptomics, metaproteomics and metametabolomics (FRANZOSA et al, 2015) (Figure 2 and Figure 3).

Figure 2 - Outline of the approaches available for studying the milk microbiota.



Source: (ADDIS et al, 2016)

Figure 3 - Definitions and methods of analysis of the microbiome. Terminology and technical aspects related to the analysis of microbiomic data

<p>The 16S ribosomal gene: A universally conserved gene region with hypervariable sequences. Amplification and sequencing of this gene region permits identification of microbial organisms</p>	<p>Metatranscriptomics: analysis the RNA transcript pool expressed by a microbial community at a specific point in time, thus allowing a simultaneous investigation of the gene expression and abundance of microorganisms.</p>
<p>Metagenomics: Advances in the sequencing technology and computational tools permit the analysis of the content of the collective microbial genome and gene expression.</p>	<p>Metaproteomics: encompasses the large-scale study of the whole protein complement of a microbiota, providing a direct measure of the</p>
	<p>Metametabolomics: the systematic analysis of the metabolite complement produced by microbial communities.</p>

Source: Figure adapted from ADDIS et al, 2016; BARKO et al, 2017.

Given the importance of the microbiome in the physiology of the normal host and its role in association with a variety of diseases (BARKO et al, 2017). Considerable effort is currently focused on understanding the natural history of microbiome development in humans in the context of health outcomes, in parallel with improving our knowledge of microbiome–host molecular interactions (DURACK et al, 2018).

3.2.2 Colostrum microbiome

The human mammary gland and milk have traditionally been considered a sterile secretion. However, this belief has recently been questioned, as a result of integrating classical culture-based microbiological methods with more sensitive molecular methods (HOOD, 2012). Several studies have revealed that colostrum and breast milk are continuous sources of commensal, mutualistic and potentially probiotic bacteria for the infant gut (MOUGHAN et al, 1992; PEREZ et al, 2007). In fact, milk and colostrum constitute one of the main sources of bacteria to the breastfed neonate gut since a baby consuming approximately 800 mL/day of milk would ingest between 1×10^5 and 1×10^7 bacteria daily (HEIKKILÄ & SARIS, 2003).

In dairy cattle, mammary gland and milk microbiota research has been carried out focusing on how the milk microbiota changes once it becomes a food product, either for direct consumption or for transformation into dairy products, that is, by considering the microbial ecology of raw milk, rather than how the milk microbiota behaves in the context of animal health and physiology (QUIGLEY et al, 2013). However, as in humans, the bovine colostrum and milk microbiota exerts many influences in the short and long term on the physiology of the dams and their offspring (MALMUTHUGE et al., 2017).

The potential origin of the intramammary microbiota involves the existence of an entero-mammary pathway in humans and mice, through which live bacteria can be transferred from the intestine to the mammary gland (RODRÍGUEZ, 2014). This hypothetical endogenous pathway proposes that upon internalization of live bacteria by intestinal dendritic cells (DC) and macrophages, these bacteria can be transferred to the mammary gland via lymphatic and peripheral blood circulation.

Although there are no studies to differentiate the effect of farm managements and of other environmental factors on the composition of udder microbiota (e.g., management practices, nutrition, herd size, and so on), DERAKHSHANI et al, (2018) inferred that microbiota of bedding materials may also have a pivotal role in shaping the overall composition of the mammary microbiota. Generally, based on information from previous studies, the intramammary environment, and specifically the milk, seems to offer a welcoming ecosystem for a wide range of environmental bacteria (DERAKHSHANI et al, 2018).

Several studies investigating microbial communities present in milk and colostrum have deduced that, regardless of the inflammatory state of the udder, the breast secretion contains several bacterial communities (BHATT et al, 2012; KUEHN et al, 2013; OIKONOMOU et al, 2014; GANDA et al, 2017; BONSAGLIA et al, 2017; LIMA et al, 2017). *Staphylococcus*, *Ruminococcaceae*, *Lachnospiraceae*, *Propionibacterium*, *Stenotrophomonas*, *Corynebacterium*, *Pseudomonas*, *Streptococcus*, *Comamonas*, *Bacteroides*, *Enterococcus*, *Lactobacillus*, and *Fusobacterium* are the most frequently detected bacterial taxa within the microbiota of milk samples obtained from clinically healthy quarters (BHATT et al, 2012; KUEHN et al., 2013; OIKONOMOU et al., 2014; GANDA et al., 2017; BONSAGLIA et al., 2017).

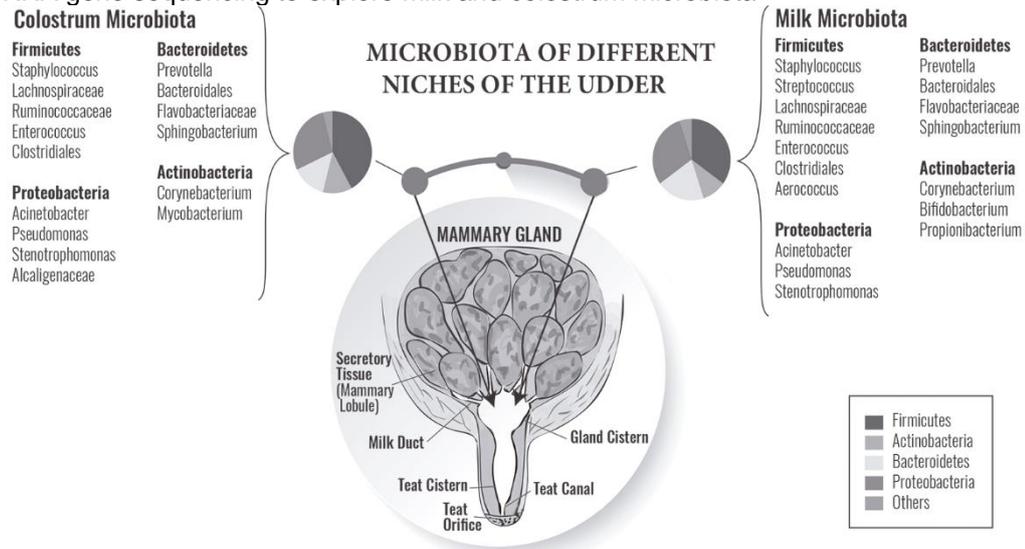
To date, only a few studies have used high-throughput sequencing technologies to determine global diversity of colostrum microbiota in relation to udder health and disease. Lima et al, (2017) used high-throughput sequencing of the 16S rRNA gene to investigate the bovine colostrum microbiome and its potential associations with early-lactation clinical mastitis from Holstein cows. Colostrum samples were dominated by Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Tenericutes phyla, with the 6 most common taxa being genera *Staphylococcus*, *Prevotella*, *Pseudomonas*, family *Ruminococcaceae*, and order *Bacteroidales* and *Clostridiales*. The colostrum microbiota of primiparous cows was significantly richer than that of multiparous cows, and differences in colostrum taxonomic structure between parities were also observed. The microbial community of healthy udder samples of primiparous cows was more diverse than that of udders diagnosed with mastitis samples. These results indicate that the colostrum microbiome of primiparous cows differs from that of multiparous cows, and it harbors some diversity and taxonomic markers of mammary gland health specific to primiparous cows only (LIMA et al, 2017).

In a study that examined calf gut microbiota, fecal samples from newborns repeatedly during the first two days of life using the 16S rRNA gene in order to evaluate possible sources of the calf faecal microbiota. In this investigation, samples from the dams (vaginal, colostrum and feces) were included to evaluate the possible sources of the fecal calf microbiota. Colostrum samples were dominated by phylum Proteobacteria and the *Enhydrobacter* genus was the most abundant (KLEIN-JOBSTI et al, 2019). In contrast, bovine colostrum samples from other similar studies were dominated by

Lactococcus, *Staphylococcus*, *Pseudomonas*, *Streptococcus*, and *Escherichia* (LIMA et al, 2017; YEOMAN et al, 2018)

A review published by DERAKHSHANI et al. (2018) compared the microbiota of milk and healthy bovine colostrum showing a broad predominance of the phylum Firmicutes and Proteobacteria in both sample types, but with higher abundance of Firmicutes in milk, and of Proteobacteria in colostrum (Figure 4).

Figure 4 - Proportions of main bacterial phyla were estimated based on studies that used 16S rRNA gene sequencing to explore milk and colostrum microbiota



Source: Adapted from DERAKHSHANI et al. (2018).

The defense mechanisms of the udder against microbial colonization are modulated by several host and environmental associated-factors that include: genotype of cows in coding of particular genes several components of the innate and adaptive immune systems (HERINGSTAD et al, 2000; RUPP; BOICHARD, 2003), changes in the physiological state and metabolic profile of cows during the different stages of the lactation cycle (LESLIE et al, 2000) and implementation of mastitis control practices, such as dry cow antimicrobial therapy, post milking/teat milking disinfection and hygiene (BARKEMA et al., 1999; GREEN et al., 2006).

Although still in its infancy, the current understanding of the mammary gland and colostrum gland suggests that the optimal diversity of the intramammary microbiota, composed of a healthy balance between commensal bacteria and opportunistic groups, is essential to maintain a balance between pro and anti-inflammatory, and thus maintaining udder homeostasis (DERAKHSHANI et al, 2018).

3.2.3 Vaginal microbiome

After birth, mammals are exposed to numerous bacteria. As of this moment, many factors influence the microbiome colonization process, among the main ones are the composition of the maternal microbiota, as well as the place and the form of birth (JERNBERG et al, 2010). This concept is supported by studies demonstrating an association between the composition of the infant intestinal microbiome and the route of delivery. In humans, infants delivered vaginally harbor microbial communities, dominated by *Lactobacillus spp.* and *Bifidobacterium spp.*, which are similar to their mother's vaginal canal. Conversely, baby's delivered by cesarean section are colonized by microbial communities composed of common skin microbes with *Staphylococcus* as the predominant genus (BIASUCCI et al, 2010; DOMINGUEZ-BELLO et al, 2010).

Studies involving cattle microbiota are focused on the gastrointestinal tract, and little is known about the vaginal microbiota. Some studies have attempted to identify the vaginal microbiota of cattle by bacterial culture and showed a dominance of Enterobacteria. In one study applying metagenomics techniques, the vaginal microbiome in Nellore cattle, heifers and cows, pregnant and non-pregnant was evaluated, and it was observed that the three most abundant bacterial phyla were Firmicutes, Bacteroidetes and Proteobacteria (LAGUARDIA-NASCIMENTO et al., 2015). A tendency for the reduction of bacteria and increase of archaea populations in pregnant animals was also noted.

A study recently published by Deng et al (2019) that evaluated the microbioma vaginal in beef heifers through gestation to examine the dynamics of vaginal microbial composition throughout pregnancy. The vaginal microbiome is dominated by an unclassified Enterobacteriaceae, followed by *Ureaplasma* and unclassified Bacteroidaceae. In this study, the top 2 dominating features of the vaginal microbiota are affiliated with *Escherichia/Shigella* and *Ureaplasma*, bacterial genus considered vaginal pathogens, due to its ability to establish residency in the reproductive tract from contamination by feces, rise the reproductive axis and maintain a presence in a contaminated uterus. The authors concluded that no change in community membership or structure is observed in the vaginal niche of the bovine female through gestation, suggesting the bovine vaginal microbiome is stable (DENG et al, 2019).

Bicalho et al, (2017) investigated the vaginal microbiota of Holstein dairy cows during the transition period, association with disease and fertility, using next-generation DNA sequencing of the bacterial 16S rRNA gene. The phyla associated with uterine disease and related risk factors were Proteobacteria, Fusobacteria, and Bacteroidetes. This study also reported the presence of 2 genera that have not been commonly found in the vagina of dairy cows: *Gallibacterium* and *Mannheimia*. This study demonstrated that the microbiota composition differed between health cows and cows that were diagnosed with uterine diseases. The results suggest that the microbial composition in the vaginal microbiome of dairy cows at calving and after parturition was predictive of uterine diseases and associated with subsequent decreased reproductive performance.

Other research conducted to evaluate similarity of taxa between the mother and the offspring, showed that in vaginal vestibule samples of cows at the time of calving there was a predominance of Firmicutes, followed by Bacteroidetes and Actinobacteria, and that this profile was similar to the profile of fecal samples of newborn calves (ALIPOUR et al, 2018).

A similar investigation, evaluated the impact of maternal microbial influences on the early choreography of the neonatal calf microbiome. The vaginal microbiota at time of calving was found to harbor and share many common and well-described fibrolytic rumen bacteria: *Bacteroides*, *Ruminococcus*, *Butyrivibrio*, *Prevotellaceae*, and *Pseudobutyrvibrio*, as well as methanogenic archaea. It was concluded that potentially indicating a role for the vagina in populating the developing rumen and reticulum with microbes is important to the nutrition of the adult animal (YEOMAN et al, 2018).

Although a number of common reproductive disorders in livestock involve bacterial infection, very little is known about their normal vaginal microbiota. Swartz et al, (2014) determined the species composition of sheep and cattle vaginal microbiota. Cow and ewe vaginal microbiota displayed few differences. Both ewes and cows were predominately colonized by the bacterial phyla Bacteroidetes, Fusobacteria, and Proteobacteria. The genus *Aggregatibacter* spp., *Streptobacillus* spp., *Phocoenobacter* spp., *Sediminicola* spp., *Lactobacillus* spp., and *Sporobacter* spp. were the major genera in cow samples. The relative dominance of *Aggregatibacter* spp. and *Streptobacillus* spp. is interesting and draws parallels to the dominance of lactobacilli often seen in the human vagina, some described members include important human pathogens such as *A. aphrophilus* and *A. actinomycetemcomitans*

that have been linked to periodontal disease, infective endocarditis, and brain abscess formation (RAVEL et al, 2011).

Microbial symbionts are essential for the development and physiology of mammals. The first microbial inoculums are vertically transmitted from the mother to the offspring. Major colonization starts at birth and is complemented during lactation and later in life (FUNKHOUSER et al, 2013; POWER et al, 2017). Therefore, understanding the maternal microbiota at the time of calving and how it is related to the mother's health parameters, can guarantee a colonization and proper health status of the offspring.

3.2.4 Fecal microbiome

Currently, there are currently limited comprehensive reviews of the intestinal microbiome in veterinary medicine. The intestinal tract microbiota is of fundamental importance for the processes of nutrition, health and development of all mammals, including cattle and other ruminants (ROUND and MAZMANIAN, 2009; YEOMAN et al, 2018).

Most studies regarding the gastrointestinal microbiome in mammals have focused on the fecal microbiome, although it is unclear how well fecal samples reflect the microbiomes of other intestinal regions. One study in humans demonstrated a strong positive association between fecal and mucosa associated with microbial communities for some genera (e.g, *Bifidobacteria spp.*), while other studies in neonatal bovines have revealed relevant differences between the microbial populations in feces and gastrointestinal tract mucosal biopsy samples (OUWEHAND et al, 2004; YEOMAN et al, 2018).

The bovine adult gastrointestinal (GI) tract microbiota has an important influence on animal health and production. A recent meta-analysis study of the bovine gastrointestinal tract was conducted by HOLMAN and GZYL (2019) to determine common taxa in the microbiota samples from the bovine GI tract. The methanogenic genera *Methanobrevibacter* and *Methanosphaera* were identified in nearly all fecal and rumen samples (>99.1%), as were the bacterial genera *Prevotella* and *Ruminococcus* (≥92.9%). Methanogens constitute a large majority of archaea in the cattle rumen and GI tract where they produce methane from substrates such as acetate, formate, methanol, hydrogen (H₂) and carbon dioxide (CO₂) that are produced by other

microorganisms during fermentation (DRIDI et al. 2009). Bacterial genera such as *Alistipes*, *Bacteroides*, *Clostridium*, *Faecalibacterium* and *Escherichia/Shigella* were associated with feces and *Fibrobacter*, *Prevotella*, *Ruminococcus* and *Succiniclaticum* with the rumen (HOLMAN; GYZL, 2019).

Faecalibacterium and *Clostridium spp.* produce butyrate, a SCFA, which is used as an energy source by epithelial cells in the mammalian GI tract, however, the *Clostridium* genus is highly heterogeneous with many of the 229 named species (PRYDE et al. 2002). *Ruminococcus spp.* are cellulolytic, as are *Fibrobacter spp.*, and members of both genera can break down cellulose, a large component of the typical plant-based diet of cattle, into energy that is utilized by animal or other bacteria in the rumen (KOIKE; KOBAYASHI, 2001).

JAMI et al (2014) have evaluated a connection between the composition and abundance of resident GI bacterial taxa and the physiological parameters in healthy Holstein Friesian lactating cows. One interesting finding was a strong correlation between the ratio of the phyla Firmicutes to Bacteroidetes and milk-fat yield. These findings are similar to the ones on human studies showing similar trends of increased adiposity with an increase in Bacteroidetes population. This correlation remained evident at the genus level, where several genera indicated parallels with the animals' physiological parameters. *Prevotella* was the most abundant genus in the samples and showed a significantly negative correlation with milk-fat yield, explaining most of the Bacteroidetes (JAMI et al, 2014). One species of *Prevotella*, *P. bryantii*, has been associated with probiotic activities: cows inoculated with *P. bryantii* strain 25A had decreased lactate production. That same study also showed an increase in milk fat during the weeks following inoculation (CHIQUETTE et al 2008).

An investigation on the association between the microbiome in dairy cattle in the peripartum period and the elimination of *Salmonella* was carried out by Munoz-Vargas et al, (2018) who concluded that individual cow fecal microbiomes, are predominated by Bacteroidetes, Firmicutes, Spirochaetes, and Proteobacteria phyla, observing significantly changes before and after parturition. At family-level taxa, *Bacteroidaceae* was predominant over other bacteria, followed by *Ruminococcaceae*, *Clostridiaceae* and *Prevotellaceae*. The relative abundance of major bacterial phyla, with the exception of Bacteroidetes, significantly changed through periparturient. At post-calving, they observed an increase in the abundance of *Spirochaetes* and

Actinobacteria, and a lower abundance of Proteobacteria, Verrucomicrobia, compared to the pre-calving period (MUNOZ-VARGAS et al, 2018).

Recent research evaluated the impact of maternal microbial influences in the time of calving, on the early colonization of the neonatal calf microbiome. They collected Holstein dairy cattle fecal sample in the calving time and it showed that family *Ruminococcaceae*, *Bacteroidetes*, *Clostridiales* and *Lachnospiraceae* were most the abundant ones. These studies concluded that the cow vaginal and the calf faecal microbiota were more similar, suggesting that some of the calf faecal microbiota may derive from inoculation from the birth canal during birth (ALIPOUR et al, 2018; KLEIN-JÖBSTL et al, 2019)

Current studies on the gastrointestinal microbiome have focused on the rumen, due to its importance in food conversion. Another focus of study has been the development of intestinal microbial colonization in calves. However, there are limited studies on the composition of the cow's microbiota at the time of calving and their effect on health status.

In ruminants, nutritional challenges during the periparturient period can modulate composition and functionality of GIT microbiota, resulting in altered microbial-driven metabolites along the GIT. An altered GIT microbiome can in turn affect systemic metabolic profiles. As such, changes in the GIT and circulating metabolite profiles may extend far beyond the GIT and affect overall physiology and immune homeostasis, resulting in modulation of microbiota profiles at various body sites (WANG et al, 2012; LIMA et al, 2015; DERAKHSHANI et al, 2017; SHABAT et al, 2016)

4 MATERIAL AND METHODS

4.1 Animals and management

The experimental procedures used in this study were approved by the Committee on Ethics in the Use of Animals of FMVZ-USP (CEUA N° 2329260218). This research was carried out between winter and spring of 2018 (from July up to November) in a commercial farm in Descalvado- Sao Paulo, Brazil, which geographic coordinates are Latitude: 21° 54' 14" South, Longitude: 47° 37' 12" West.

Twenty Holstein multiparous cows from 2nd up to 5th lactation (median = 3.00) were included in this research using the same criteria as expected on the date of calving, eutocic parturition and produce a minimum volume of colostrum at least three liters (median= 6.00 liters) with good quality IgG \geq 45 g/L (median= 60.00 g/L), measured by using colostrometer.

Historical data regarding dams was collected from dairy registries such as: weight of cows was estimated immediately after calving by using the thoracic perimeter, with 607.00 minimum and 856.00 maximum (median= 778.00 kilograms), duration of the dry period between 21 to 156 days (median= 57 days), number of lactations between 1 to 4 lactations (median= 2.00 lactations) and milk production in the last lactation from 10,067 to 19,008 liters (median= 13,543.00 liters). The parameters are described in Table 1.

Dry-cow treatment was made in the last milking of the previous lactation around -60 days before expected calving by using an intramammary antibiotic based on anhydrous cephalonium (Cepravin®, MSD Animal Health), with a withholding period of 51 days. At the same time, teat sealant (Teat Seal®, Zoetis) was also applied following the farm routine.

Table 1 - Descriptive statistical of the parameters of Holstein cows (n= 20) at calving – Sao Paulo-2020.

Parameters		Average	Standard Deviation	Median	Minimum	Maximum
Parity number	Number	3.05	0.97	3.00	2.00	5.00
Volume of colostrum	Liters	6.11	2.60	6.00	3.00	12.00
Colostrum quality	g/L	59.75	10.45	60.00	45.00	85.00
Duration of the dry period	Days	72.68	36.46	57.00	21.00	156.00
Number of lactations	Number	1.95	1.05	2.00	0	4
Milk production	Liters	13,491.21	2,305.15	13,543.19	10,067.33	19,008.37
Weight	Kg	756.70	76.55	778.00	607.00	856.00

Source: (CASTRO-TARDÓN 2020)

Around 60 and 30 days from the expected date of calving, the animals received diet A (Table 2) and concentrate (Table 3), while between 30 days from the expected date of calving until the real time of calving the cows receive diet B (Table 2). A common close-up diet was offered twice a day, considering the consumption of 13 to 14 kg of dry matter (DM) per animal. The proportion of the diet was calculated based on the percentage of materials in the roughage supplied (corn silage). Minerals and vitamins were added in the feed manufacturing and mineral salt and water were provided ad libitum. The diet met or exceed nutrients and energy demand requirements for pre calving dairy cows (NRC, 2001).

Table 2 – Diet A provided to dams between 60 and 30 days and dieta B provided to dams between 30d to calving time during the experiment.

Diet A (60-30d from expected calving)		Diet B (30d to calving)	
Item	% DM	Item	% DM
Concentrate A	5.92	Anionic concentrate	9.06
Soy bran	8.33	Wheat straw	12.41
Wheat straw	7.80	Soybean meal	19.82
Tifton grass	13.25	Corn silage	58.71
Corn silage	64.70	-	
Total	100	Total	100

Source: SANTOS (2020)

Table 3 - Concentrate A provided to dams between 60 and 30 days and Anionic concentrate provided to dams between 30 days to calving time during the experiment (recipe to 1000.9 kg)

Concentrate A		Anionic concentrate	
Item	Quantities (Kg)	Item	Quantities (Kg)
Vitamin E	2	NutriCAB®	25
Corn grain	1086	Colina	15
570 ca (Nutron)	510	Vitamin E	1
Urea	200	Salus NA	440
OmniGen® (Phibro)	100	Corn grain	425
Yeast	50	Vitamin AD3	250
Magnesium oxide	25	OmniGen® (Phibro)	40
Mycofix® (Biomin)	20	Yeast	25
Sel-plex® (Alltech)	2	Urea	15
Difly® (Champion)	1.5	Mycofix® (Biomin)	7
Biotin	1.0	Nexulin® (Pancosma)	41
-	-	Biotin	1
-	-	Sel-plex® (Alltech)	1
-	-	Difly® (Champion)	1
-	-	Probios® (Ourofino Saúde Animal)	0.5

Source: SANTOS (2020).

Cows were kept in pastures from d-60 up to d-31 (Figure 5A) and transferred to a compost barn system on D-30, where they were maintained until calving. Compost barn is a relatively new loose housing system (“freewalk housing”) for dairy cows that seems to increase their comfort. In this system, cows are provided with an open bedded pack area rather than the individual stalls and concrete alleys found in freestall systems

Births were monitored by the farm employees and our research team 24 hours a day. Cows were transferred to the maternity pens (Fig. 5C) within the compost barn system at the first signs of calving such as behavioral changes, isolation, mucous discharge, vulva edema and relaxing of sacral ligaments. Calving pens were maintained in hygienic conditions, dry, with good drainage and with wood shavings cover. The bed material was changed after each calving.

Figure 5 - Housing system used to maintain dams from d-60 up to calving time. (A) Pre-partum pasture (B) Compost barn system (C) maternity pens.



Source: Castro-Tardón (2020).

Calves were transferred for a *cuddle box* to stimulate maternal contact (Figure 6), while dams were contained in front of their offspring to be milked using an individual and mobile machine within of the maternity pens.

Figure 6 - The use of cuddle box to stimulate the maternal contact and colostrum secretion during the first milking time after calving.



Source: (CASTRO-TARDÓN 2020)

4.2 Mammary gland examination

The initial hygiene and antisepsis of teats were made following farm routine, removing the first jets of mammary secretion to eliminate the teat sealer used in the dry moment. After that, teats were dipped in a 2% chlorinated solution and dried with individual paper towels. During this procedure, the physical examination of the mammary gland (MG) was done by using the semiological techniques of inspection and palpation of structures following the procedures described by Rosenberger (2008). Then, each parameter was classified in a score scale according to the clinical finding severity. After the initial cleaning and disinfection by the dairy operator, the macroscopic characteristic of colostrum by using strip cup was evaluated (Table 4).

After all these procedures, cows were milking and the volume and quality of colostrum was also measured by using colostometer, Brix refractometer and colostrum balls.

Table 4 - Methods, parameters and score adopted to realize the mammary gland examination immediately after calving. Sao Paulo – 2020.

Methods	Parameters	Classification	Score
Inspection	Generalized Volume	Normal	0
		Increased	1
		Diminished	2
	Localized Volume	Absent	0
		Present	1
	Coloration	Normal	0
		Reddish	1
		Purplish	2
	Palpation	Mammary Gland Consistency	Normal/ Soft
Soft with small nodules			1
Soft with median nodules			2
Slightly hardened			3
Edemaciated			4
Hardened			5
Temperature		Normal	0
		Increased	1
Skin Elasticity		Normal	0
	Diminished	1	
Lymph nodes Retromammary	Volume	Normal	0
		Increased	1

Source: (CASTRO-TARDÓN 2020)

4.3 Body condition score (BCS) and weight at calving

The BCS was evaluated immediately after calving by using five-point and scale 0.25 increments, according to the criteria established by Edmonson et al, (1989) and adapted by Rosenberger et al. (2008).

4.4 Sampling

Before sampling, our team executed a 2nd preparation of the mammary gland to guarantee the microbiological quality of colostrum samples to the microbiome analysis. Using sterile gloves, the sampling protocol was made following these steps: 1. Udder was dry-cleaned using a soft brush; 2. teats antiseptis was sprayed by using povidone iodine (Riodeine®, Rioquimica), followed by drying with sterile gauze; 3. the final antiseptis was made by rubbing sterile gauze with ethanol 70%. 50 mL of colostrum in sterile tubes (Falcon®, BD Biosciences, San Jose, CA, EUA) was obtained per mammary quarter.

For vaginal and fecal sampling, the recto-vulvar region was also cleaned and disinfected. Initially, the region was cleaned with dry paper towel, followed by antiseptis by using povidone iodine and ethanol 70% disinfection in the separated steps. Finally, the area was dried with sterile gauze. Vaginal secretion was obtained close to the cervix using a sterile long swab covered by a plastic sheath. Swabs were placed in sterile and DNase-free microtubes. Fecal samples were obtained manually and immediately deposited in sterile universal collectors. Sterile gloves were used for all sampling processes.

The vaginal and fecal samples used for the microbiome analysis were kept in dry ice (-70°C) immediately after sampling and during transportation from the farm to the laboratory, where they were placed in storage at -80°C until further processing. Colostrum samples were maintained in the temperature around 4°C from harvesting time and during transportation to the laboratory, in the College of Veterinary Medicine and Animal Science at the University of São Paulo.

In the laboratory, fecal samples were unfrozen and divided in aliquots in DNase free tubes in sterile conditions within the laminar air flow. Fecal samples and vaginal swabs were stored in freezer at -80°C.

Colostrum samples were divided in aliquots per quarter in aseptic conditions in the following manner: 1mL of the total fraction of colostrum for microbiological examination and 5mL for cytological analysis. A colostrum pool per cow was used for the colostrum cells isolation and to perform the microbiome analysis. For this, 5mL of each quarter was mixed in a new sterile falcon tubes to complete a total volume equal 20mL and was diluted 1:1 with 0.9% sterile saline solution and subjected to centrifugation at 1500 g for 20 minutes, this process was repeated twice. The cell fraction was aliquoted into sterile DNase free microtubes and stored at -80°C.

For this investigation, negative controls (blanks) were included for each type of samples. In the farm, two sterile Falcon tubes were filled with molecular biology water (Molecular Biology Reagent, Sigma-Aldrich®) and processed in the lab following the same steps used to isolate the colostrum cells fraction. Two sterile swabs were added in sterile and DNA free microtubes as a negative control of vaginal swabs Also, two bed samples from the compost barn area were harvested. The objective of this process was to try to estimate the possible background in the microbiome analysis due to potential background contamination during the sampling process.

Blood samples from each cow were harvested by puncturing coccygeal vein in vacutainer tubes containing sodium fluoride (3 to 5 ml) and without anticoagulant (10ml) to obtain plasma and serum, respectively, to measure the glucose and other energetic metabolic parameters. The tubes containing blood without anticoagulant and with sodium fluoride were centrifuged at 1200 g for 10 minutes to obtain serum and plasma. After, the clinical material was transferred to the microtubes (1 mL) and stored in freezer -20°C until the moment of data analysis process.

4.5 Biochemical parameters and Haptoglobin

Plasma was used to measure NEFA, BHB and glucoses, while albumin, total protein, iron, triglycerides and cholesterol concentrations were determined from serum samples. Before measuring, plasma and serum samples were thawed overnight and vortexed. Subsequently, biochemical tests were performed on an automatic biochemical analyzer (Rx Daytona, Randox®) by using commercial kits, according to the manufacturer instructions (Table 5).

The concentration of haptoglobin (Hp) was determined by using the principle of binding between Hp and meta-hemoglobin (BASTOS et al., 2013). The standard

curve was prepared using a serial dilution of control serum. The determination of serum haptoglobin concentration was calculated by interpolation of the linear regression of the standard curve for each assay after reading of the absorbance with a microplate reader at a wavelength of 450 nm.

Table 5 - Reference range of biochemical parameters in Holstein cows immediately postpartum - São Paulo - 2020

Parameters	Mark	Reference
Total Proteins	Labtest	99-250
Albumin	Labtest	19-1/250
Triglycerides	Labtest	87-2/250
Cholesterol	Labtest	76-2/100
Iron	Randox	SI250
Glucose	Labtest	133-1/500
NEFA	Randox	FA115
Beta hydroxybutyrate	Randox	RB1007

Source: (CASTRO-TARDÓN 2020)

Subtitle: NEFA: non-esterified fatty acids

To determine the frequencies of metabolic disorders during the transition period, cohort points were selected according to the reference ranges available in the literature (Table 6).

Table 6 - Reference intervals of the biochemical parameters used to determine the frequencies of metabolic disorders in Holstein cows immediately postpartum - São Paulo - 2020

Reference Values of Biochemical Analysis			
	Units	Reference values	Authors
Glucose	mg/dL	45-75	Kaneko, 2008
NEFA	mmol/L	≤1.0	Ospina et al, 2010
BHB	mmol/L	≤1.2	Kaneko et al, 2008
Cholesterol	mg/dL	80-120	Kaneko, 2008
Triglycerides	mg/L	0-140	Kaneko, 2008
Total Protein	g/dL	6.8-8.6	Smith, 2006
Albumin	g/dL	2.7-3.8	Gonzales; Silva, 2006
Iron	µmol/L	17.9 -35.8	Radostits et al, 2007
Haptoglobin	mg/dL	≤20 mg/dL	Crawford et al, 2005; Pohl et al, 2015

Source: (CASTRO-TARDÓN 2020)

Subtitle: NEFA: non-esterified fatty acids; BHB: Beta hydroxybutyrate

4.6 Colostrum analysis

4.6.1 Cytology

Somatic cells count (SCC) was determined using the direct microscopic counting technique according to the methodology described Prescott & Breed (1910) adapted by Gomes (2011). Briefly, colostrum samples were diluted 1:1 in Phosphate Buffer Solution (PBS) distributed over an area of one cm². Then, they were dried in the room temperature for 24 hours. After that, slides were fixed in methanol for 15 minutes and stained with the Rosenfeld dye (ROSENFELD, 1947). The number of somatic cells were counted in 100 microscopic fields using an optical microscopy with immersion objective (x1000). The total cells counted were multiplied by the factor of the optical microscope (17,000) and dilution of the sample (x2), obtaining the number of somatic cells/mL of colostrum.

The differential exam of SCC was performed using the cytocentrifugation technique following the procedures described by Gomes et al. (2011). 15 mL of colostrum from each quarter was diluted in 5 mL of PBS and centrifuged at 1200 g for 5 minutes. After centrifugation, colostrum presented three distinct phases: the cell pellet, a portion of intermediate fluid and a layer of fat. The process of centrifugation was repeated in the same condition 3 times. Finally, cellular pellet was diluted in 1mL of cellular culture medium (RPMI 1640-GIBCO, Sigma-Aldrich®). Cellular suspension was centrifuged in a cytospin centrifuge (CITOSPIN, INCIBRAS®). Slides were also stained (ROSENFELD, 1947) and examined in an optical microscopy (x1000) to differentiate leukocytes classified in lymphocytes, monocytes, neutrophils, eosinophils and basophils, according to the cellular morphology particularities described by Gomes, 2008. The results for this variable were expressed in proportion of cells (%).

4.6.2 Bacterial culture

To identify the bacterial infection of mammary gland, we used an agar-plate culture system for identification of main pathogens or confirmation of pathogen-free colostrum. Samples of colostrum were plated on 5% sheep blood agar plates. The plates were aerobically incubated at 37°C, and making readings at 24, 48 and 72 hours. Samples that showed growth of three or more colonies of the same

microorganism were considered positive (NATIONAL MASTITIS COUNCIL, 1999). The bacterial identification was due to its morphological and Gram coloration characteristics, followed by the catalase test.

4.6.3 Immunoglobulin G (IgG) concentration

Concentrations of IgG in serum samples from cows were measured using an “in-house sandwich ELISA” (REBER et al, 2008). Rabbit anti-bovine IgG antibody (B5645; Sigma, St. Louis, MO) diluted 1:400 in sodium carbonate buffer at pH 9.7 was used to coat Immulon 4HBX plates (Thermo Corp., Milford, MA) at 4-8°C overnight. The colostrum samples were diluted in 1: 1,000,000 and 1:10,000,000 in diluent (phosphate-buffered saline containing 0.5% Tween 20) for screening and IgG positive samples were further diluted to determine titer endpoint. The previously prepared colostrum samples were centrifuged at 500g for 10 minutes forming the colostrum serum samples, which were used in the assay diluted 1: 1,000,000 and 1:10,000,000. The samples were placed in duplicate wells for assessment, and incubated for 1 h at room temperature. The plates were again washed three times, followed by IgG detection using a horseradish peroxidase - rabbit-anti-bovine IgG conjugated (A5295; Sigma, St. Louis, MO). The detection antibody was diluted 1:1000 in diluent and the wells were incubated with detection antibody for 30 min. The plates were washed three times with wash buffer, after which bound detection antibody was detected using 2,20 -azino-bis(3-ethylbenzthiazoline-6- sulfonic acid) substrate (A-9941; Sigma, St. Louis, MO). The plates were incubated for 30 min to allow color development and measured using a plate reader with a 405 nm filter. The quantity was determined relative to a six-point serial dilution of bovine gamma globulin standard (I5506; Sigma, St. Louis, MO).

4.7 DNA extraction, 16s rRNA gene amplification and high throughput sequencing

The samples were set on dry ice (-70°C) to execute this stage. The services were performed by the company Neopropecta, microbiome technologies, located in the state of Santa Catarina, Brazil

Total DNA was extracted using from all samples utilizing the PowerSoil 96-well DNA Isolation Kit (MoBio Laboratories, Inc.,) strictly adhering to the manufacturer's

instructions. The V3-V4 region of the 16S rRNA gene was amplified using PCR. The sequencing library preparation was carried out in a twostep PCR protocol, following Illumina's recommended protocol. In the first PCR reaction, we used the V3-V4 primers 341F-806R (WANG et al, 2009; CAPORASO et al, 2011), since this pair has great taxonomy coverage in bacteria and archaea (Takahashi et al., 2014).

The PCR reactions were always carried out in triplicates using Platinum Taq (Invitrogen, USA) with the conditions: 95°C for 5 min, 25 cycles of 95°C for 45s, 55°C for 30s and 72°C for 45s and a final extension of 72°C for 2 min for PCR 1. In PCR 2 the conditions were 95°C for 5 min, 10 cycles of 95°C for 45s, 66°C for 30s and 72°C for 45s and a final extension of 72°C for 2 min. The final PCR reaction was cleaned up using AMPureXP beads (Beckman Coulter, Brea, CA) and samples were pooled in the sequencing libraries for quantification. The pool amplicon estimations were performed with Picogreen dsDNA assays (Invitrogen, USA), and then the pooled libraries were diluted for accurate qPCR quantification using KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems, Woburn, MA).

The libraries were sequenced in a MiSeq 300 cycle run system, using the standard Illumina primers provided in the kit.

4.8 Bioinformatic analyses

The 16S sequences obtained from the microbiome were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) 2 2019.1. Unprocessed data was separated by each sample using the q2-demux plugin. The sequences were filtered by their quality and the sequencing noise was eliminated using the DADA2 program. For this, the direct sequences were cut at position 223 and the reverse sequences at position 98. These positions were chosen because the first quartile of the Sequence quality score was greater than or equal to 20. The "clustered" method was used to detect amplicon sequence variants or "amplicon sequence variants" (ASV) with low frequency in several samples. Chimeric sequences were filtered out using usearch61 within the QIIME platform, Sequence data was grouped in Operational Taxonomic Units OTU's at 97% similarity for bacterial sequences, and was considered for further analysis if they had a minimum of 5 sequences detected across all samples. Taxonomy was assigned to OTU representative sequences using the RDPclassifier. All subsequent analyzes were performed with R version 3.6.1.

The abundance of OTUs and taxon was calculated with the phyloseq package. The proportions of each OTU per sample were used to build heatmaps, clustering samples and OUT's using Ward's criterion. For heatmap representation OTUs were filtered with minimum threshold = 1000.

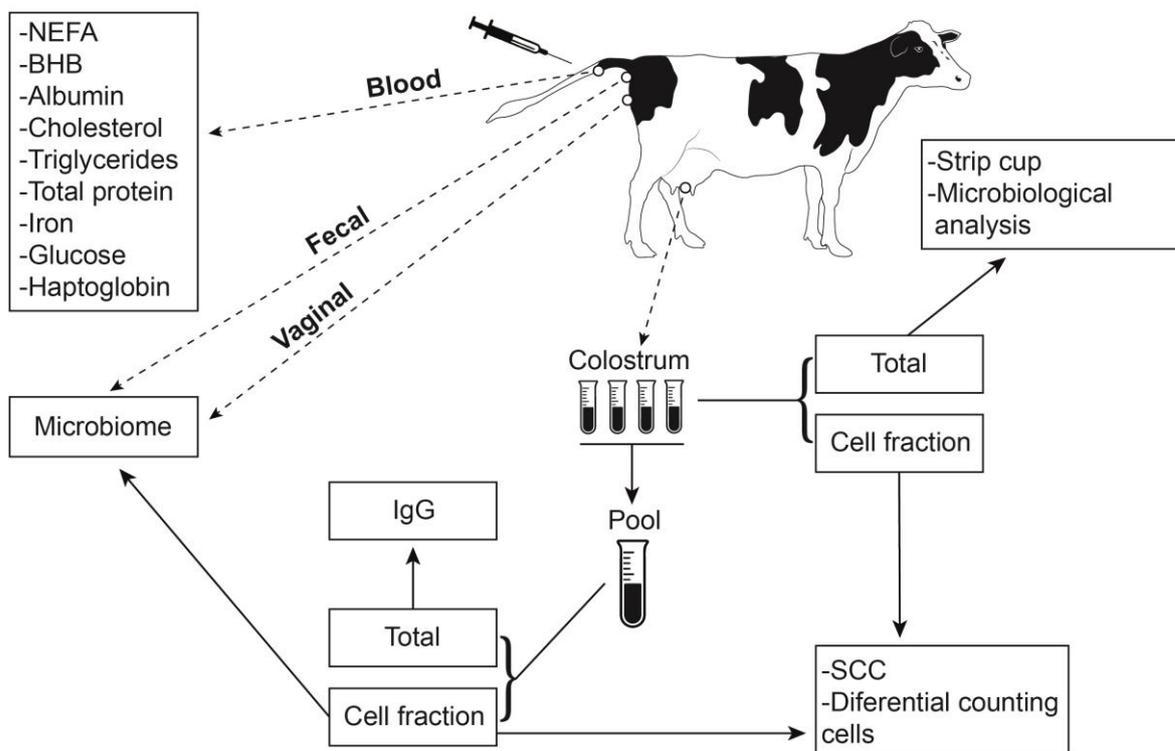
Alfa-diversity analysis rarefaction was applied as a standardization for the diversity values derived from different numbers of sampling units Sequences were subsampled to the lowest number (min. 100) of reads for the alpha diversity analysis, it was determined as Shannon (diversity) and Chao1 (richness) index. Beta diversity was calculated using Bray Curtis and Jaccard distance metrics: principal coordinate analysis was carried out using the OTU-based Bray-Curtis metric that takes into account both the presence/absence and the abundance of OTU in the samples. The Jaccard metric was also calculated, taking into account the presence/absence.

Spearman correlations were calculated between OUT's from the colostrum, fecal and vaginal samples with the metabolic and health parameters only for OUT's present in at least 10% of the samples, to reduce multiple comparisons. P values were corrected using False Discovery Rate method implemented in the p.adjust function, already available in the R base library. Only correlations with corrected $p < 0.05$ were selected for the preparation of heat maps and network analysis.

4.9 Flowchart of the experiment

In order to facilitate the understanding of the methodology used in this investigation, a representative flowchart is presented in Figure 7.

Figure 7 - Flowchart of the method used in this investigation from sample collection to laboratory procedures- São Paulo – 2020.



Source: (CASTRO-TARDÓN 2020)

5 RESULTS

This research evaluated the correlation between metabolic biomarkers, mammary gland health status and cow's microbiome immediately after calving. This topic will be present in the descriptive results of metabolic parameters and mammary gland health status and its correlation with cow's microbiome.

5.1 Body condition score metabolic biomarkers and haptoglobin

BCS of cows had individual variations at calving time between 2.50 and 3.75 (Median= 3.25) (Table 1). In addition, only 5% (1/20) of the cows presented a BCS over 3.5. In relation to the energetic biomarkers, NEFA and BHB concentrations had a variation between 0.19 -1.17 mmol/L (Median= 0.64 mmol/L) and 0.01-0.47 mmol/L (Median= 0.03 mmol/L), respectively. Glucoses concentration presented a minimum and maximum values of 52–145 mg/dL (Median= 77.50 mg/dL), respectively. Cholesterol (mg/dL) and triglycerides (mg/L) concentrations had a range of variation from 38.10-105.90 (Median= 70.20) and 3.6-81.5 (Median = 29.55), respectively.

According to the reference cut-off values adopted in this research, the frequency of dairy cows presenting normal values for NEFA was of 90% (18/20). On the other hand, 10% (2/20) of cows had NEFA values higher than the cut-off established. All dairy cows (100%, 20/20) presented BHB values below of the cut-off point. 100% of them (20/20) had normal glucoses concentration. Regarding the interpretation of triglycerides and cholesterol concentration, it was possible to observe that 85% (17/20) and 70% (14/20) of cows, respectively, had values below of the cut-off used to interpret the results in this research.

In relation to protein biomarkers, it was observed a range of 2.87- 8.4 (Median = 6.40) of total protein (g/dL) and 1.66 – 3.03 (Median= 2.74 for albumin (g/dL)). It was observed 65% (13/20) of the animals presenting normal values for albumin, while 35% (7/20) had values below of the established range. For total protein, on which most of the animals (70%, 14/20) had low concentration, and 30% (6/20) were within normal ranges. The iron presented variations between 5.5 μ mol/L minimum and 37.90 μ mol/L maximum (Median= 23.15). According to the established cutoffs, 80% of cows (16/20) had normal values, 15% (3/20) low values and only 1 animal was on the cut-off (5%). Finally, haptoglobin showed values between 13 mg/dL and 59.03 mg/dL (Median=

22.92 mg/dL) and 80% of the animals (16/20) had values over of the established range. The results are shown in Table 7, Figure 8 and Table 8.

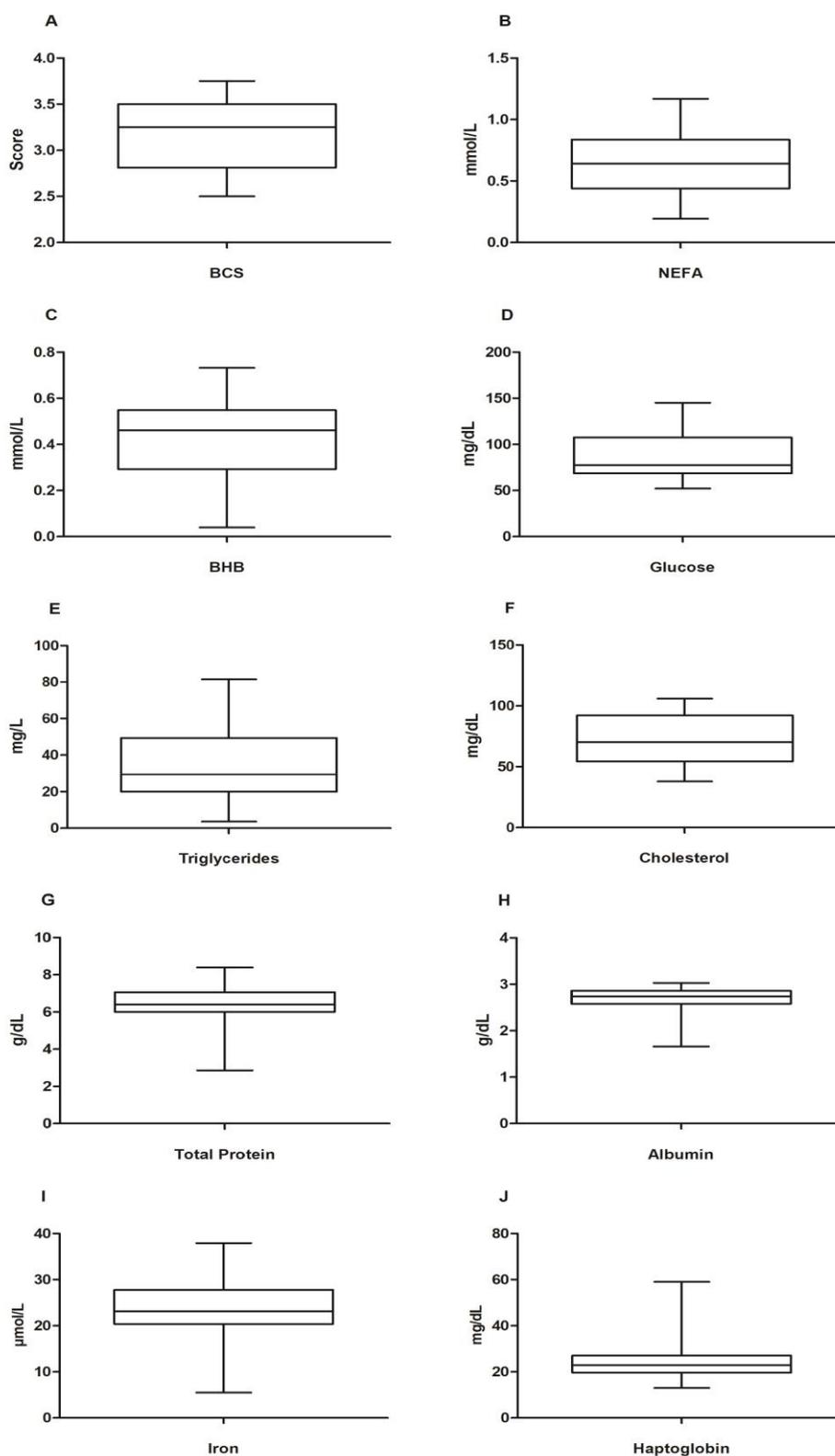
Table 7 - Descriptive statistical of the metabolic biomarkers and body condition score of Holstein cows (n= 20) at calving - Sao Paulo-2020.

Parameters	Cut-off	Average	Standard Deviation	Median	Minimum	Maximum
BCS (1-5 scale)	3.0-3.5	3.16	0.41	3.25	2.5	3.75
NEFA (mmol/L)	≤ 1.0	0.62	0.27	0.64	0.19	1.17
BHB (mmol/L)	≤ 1.2	0.14	0.15	0.03	0.01	0.47
Glucose (mg/dL)	45-75	88.25	25.66	77.5	52	145
Triglycerides (mg/L)	1-140	33.61	19.4	29.55	3.6	81.5
Cholesterol (mg/dL)	80-120	70.92	20.72	70.2	38.1	105.9
Total protein (g/dL)	6.8-8.6	6.07	1.49	6.4	2.87	8.4
Albumin (g/dL)	2.7-3.8	2.64	0.36	2.74	1.66	3.03
Iron (µmol/L)	17.9-35.8	23.45	6.81	23.15	5.5	37.9
Haptoglobin (mg/dL)	≤ 20	24.59	10.01	22.92	13	59.03

Source: (CASTRO-TARDÓN 2020)

Subtitle: NEFA: non-esterified fatty acids, BHB: Beta-hydroxybutyrate, BCS: body condition score

Figure 8 - Boxplot of Body Condition Score (BCS) and metabolic biomarkers in dairy cows at calving time – Sao Paulo - 2020



Source: (CASTRO-TARDÓN 2020)

Subtitle: NEFA: non-esterified fatty acids, BHB: Beta-hydroxybutyrate, BCS: body condition score

Table 8 - Percentage of animals low, normal and high the reference interval for each biochemical parameter, in Holstein cows immediately after calving - Sao Paulo -2020

Parameters		Low	Normal	High
NEFA	sampling	0/20	18/20	2/20
mmol/L	%	0	90	10
BHB	sampling	0/20	20/20	0/20
mmol/L	%	0	100	0
Glucose	sampling	0/20	0/20	20/20
mg/dL	%	0	0	100
Iron	sampling	3/20	16/20	1/20
µmol/L	%	15	80	5
Haptoglobin	sampling	0/20	4/20	16/20
mg/dL	%	0	20	80
Total Protein	sampling	14/20	6/20	0/20
g/dL	%	70	30	0
Albumin	sampling	7/20	13/20	0/20
g/dL	%	35	65	0
Triglycerides	sampling	17/20	3/20	0/20
mg/L	%	85	15	0
Cholesterol	sampling	14/20	6/20	0/20
mg/dL	%	70	30	0
BCS	sampling	5/20	14/20	1/20
	%	25	70	5

Source: (CASTRO-TARDÓN 2020)

Subtitle: NEFA: non-esterified fatty acids, BHB: Beta-hydroxybutyrate, BCS: body condition score

5.2 Clinical examination of the mammary gland

5.2.1 Data per individual quarter

At calving, most cows in this research presented a generalized volume increase of mammary gland (71%, 56/79), while only 8% (6/79) of quarters had a localized volume increase. Also, most mammary glands were reddish (67%, 53/79) at parturition time, followed by a 33% (29/79) of quarters with normal coloration and finally any mammary quarters presented purple coloration.

Most quarters had decrease of elasticity immediately after calving (90%, 71/79). An increase of local temperature (65%, 51/79) of the mammary glands was observed. Regarding the consistence, swelling (edema) was detected in most of mammary glands during the palpation technique (57%, 45/79), while 38% of quarters were hardened (30/79) and 5% (4/79) presented normal consistency.

In relation to the examination of retromammary lymph nodes, most of the animals (95%, 19/20) did not show an increased volume, since only one animal presented a bilateral increased volume. In the quarter's colostrum examination by strip cup, no mammary quarter presented alterations. The results are shown in the Table 9.

Table 9 - Frequencies of each categories obtained by inspection and palpation of the mammary gland (N= 79) of Holstein cows immediately after calving. São Paulo 2020.

Physical Examination	Sampling	Percentage %
<i>Inspection- per Mammary Quarter</i>		
Generalized volume - Absent	23/79	29
Generalized volume - Present	56/79	71
Localized Volume - Absent	73/79	92
Localized Volume - Present	6/79	8
Normal Coloration	26/79	33
Reddish Coloration	53/79	67
Purplish Coloration	0/79	0
<i>Palpation- per Mammary Quarter</i>		
Normal Elasticity	8/79	10
Decreased Elasticity	71/79	90
Elevated Temperature	51/79	65
Decreased Temperature	28/79	35
Normal Consistency	4/79	5
Hardened Consistency	30/79	38
Edematized Consistency	45/79	57

Source: (CASTRO-TARDÓN, 2020)

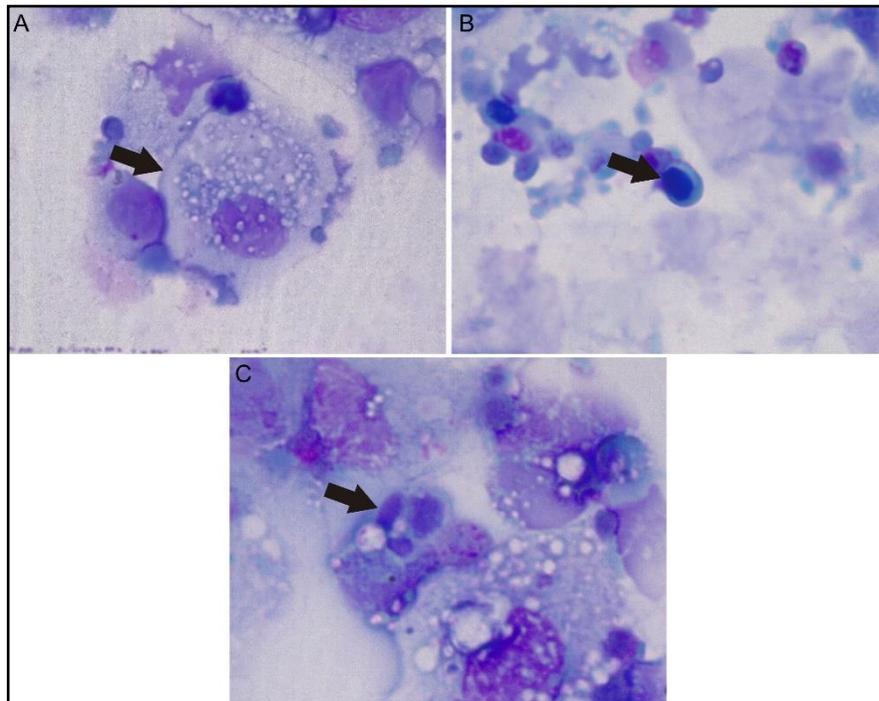
The median of SCC was 2.2 million of cells/mL (2.00-8.20 cells/mL). The proportion (%) of cells found in colostrum secretion was 76.00%, 12.91%, 10.85% and 0.06% for macrophages/epithelial cell, lymphocytes, neutrophils and eosinophils, respectively (Table 10 and Figure 9).

Table 10 - Average, median and standard deviation values of quantitative and qualitative cytological per quarter mammary analysis in Holstein cows at the time of calving- Sao Paulo-2020.

	SCC (x 10 ⁶ leukocyte/mL)	Macrophages/ Epithelial cells (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
Average	4.70	76.00	12.91	10.85	0.06
Standard Deviation	3.85	4.37	2.52	2.71	0.24
Median	2.21	76	13	10	0
Minimum	2.00	60	5	5	0
Maximum	8.20	89	19	20	1

Source: (CASTRO-TARDÓN, 2020)
Subtile: SCC: somatic cell count

Figure 9 - Photomicrograph of bovine colostrum cells identified by using morphological aspects in immersion optical microscopy (1000x).



Source: (CASTRO-TARDÓN. 2020)

Legend: A - macrophage; B-lymphocyte; C- Neutrophil

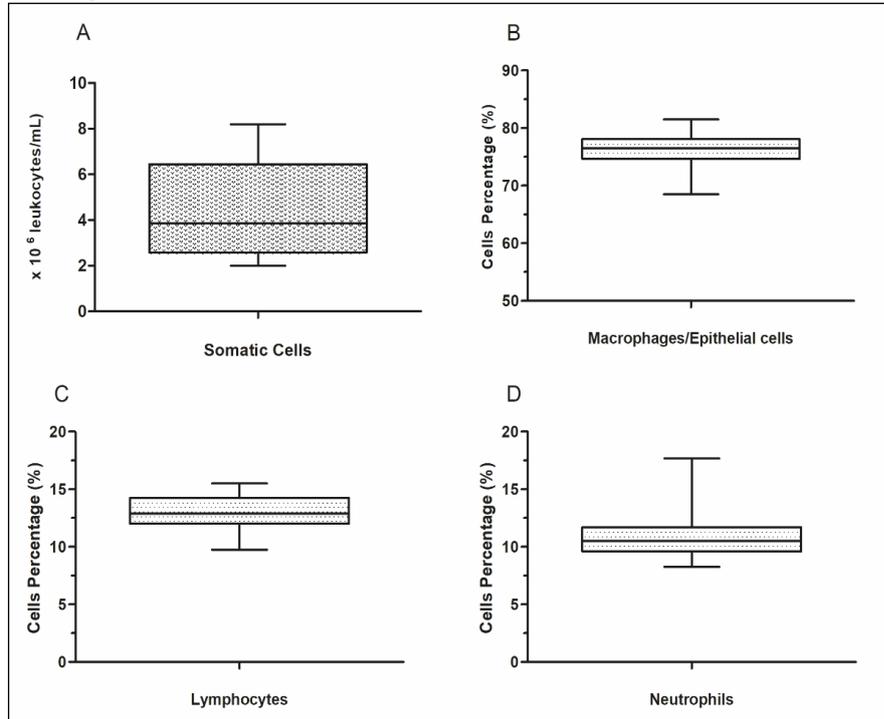
The results of bacterial culture of colostrum samples (n=79) harvested from 20 multiparous cows immediately after calving showed 16% (13/79) of positivity. While 84% had negative results (66/79).

5.2.2 Data per cow unit

In relation to the score of the evaluation of the udders, it had a score that ranged from 1.5 to 11, with a median of 9. These data are presented in the appendix A.

The range of SCC determined in colostrum pool was between 2×10^6 and 8.2×10^6 somatic cells/mL (Median= 3.85). The median values according to leukocyte types were of 77% (69% minimum and 82% maximum), 13% (10-16%), 11% (8-18%) of macrophages/epithelial cells, lymphocytes and neutrophils, respectively. In the analysis of the colostrum pool blades, eosinophils and basophils were not found (Figure 10).

Figure 10 - Boxplot represented median, minimum and maximum values of quantitative and qualitative cytological analysis of colostrum in Holstein cows at the time of calving - Sao Paulo-2020.

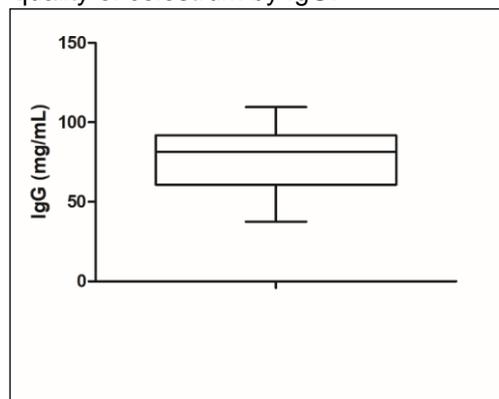


Source: (CASTRO-TARDÓN. 2020)

Legend: A- Counting Somatic cells; B- Macrophages/epithelial cells percentage; C- Lymphocytes percentage and D- Neutrophils percentage.

IgG concentration was determined from colostrum pool measured by a standardized inhouse ELISA. The range of IgG detected was 37.58- 109.55 mg/mL (Median = 81.29) (Figure 11).

Figure 11 - Evaluation of the immunological quality of colostrum by IgG.



Source: (CASTRO-TARDÓN. 2020)

5.3 Microbiome analysis

5.3.1 General characteristics of the data

The full dataset including colostrum, vaginal and fecal samples generated a total of 700.794 reads after quality control, grouped into 6.745 OTUs. From the total reads, 699.263 reads belong of the bacterial and 1.479 read belonging to Archaea kingdom. The phylogenetic analysis of the set samples from cows was distributed in six main phylum: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia, Spirochaetes and Tenericutes.

5.3.2 Reads, abundance and prevalence

5.3.2.1 *Phylum and genus from cow samples*

The most abundant phylum per cow observed in this research was Firmicutes with abundance of 61.24% (429.153 reads), followed by Bacteroidetes (27.82%; 194.955 reads) and Proteobacteria (7.37%; 51.615 reads).

In the classification of abundance according to sample type. Colostrum samples had a different profile comparing with fecal and vaginal samples. It was determined a total of 21.234 reads in colostrum samples. The predominant phylum was Proteobacteria with 15.481 reads (72.91%). The following phylum also was observed in colostrum samples: Firmicutes with 2.285 reads (10.76%); Bacteroidetes, 1.534 reads (7.22%); and Actinobacteria with 1498 reads (7.05%). Tenericutes, Verrucomicrobia and others (2.06%) phylum did not present readings in colostrum samples.

Vaginal samples had a total of 225.500 reads, observing a predominance of Firmicutes (122.555 reads; 54.35%), followed by the Phylum Bacteroidetes (57.297 reads; 25.41%), Proteobacteria (33.183 reads; 14.72%), Actinobacteria (6.774 reads; 3%). Verrucomicrobia (1269 reads; 0.56%), Tenericutes (947 readings; 0.42%) and others (4.54%).

A total of 454.060 reads in the fecal samples was detected, observing predominance of Firmicutes (304.318 reads; 67.02%), followed by Bacteroidetes (136.124 reads; 29.98%), Actinobacteria (3.060; 0.68%), Proteobacteria (2998 reads;

0.66%) Verrucomicrobia (2.918 reads; 0.66%), Terenicutetes (886 reads; 0.20%) and others phylum (0.8%).

Genus-level taxa present in a Holstein cow postpartum were represented in a relative abundance and prevalence. In the colostrum samples, the most abundant genera were *Pseudomonas* 10.84% (100% prevalence), followed by *Acinetobacter* 7.43% (70% prevalence), *Ochrobactrum* 7.10% (10% prevalence), *Staphylococcus* 6.18% (35% prevalence), *Anaplasma* 3.76% (55% prevalence), *Thaurea* 1.70% (30% prevalence), *Paracoccus* 1.02 % (20% prevalence). Other genus such as *Mogibacterium*, *Ruminococcus*, *Treponema*, *Clostridium*, *Blautia* and *Prevotella* were also identified with $\leq 0.04\%$ abundances and a prevalence of less than 35%.

In the vaginal samples the most abundant bacterial genus was identified as 5-7N15 (6.43%) followed by *Acinetobacter* (4.26%), *Ruminobacter* (2.23%), *Streptococcus* (2.15%), *Pseudomonas* (1.90%), *Ruminococcus* (1.79%) and *Oscillospora* (1.14%). The prevalence of taxa was greater than 45% in the samples. (Table 5). In addition, archaea genus was identified as *Methanovibacter* (0.15%) and *Methanocorpusculum* (0.07%).

Finally, the most abundant genera identified in the fecal samples were 5-7N15 (8.21%), *Oscillospira*, (1.95%), *CF231* (1.24%), *Ruminococcus* (1.05%), *Bifidobacterium* and *Prevotella* (0.61%), *Bacteroides* (0.39%), *Treponema* (0.29%), *Anaerostipes* and *Dorea* (0.28%) (Table 11). Other bacterial genera such as *Clostridium*, *Coprococcus*, *Succinivibrio*, *Blautia* ($\leq 0.27\%$) were identified. In relation to the prevalence of taxa, it was higher than 95% in all fecal samples.

Table 11 - Percentages of relative abundance and reads of the main genera found in colostrum, vaginal and fecal samples from Holstein cows immediately postpartum - São Paulo - 2020

Genus	Reads	Abundance %	Prevalence % (N= 20)	Genus	Reads	Abundance %	Prevalence % (N= 20)
Colostrum							
<i>Pseudomonas</i>	2301	10.84	100	<i>Paracoccus</i>	216	1.02	20
<i>Acinetobacter</i>	1578	7.43	70	<i>Streptococcus</i>	187	0.88	5
<i>Ochrobactrum</i>	1507	7.10	10	<i>Corynebacterium</i>	55	0.26	35
<i>Staphylococcus</i>	1312	6.18	35	<i>Lactobacillus</i>	29	0.14	15
<i>Anaplasma</i>	798	3.76	55	<i>Bacteroides</i>	15	0.07	15
<i>Thauera</i>	376	1.77	30	<i>Bifidobacterium</i>	8	0.04	15
Vaginal							
5-7N15	14496	6.43	65	CF231	1940	0.86	40
<i>Acinetobacter</i>	9606	4.26	90	<i>Clostridium</i>	1853	0.82	50
<i>Ruminobacter</i>	5038	2.23	45	<i>Dorea</i>	1715	0.76	45
<i>Streptococcus</i>	4848	2.15	55	<i>Bifidobacterium</i>	1096	0.49	55
<i>Pseudomonas</i>	4282	1.90	65	<i>Prevotella</i>	1038	0.46	45
<i>Ruminococcus</i>	4027	1.79	55	<i>Coprococcus</i>	655	0.29	55
<i>Oscillospira</i>	2567	1.14	65	<i>Treponema</i>	590	0.26	40
Fecal							
5-7N15	37260	8.21	100	<i>Anaerostipes</i>	1254	0.28	100
<i>Oscillospira</i>	8849	1.95	100	<i>Dorea</i>	1249	0.28	100
CF231	5641	1.24	100	<i>Clostridium</i>	1233	0.27	100
<i>Ruminococcus</i>	4777	1.05	100	<i>Coprococcus</i>	1011	0.22	100
<i>Bifidobacterium</i>	2779	0.61	95	<i>Succinivibrio</i>	887	0.20	95
<i>Prevotella</i>	2774	0.61	100	<i>Blautia</i>	798	0.18	100
<i>Bacteroides</i>	1755	0.39	100	<i>Methanovibacter</i>	636	0.14	100
<i>Treponema</i>	1322	0.29	100	<i>Mogibacterium</i>	288	0.06	100

Source: (CASTRO-TARDÓN. 2020)

5.3.2.2 Phylum and genus in controls (blank)

Table 12 represents the most abundant phylum and genera in the controls used in this investigation.

In the control samples of colostrum (molecular biology water) presented a total of 40 reads and the predominant phylum were Firmicutes, Bacteroidetes. At the level genus *Pseudomonas*, *Bacteroides* and *Ruminococcus* were the most abundant taxa.

In relation to the control of vaginal samples, a total of 42 reads were detected, being the phylum Firmicutes, Bacteroidetes, Cyanobacteria and Proteobacteria. At the taxonomic level of genus, *Dorea*, *Saccharopolyspora*, *Bacteroides* and *Ruminococcus* were the most abundant taxa.

Finally, 41,659 reads were detected in the compost barn samples. Identification by phylum showed a broad domain of Firmicutes, followed by Proteobacteria, Bacteroidetes and Actinobacteria. While the most abundant genus in the samples were *Blautia*, *Prevotella*, *Planococcus*, *Bacteroides*, 5-7N15 and *Pseudomonas*.

Table 12 - Percentages of relative abundance and reads of the main genera found in colostrum, vaginal and fecal samples from Holstein cows immediately postpartum - São Paulo - 2020

Taxon (phylum)	Read	Abundance %	Taxon (genus)	Read	Abundance %	Prevalence % (N=2)
Colostrum Controle						
Firmicutes	19	47.5	<i>Pseudomonas</i>	7	32.5	100
Bacteroidetes	10	25	<i>Bacteroides</i>	5	12.5	50
WPS-2	7	17.5	<i>Ruminococcus</i>	4	10	50
Proteobacteria	1	2.5	<i>Butyrivibrio</i>	2	5	50
			<i>Bacillus</i>	1	2.5	50
Vaginal Swab Controle						
Firmicutes	25	59.5	<i>Dorea</i>	3	7.1	50
Bacteroidetes	9	21.4	<i>Saccharopolyspora</i>	2	4.7	50
Cyanobacteria	4	9.5	<i>Bacteroides</i>	2	4.7	50
Proteobacteria	3	7.1	<i>Ruminococcus</i>	1	2.38	100
Compost barn Controle						
Firmicutes	27876	66.9	<i>Blautia</i>	3411	8.1	100
Proteobacteria	6240	14.9	<i>Prevotella</i>	1582	3.7	100
Bacteroidetes	4999	12	<i>Planococcus</i>	780	1.8	100
Actinobacteria	2481	6	<i>Bacteroides</i>	694	1.6	100
			5-7N15	282	0.6	100
			<i>Pseudomonas</i>	208	0.5	100

Source: (Castro-Tardón. 2020)

5.3.2.3 Main microorganisms in the immediately postpartum cow samples

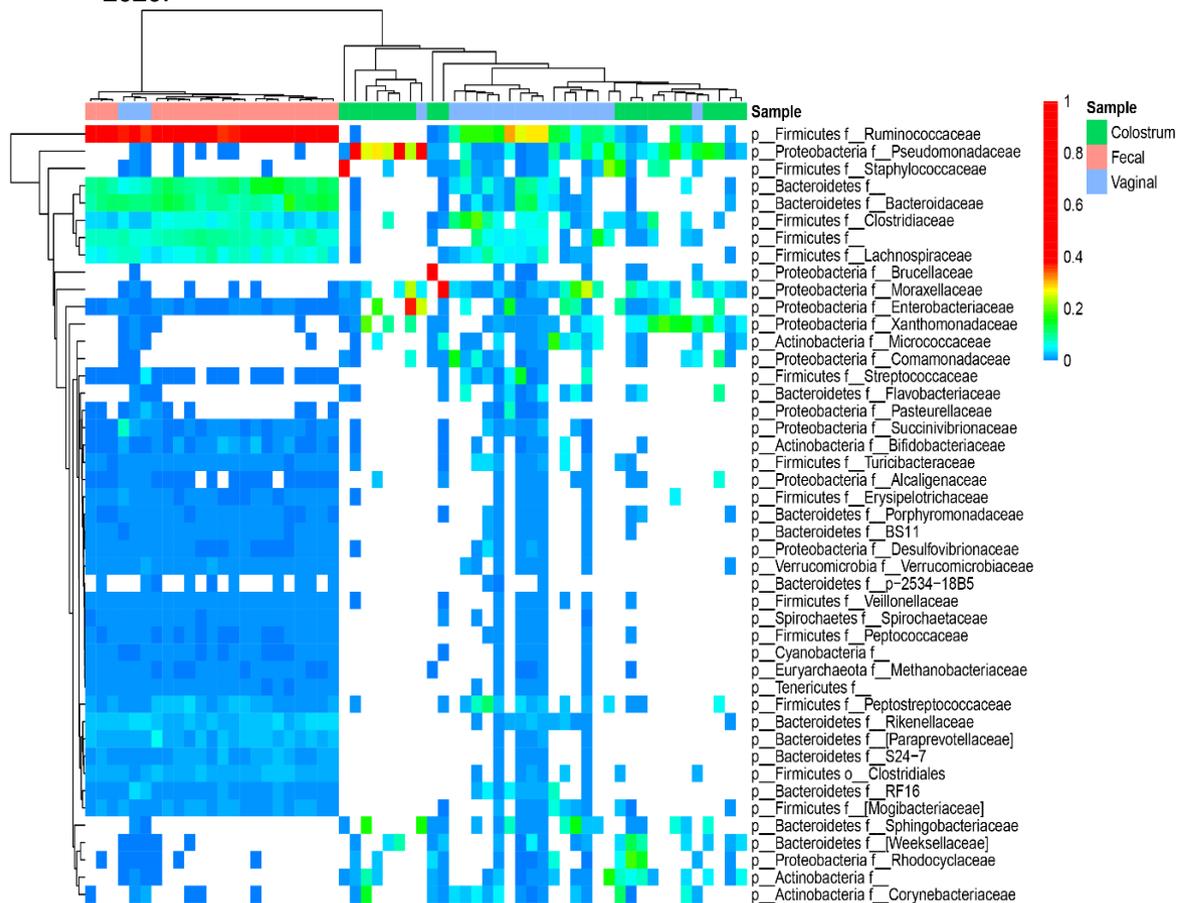
In the analysis of the proportions detected in the samples of colostrum, vaginal secretion and cow feces obtained immediately after calving showed that at phylum level the fecal samples and vaginal secretion were predominant in Firmicutes and

Bacteroidetes. While in the colostrum samples the most abundant phylum was the Proteobacteria.

At the family taxonomic level, a greater abundance of *Ruminococcaceae* was detected followed by *Bacteroidaceae* and *Clostridiaceae* in fecal and vaginal samples. Although colostrum samples showed lower abundance compared to the rest of the samples, the predominant families in this type of samples were *Pseudomonadaceae*, *Staphylococcaceae* and some samples showed a greater abundance of *Enterobacteriaceae*.

The phylum Euryarchaeota, which belongs to the Archaea kingdom, was abundant only in fecal and vaginal samples. These abundances are shown in Figure 12.

Figure 12 - Heat map showing the main bacterial taxa found in samples of Holstein cows. Each row refers to taxon's (phylum and family) and each column represents the samples. The legend at the top of the graph indicates the type of sample (colostrum, feces, vaginal)– Sao Paulo – 2020.



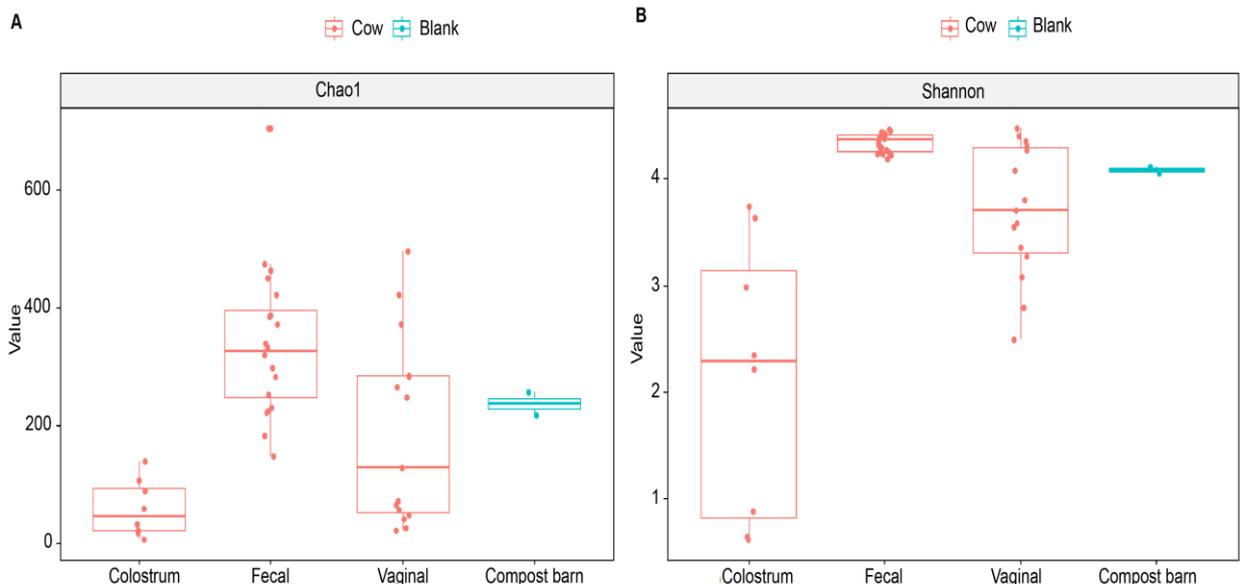
Source: (CASTRO-TARDÓN. 2020)

5.3.3 Alfa-diversity and Beta-diversity

The Chao 1 richness and Shannon diversity indexes were calculated to obtain estimates of community diversity. Measures of Chao1 richness varied with type sample, it was greater in fecal samples with median = 324.98, 127.25 standard deviation and n = 20 cows, followed by compost barn samples (median = 236.24; 27.08 standard deviation; n = 2 samples). The vaginal samples showed less richness with median = 127.10, 159.50 standard deviation and n = 16 cows. Finally, colostrum samples exhibited the lowest richness value, showed a median = 23.78 with 48.49 standard deviation and n = 8 cows (Figure 13).

Alpha diversity was assessed by measuring Shannon index. The values were also higher for fecal samples exhibited median values 4.34, 0.08 standard deviation, and n = 19 cows. The compost barn samples showed median 4.07; 0.04 standard deviation and n = 2 samples. The Shannon index in the vaginal samples was lower (median = 3.74; 0.58 standard deviation; n = 14 cows). Finally, the colostrum samples showed the lowest diversity values (median = 2.28; 1.28 standard deviation; n = cows). These values are represented in figure 13

Figure 13 - Species richness measured with Chao1 index (A) and Shannon diversity (B) in of Holstein cows immediately after calving. Data are visualized as box-and-whisker plots showing the median and the interquartile (midspread) range (boxes containing 50% of all values), the whiskers (representing the 25 and 75 percentiles) and the extreme data points.

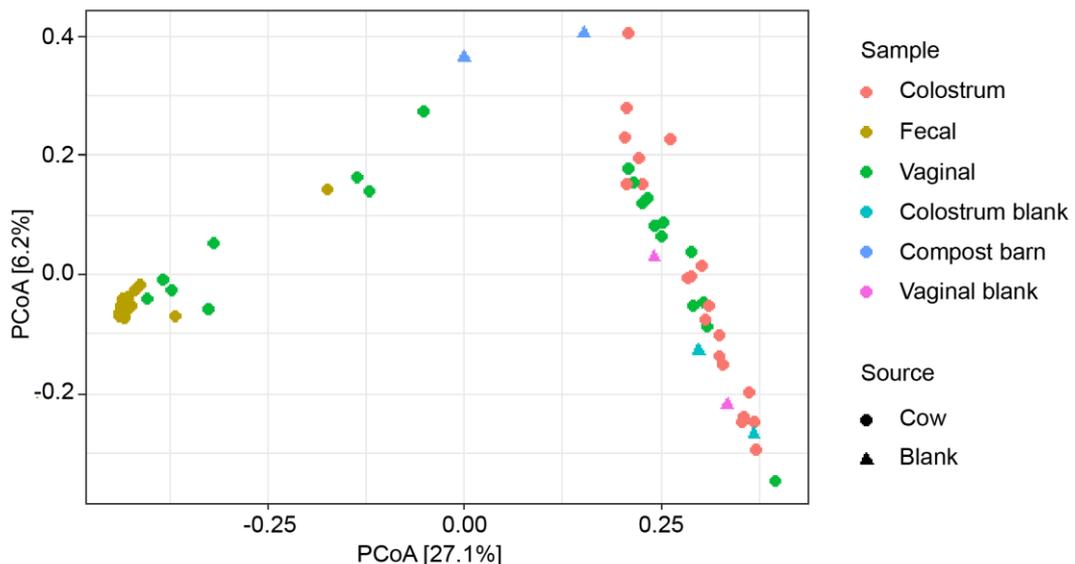


Source: (CASTRO-TARDÓN. 2020)

The Principal Coordinate Analysis (PCoA) was applied to compare the similarities among communities membership and structure and the analysis. The beta-diversity of samples harvested immediately after calving was made by using Bray-Curtis and Jaccard tests. It was possible to observe a different profile of bacterial DNA according to the sample type. Colostrum samples bacterial DNA had profile similar to vaginal secretion in terms of abundance and presence or absence, while the fecal samples showed a greater distance from the colostrum samples. Some vaginal samples showed a greater distance from the colostrum samples. Some vaginal samples had a similar profile of fecal samples, while others showed greater similarity with the colostrum samples.

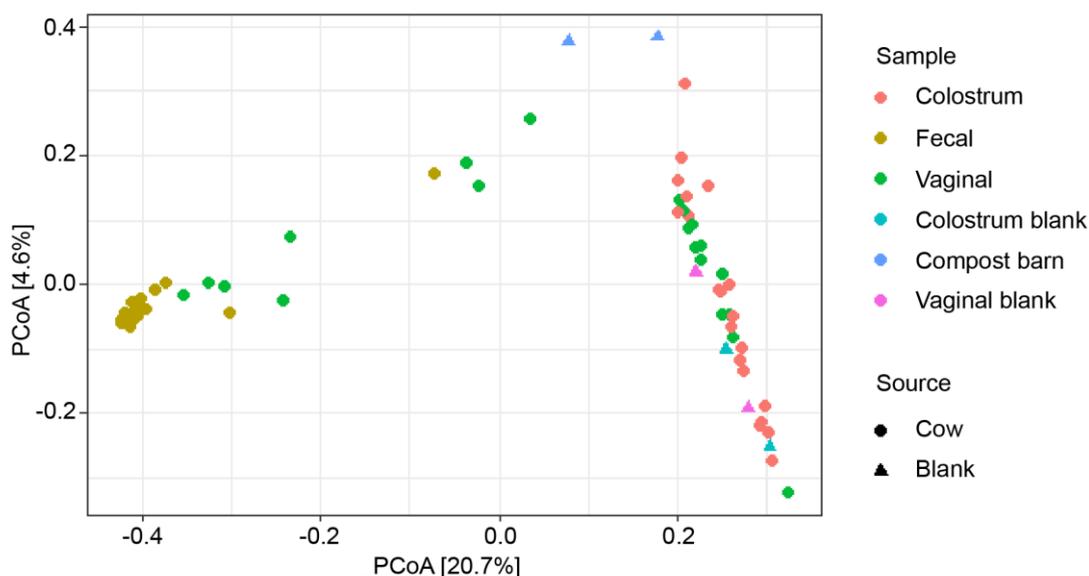
Regarding the distances of the control samples, the blank colostrum blanks and vaginal swab showed similarity with the colostrum and vaginal samples. The results of these analysis are outlines in Figures 14 and 15.

Figure 14 - Global microbiome analysis of the different samples of Holstein cows immediately after calving using the Bray Curtis metric to plot distances – Sao Paulo – 2020.



Source: (CASTRO-TARDÓN. 2020)

Figure 15 - Global microbiome analysis of the different samples of Holstein cows immediately after calving using the Jaccard metric to plot distances – Sao Paulo – 2020.



Source: (CASTRO-TARDÓN. 2020)

5.3.4 Correlations between metabolic biomarkers, mammary gland health status and colostrum microbiome

The results regarding this topic are shown on Figure 16. Colostrum microbiome had only significant associations with Bacteria kingdom. The *Coprococcus* genus showed a negative correlation with metabolic markers like triglycerides ($P= -0.024$), cholesterol ($P= -0.028$), albumin ($P= -0.013$) and total protein ($P= -0.045$), and also with the total clinical score of mammary glands ($P= -0.026$). At the family level, *Lachnospiraceae* presented a negative correlation with parameters related to the mammary gland such as: milk production, number of lactations, percentage of macrophages/epithelial cells and colostrum IgG. In addition, NEFA, BHB and cow weight also had a negative correlation with *Lachnospiraceae*. The *Treponema* genus showed a significantly negative correlation with the metabolic parameters: cholesterol ($P= -0.054$), triglycerides ($P= -0.048$), albumin ($P= -0.044$), total protein ($P= -0.050$) and with the volume of colostrum produced ($P= -0.030$). The genus identified *L7A E11* was positively associated with the SCC ($P= 0.021$) and negative correlation with NEFA, BHB ($P= -0.019$), BSC ($P= -0.013$), weight ($P= -0.011$) and number of lactations ($P= -0.055$).

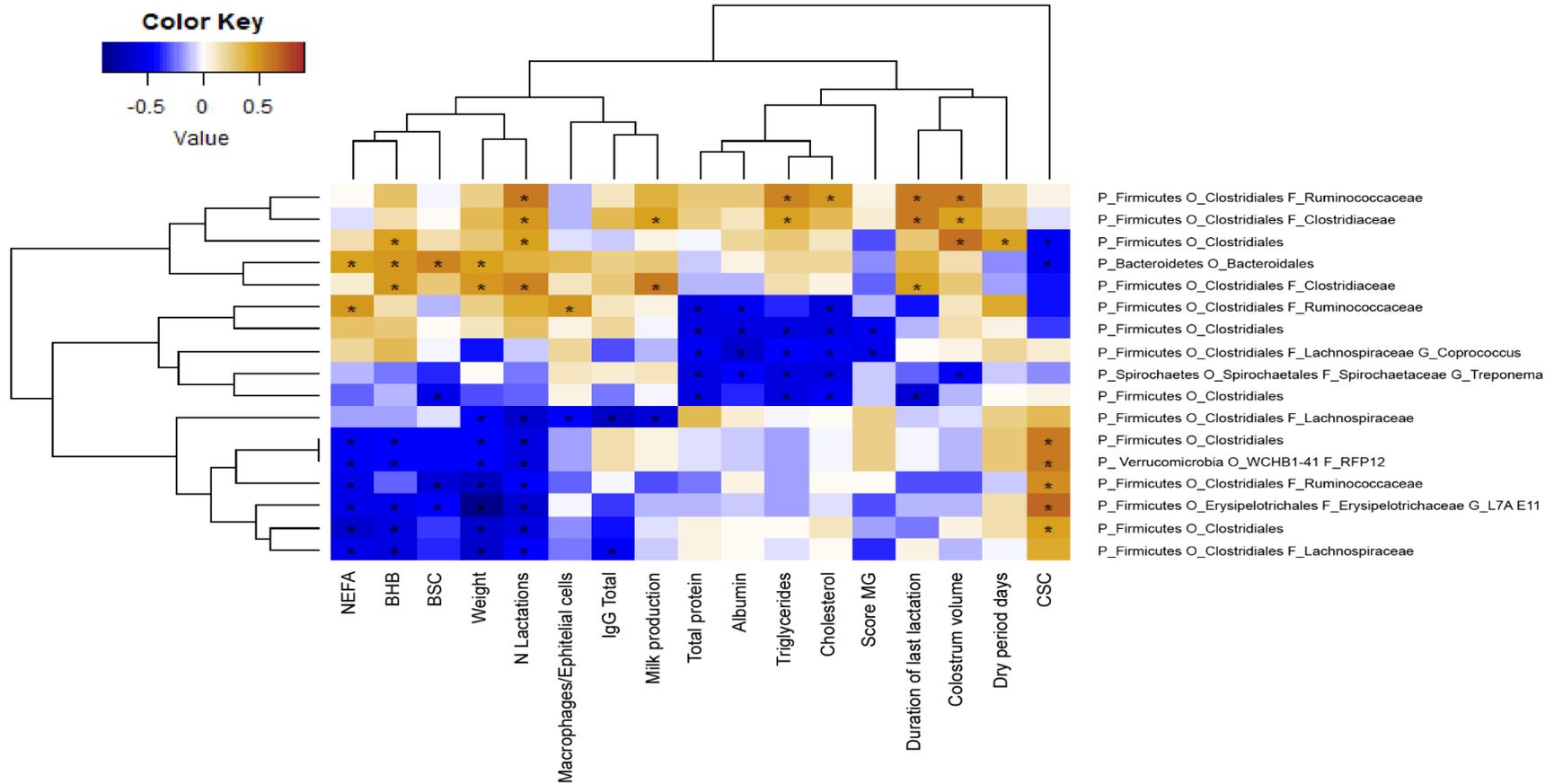
At the family level, *Ruminococcaceae* had a negative correlation with cholesterol, albumin, total protein, NEFA, BHB, weight and number of lactations. On

the other hand, this family had a positive correlation with total score of mammary glands, SCC, previous lactation duration, volume of colostrum produced and also triglycerides. The *Clostridiaceae* family presented a positive correlation with parameters related to the productivity of the cows, such as volume of colostrum produced, duration of the previous lactation, number of lactation and milk production. Also, it was possible to observe positive correlation among *Clostridiaceae* family with BHB and triglycerides.

The order *Bacteroidales* indicated a positive correlation with markers of energy metabolism BHB, NEFA, weight and BSC. While a negative correlation was detected for e CSC.

Some parameters associated with the health of the mammary gland showed no significant correlation, such as the microbiological status and quality of colostrum measured by colostrometer. Other metabolic parameters such as Glucose, iron and haptoglobin also showed no significant correlation Spearman in the colostrum microbiome of cows immediately postpartum.

Figure 16 - Spearman correlations between colostrum bacterial and health parameters. The correlations between the colostrum bacteria and these variables are indicated by colors (brown: positive; blue: negative). The significant correlations ($P \leq 0.05$) are indicated by *; only the variables and bacteria with at least one significant correlation are shown.



Source: (Castro-Tardón. 2020)

Subtitle: NEFA: non-esterified fatty acids. BHB: Beta-hydroxybutyrate. BSC: body score condition. CSC: Counting somatic cells. Score MG: score mammary gland.

5.3.5 Correlations between metabolic biomarkers, mammary gland health status and vaginal microbiome

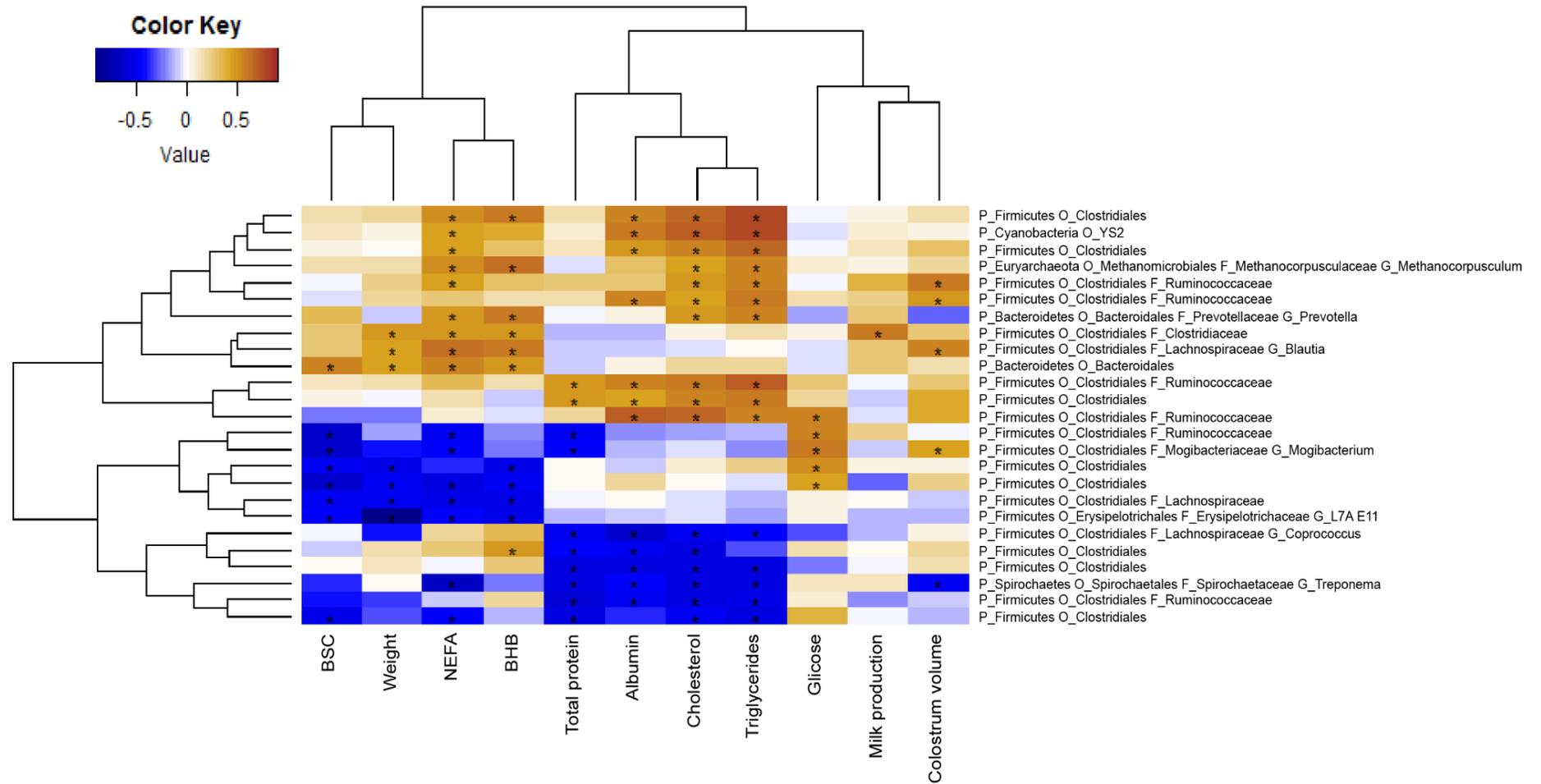
The results regarding this topic are shown on Figure 17. The primary association between vaginal microbiome and the variables studied in this experiment was observed for Bacteria kingdom. An exception was observed for *Methanocorpusculum* genus from the Archaea kingdom that presented a positive correlation with some energetic biomarkers such as cholesterol ($P= 0.045$), triglycerides ($P= 0.055$), BHB ($P= 0.061$) and NEFA ($P= 0.062$). The bacterial genus *Prevotella* also proved to be negatively associated with these markers ($P= 0.021$; $P= 0.033$; $P= 0.042$ and $P= 0.046$ respectively).

The *Blautia* genus was positively associated with energetic biomarkers like NEFA ($P= 0.010$) and BHB ($P= 0.052$), weight ($P= 0.020$) and colostrum volume ($P= 0.035$). On the other hand, the *Coprococcus* genus had a negative association with cholesterol ($P= -0.028$), triglycerides ($P= -0.026$), albumin ($P= -0.029$) and total protein ($P= -0.054$).

In the family level, *Lachnospiraceae* presented a negative correlation with NEFA, BHB, weight and BSC. The genus *Mogibacterium* showed a positive correlation with glucose ($P= 0.026$) and colostrum volume ($P= 0.022$), however, this group of bacteria was negatively associated with total protein ($P= -0.016$), NEFA ($P= -0.043$) and BSC ($P= -0.065$). The genus identified *L7A E11* also was negatively associated with the markers of metabolism BHB ($P= -0.052$), NEFA ($P= -0.053$), weight ($P= -0.011$) and BSC ($P= -0.044$). Finally, the *Treponema* genus presented a negative correlation with colostrum volume produced ($P= -0.028$), NEFA ($P= -0.013$), triglycerides ($P= -0.057$), cholesterol ($P= -0.054$), albumin ($P= -0.044$) and total protein ($P= -0.050$).

In addition, the family level, *Ruminococcaceae* showed mostly positive correlation with cholesterol, triglycerides and NEFA and negativity associated with total protein. Finally, the variables associated with the health of the mammary gland, iron, haptoglobin and some the cow's historical data showed no significant correlation.

Figure 17 - Spearman Correlations between vaginal bacterial and health parameters. The correlations between the vaginal bacteria and these variables are indicated by colors (brown: positive; blue: negative). The significant correlations ($P \leq 0.05$) are indicated by *; only the variables and bacteria with at least one significant correlation are shown.



Source: (Castro-Tardón. 2020)

Subtitle: NEFA: non-esterified fatty acids. BHB: Beta-hydroxybutyrate. BSC: body score condition. CSC: Counting somatic cells. Score MG: score mammary gland.

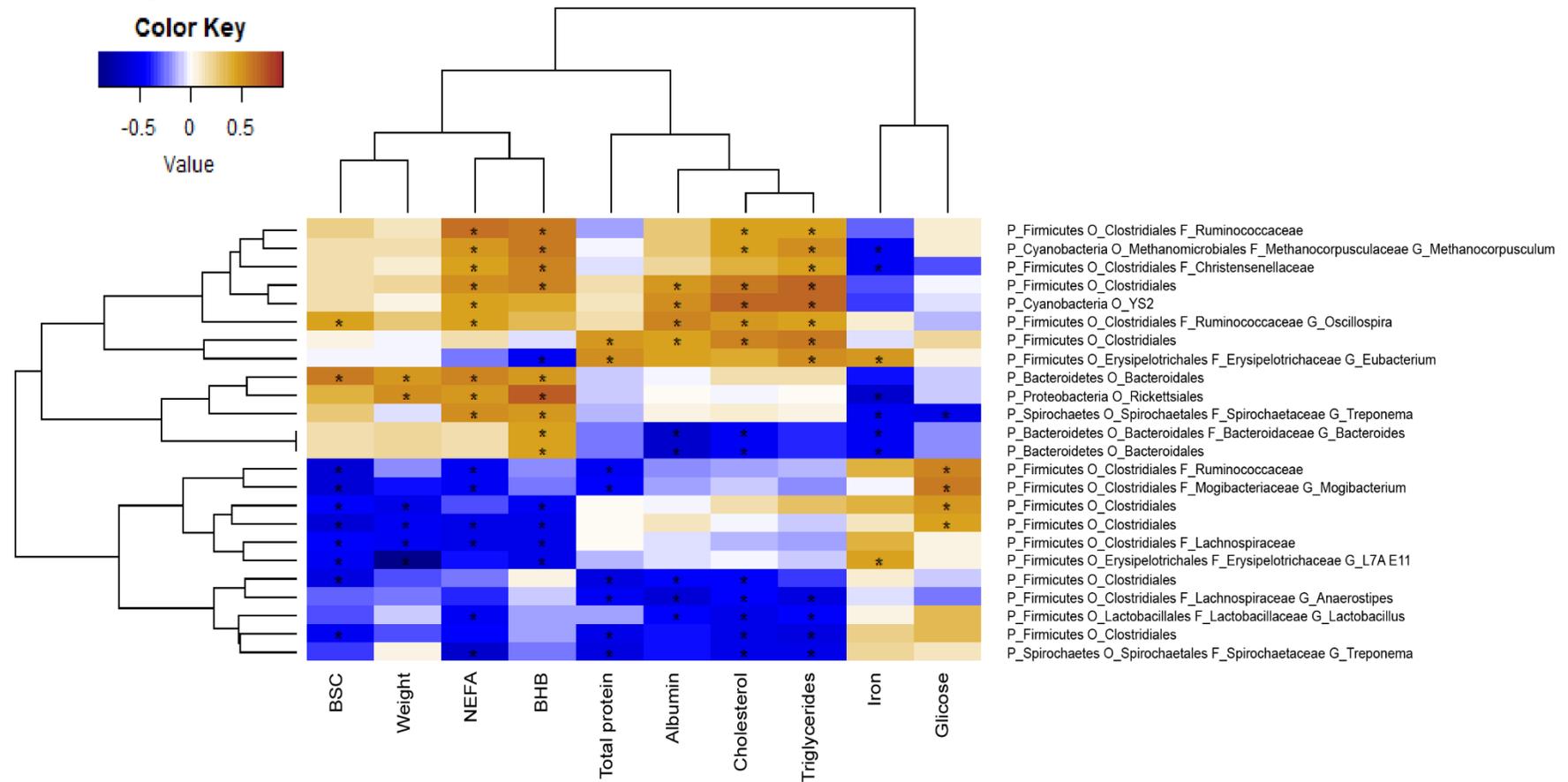
5.3.6 Correlations between metabolic biomarkers, mammary gland health status and fecal microbiome

Spearman's correlation was used to assess the relationship among metabolic parameters, the health of the mammary gland and the microbiome.

The results regarding this topic are shown on Figure 18. The Archaea, specifically, the *Methanocorpusculum* genus was strongly associated with metabolic biomarkers. This genus had a positive correlation with NEFA ($P= 0.026$), BHB ($P= 0.017$), cholesterol ($P= 0.045$) and triglycerides ($P= 0.055$), while it was possible to observe a negative correlation between *Methanocorpusculum* genus and iron concentration ($P= - 0.043$). The *Oscillospira* genus was positively associated with NEFA ($P= 0.002$), triglycerides ($P= 0.028$), cholesterol ($P= 0.034$) and albumin ($P= 0.048$); but the family *Ruminococcaceae* was associated negatively with total protein and BSC. The genus of *Eubacterium* showed a positive association with iron ($P= 0.049$), triglycerides ($P= 0.018$) and total protein ($P= 0.016$) of the cows; while at the family level, *Erysipelotrichaceae* had a negative correlation with NEFA, BHB, BSC and weight of the cows. The genus *Treponema* was positively associated with energy metabolism markers like NEFA ($P= 0.047$) and BHB ($P= 0.050$), and showed a negative association with glyucose ($P= -0.024$) and iron concentration ($P= -0.043$). The genus *Lactobacillus* had a negative correlation with NEFA ($P= -0.012$), cholesterol ($P= -0.024$), triglycerides ($P= -0.044$) and albumin ($P= -0.057$). The genus *Bacteroides* showed a positive correlation only with BHB ($P= 0.013$), while it showed a negative correlation with iron ($P= -0.014$), cholesterol ($P= -0.021$) and albumin ($P= -0.024$).

Finally, the *Anaerostipes* genus showed a negative correlation with cholesterol ($P= -0.010$), triglycerides ($P= -0.029$), albumin ($P= -0.007$) and total protein ($P= -0.004$). While the *Lachnospiraceae* family also showed a negative correlation with BHB, NEFA, weight and BSC. The parameters associated with the mammary gland, historical data of the cows, iron and hapoglobin did not present significant values in the Spearman correlation.

Figure 17. Spearman correlations between fecal bacterial and health parameters. The correlations between the fecal bacteria and these variables are indicated by colors (brown: positive; blue: negative). The significant correlations ($P \leq 0.05$) are indicated by *; only the variables and bacteria with at least one significant correlation are shown.



Source: (Castro-Tardón. 2020)

Subtitle: NEFA: non-esterified fatty acids. BHB: Beta-hydroxybutyrate. BSC: body score condition.

6 DISCUSSION

This investigation, evolved the influence of some parameters of energetic metabolism and health status of the mammary gland on cow's microbiota detected by metagenome analysis of the fecal, vaginal and colostrum samples from Holstein cows harvested immediately after calving.

6.1- Metabolic status of dairy cows at calving

The metabolic profile of the cows was analyzed by biomarkers measuring BHB, NEFA, glucose, triglycerides, cholesterol, total protein, albumin and iron. In order to establish the health status of the mammary gland, the specific clinical examination of the udder, cytological (total and differential) bacterial colostrum culture and measurement of total IgG by ELISA was also performed. These health parameters were associated with the microbiome profile of the cows immediately after calving.

In relation to the body condition, 95% (19/20) of the cows of this research presented a score between 2.5-3.5 (Median = 3.25) at the time of calving, and only one cow (5%) presented a body condition over the cohort point. The BCS at birth is directly related to the consumption of the animal at the time of lactation, the optimal body condition is 3-3.5 (5-point scale) at calving (ROCHE et al, 2009). However, the measurement of the body condition does not provide information on the process of mobilization of body reserves (JORRITSMA et al., 2001). Although modern BCS systems are more definitive than the early versions proposed, limitations of these scoring systems must still be recognized (ROCHE et al, 2009).

In the analysis of energy biomarkers, the NEFA and BHB concentrations varied between 0.19-1.17 mmol/L (Median = 0.64 mmol/L) and 0.01-0.47 mmol/L, respectively. One hundred % of the cows had normal concentrations of BHB at the time of delivery, however, 10% (2/20) of the animals presented values above to the cohort point established in the NEFA measurement. The dosage of the NEFA and BHB concentrations is considered the best way to assess whether an animal is in a negative energy balance, being able to determine its intensity and the adaptation of the animal to that process (DUFFIELD et al, 2009; LEBLANC et al, 2010). The variation in NEFA is a good marker for assessing dry matter intake before and after calving (LEBLANC et al., 2005, 2010). The plasma concentration of NEFA begins to increase two to four

days before calving, but in animals at risk of metabolic disorders it may begin to increase earlier. The peak usually occurs approximately three days after calving (Le Blanc et al., 2005). Ospina et al, (2010b) indicated that herds with more than 15% of cows with NEFA values ≥ 0.7 mmol/L have less productive, reproductive performance and a greater chance of acquiring disease. In this investigation the parameters associated to metabolism were evaluated, only at the time of delivery, therefore clinical postpartum data are necessary to assess the health status of cows that presented high concentrations (2/20). A considerable limitation of this research was the moment at which these metabolic biomarkers were measured, mainly in the BHB concentration. Samples for BHB measurement should be collected between 3 to 50 days postpartum (OETZEL, 2004). This explains the low values obtained in this study.

Regarding energy metabolism, it was possible to observe an increase in NEFA values as previously reported, this phenomenon was associated with a decrease in cholesterol and triglycerides levels. Concentrations of cholesterol and triglycerides had a range of variation from 38.10-105.90 mg/dL (Median= 70.20) and 3.6-81.5 mg/L (Median = 29.55), respectively. This may be explained since in situations where there are low concentrations of insulin and high glucagon, cholesterol synthesis decreases. These situations are usually caused by ingest deprivation. For this reason, cholesterol concentration is generally closely related to aliment intake. As calving approaches, there is a drop in intake and, consequently, a reduction in cholesterol concentrations (GURETZKY et al, 2006). The low levels of triglycerides of calving time correspond with other authors, who postulate that they are used by mammary gland as fat precursors and the decrease in blood concentrations may be a consequence of the catabolism that occurs in the mammary gland in the transition period (SCHWALM; SCHULTZ, 1976; BAUMAM; GRIINARI, 2003; MOREIRA, 2013).

Following the lipidogram profile, 100% (20/20) of the cows included in this study had high glucose concentrations, a minimum and maximum concentration of 52-145 mg / dL, respectively. The increase in glucose on the day of calving is widely reported in the literature (SCHWALM; SCHULTZ, 1976; PARK et al., 2010; MOREIRA, 2013). This elevation is a physiological process, resulting from an increase in the concentration of glucagon, catecholamines and glucocorticoids. They promote the elevation of circulating glucose levels due to gluconeogenesis and hepatic glycogen stores. At the beginning of lactation, the endocrine mechanisms that prioritize the use

of glucose for the mammary gland are activated, in this process there is a high demand for this monosaccharide for the production of milk lactose (BUSATO et al., 2002).

In relation to the results of protein metabolism, a median = 6.4 g/dL total protein was observed and 70% (14/20) of the cows presented a concentration under the established cohort point, these results are consistent with a study conducted by MOREIRA (2013) that observed a decrease in concentration of this biomarker two weeks before calving, these levels remained low until 10 days postpartum. This drop in blood concentration at birth is also supported by other authors and it can be explained by transfer of IgG from blood to mammary gland during the colostrogenesis process (BLUM et al., 1983; SAUT, 2008). On the other hand, the median value was 2.74 g/dL for albumin concentrations, and most of the animals (65%) were within the ranges established by Kaneko, 2008. As the concentration of albumin was variable in the cows immediately after calving, the causes of these variations are mainly due to changes in the animal's management or physiological state. In the literature, several results are described concerning the behavior of albumin in the peripartum, without agreement or typical behavior. Feitosa and Birgel (2000) report lower albumin values on calving day with a subsequent increase, while Gonçalves and Kozicki (1997) report a decrease in serum albumin levels after calving. According to some authors, the decrease in albumin concentrations at the beginning of lactation can also occur due to the demand for amino acids for the production of milk proteins, which reduces the synthesis of albumin and other proteins (CONTRERAS, 2000).

The iron concentration values in the cows varied between 5.5 $\mu\text{mol/L}$ minimum and 37.90 $\mu\text{mol/L}$ maximum (Median = 23.15). Most of the animals (80%) was within the normal range, however, 15% presented values below of the cutt-offs and only one animal showed higher values. Research led by Taunturier et al, (1984) showed that serum iron concentration is relatively high from the third to the seventh month of pregnancy although, afterwards, it decreased steadily to a minimum value (20.4 $\mu\text{mol/L}$). This light decrease in calving day was also described by other authors (MILTENBURG et al, 1991; WEISS et al, 2010). The decrease in serum iron concentration at the end of pregnancy may be due to the incorporation of this element into fetus and persisted in the first month of lactation (TAUNTURIER et al, 1984).

The serum concentration of haptoglobin in cows at calving was increased in 80% (16/20) of the animals. However, this is consistent with other previously reported that measured postpartum haptoglobin in dairy cows (USHIDA et al, 1993;

CRAWFORD et al, 2005; POHL et al, 2015). Haptoglobin is an acute phase protein synthesized in the liver in response to inflammation (CRAWFORD et al., 2005) and, in the bovine, haptoglobin appears to be one of the best indicators of acute phase protein (CONNER et al, 1988). Parturition is generally an inflammatory event, as is the involution of the reproductive tract in preparation for rebreeding. It is, therefore, logical that average haptoglobin levels would be higher at this time, even accounting for disease or health relation (CRAWFORD et al., 2005). Thus, an increase in serum haptoglobin concentration in dairy cows at calving is a physiological event.

6.2 Mammary gland health at calving

In the specific analysis of the mammary gland immediately after calving it was noticed that most of the cows presented alterations such as volume increase, edematized consistency and decrease in the elasticity of the udder skin, these alterations may be related to physiological edema. This phenomenon occurs by the transfer of redirected blood flow from the fetus to the mammary gland and colostrum production. The process of colostrogenesis begins weeks before delivery and ceases at the time of delivery due to hormonal influences. (DENTINE; MC DANIEL, 1983; HIBBIT et al, 2001; BARRINGTON ET AL., 2001).

The quantitative cytological analysis (somatic cells count) of the colostrum from Holstein cows immediately after calving, showed values between 2×10^6 minimum and 8.2×10^6 maximum somatic cells/mL. The median value (3.85×10^6 somatic cells /mL) found in this study was greater than the values observed by McDonald and Anderson (1981), Miller et al, (1990), Estrella (2001) and Gomes et al, (2011). Probably these divergences are consequences of the differences in the practices implemented in the herds studied, especially since the cows included in this study were housed in a *compost barn*, this system is a mixture of organic bedding (sawdust and wood shavings, and ground soybean straw) and cattle excreta, is cultivated frequently to incorporate fresh manure and air into the pack, thus promoting an aerobic composting process (LESO et al., 2020). Another interesting finding in this study was that only 13% (13/79) of mammary quarters was positive for bacterial growth. Bacterial infection taxa were similar to the frequencies of 12% reported by Pinedo et al, (2012) and lower than those found by Gomes et al, (2011). The differences obtained may be associated with the health status of the cows. In addition,

studies have indicated that compost barn, compared with conventional systems such as free-stall barns, have the potential to improve the welfare of dairy cows. In particular, the main reported benefits include improved comfort during resting, better foot and leg health, more natural animal behavior and also indicated that adequate udder health can be achieved in Compost-bedded pack barns (CBP) (LESO et al, 2020). To our understand, this is the first report of the Holstein cow microbiome at calving, housed in a compost barn system. Therefore, further studies are needed to verify this hypothesis.

The differential count analysis of bovine colostrum leukocytes showed a domain in the ratio of macrophages/epithelial cells (77%) followed by decreasing proportions of lymphocytes (13%) and neutrophils (11%), eosinophils and basophils were not found in this study. The use of this test for the diagnosis of mastitis in cows in the colostrum phase is important, the SCC can present normal values when it comes to colostrum, because it presents physiologically increased cellularity, even if it is in the absence of udder inflammation (RAIMONDO, 2006). Although a few articles were found evaluating the cell types of bovine colostrum, the results found in this study are consistent with the studies performed by Schalm et al, (1971) and Salmon (1999). In a similar study led by Gomes et al, (2011) the cytological analysis of bovine colostrum revealed a predominance of macrophages and epithelial cells (69.5%), followed by lymphocytes (16.4%), neutrophils (13.3%) and eosinophils (0.27%). On the other hand, immunophenotyping of colostrum cells also demonstrated a higher percentage of macrophages (32.7%), in addition to T cells (25.4%) and B cells (2.9%) (MEGANCK et al., 2014).

The results obtained in this study are different from those reported by Miller et al, (1991), where they observed higher proportions in polymorphonuclear cells (neutrophils), lower for macrophages and epithelial cells and similar proportions in lymphocytes, in the bovine colostrum analysis. This may be justified because this study chose not to classify epithelial cells and macrophages, since it was often impossible to differentiate them due to their morphological similarity, in addition, the literature remains controversial on the subject, since some authors consider that the macrophages can suffer morphological variations, depending on the different degrees of activation and can be classified as epithelial cells (SCHALM et al., 1971; LEE, 1980;; SANDGREN, 1991).

Immunoglobulin G (IgG) is the most abundant isotype found in bovine colostrum and represents over 75% of the total immunoglobulin (Ig) concentration and,

consequently, the quality of colostrum is assessed with reference to the content of this specific Ig class (KORHONEN et al. 2000). In this investigation, the concentration of IgG was determined from a standardized in-house ELISA and the median detected 81.29 mg/mL (average 76.62mg/L). Concentrations of IgG in colostrum vary among cows. In general, concentrations of IgG >50 g/L indicate good quality colostrum (GODDEN 2008), therefore, our values are over the recommended concentrations. Similar studies measured the total IgG of bovine colostrum with different result; Gelsinger et al, (2015) observed average values of 40.08 mg/mL, similar results (45.04 mg/mL average) were obtained by Dunn et al, (2018) in the analysis of bovine colostrum from Holstein and Limousin × Friesian cows. The differences in IgG concentrations may have been due to factors such as breed, timing of colostrum collection, parity, month of calving, nutrition, environment and samples status.

6.3 Dairy cow microbiome at calving

Several studies have previously described the microbiota of the cattle's vagina (SWARTZ et al, 2014; LAGUARDIA-NASCIMENTO et al, 2015; ALIPOUR et al, 2018), fecal (ALIPOUR et al, 2018; HOLMAN; GZYL,2019; KLEIN-JÖBSTL et al, 2019) and colostrum (LIMA et al, 2017; DERAKHSHANI, et al, 2018; YEOMAN et al, 2018; KLEIN-JÖBSTL et al, 2019). However, these studies have focused on the characterization of bacterial communities. In our concern, it is the first study that evaluated the correlation between the health of the mammary gland and metabolic profile with the microbiome in Holstein cows at the time of calving.

The descriptive evaluation of the microbiota of the cow's samples (colostrum, vaginal and fecal) showed that the fecal samples presented a greater number of reads, however, vaginal and colostrum samples presented a lower amount of bacterial DNA. In addition, in species richness (Chao1) and diversity (Shannon index), as well as the number of OTUs analysis, differed among type samples. These results were similar to those shown by YEOMAN et al, (2018) in the analysis of the samples of the cows at the time of calving. The mean alfa-diversity indices for maternal sources of microbiota indicated the udder skin had the greatest richness and diversity, followed by vaginal, and then colostrum.samples

Colostrum and vaginal secretion in this research presented a low-abundance microbiota, and working with this kind of sample is a challenging task due to microbial

DNA contamination present in different niches (place of collect, equipment and reagents) (ALIPOUR, ET AL 2018). Consequently, in this investigation we included negative controls (blanks), as previously reported, to evaluate the possible contamination during sample harvesting. Molecular biology water was added in a 50mL tube, following the same steps established in the lab to isolate the colostrum cells, source of bacterial DNA in the metagenome protocol. As a vaginal and rectal negative control, a swab was used and processed in the same way of these clinical material. In addition, samples also were collected from the housing of the cows, to assess the possible influence of the calving bedding on the contamination of the samples.

The profile of the colostrum and vaginal blanks, showed similar profiles and readings (40 and 42 reads, respectively), at the level of genus *Pseudomonas*, *Ruminococcus*, *Bacteroides* were the abundant identified genera. The prevalence of these genres was 50% (1/2 samples). In the colostrum control samples *Pseudomonas* was the dominant genus with 32,5% relative abundance and 100% prevalence. This finding coincides with contaminating sequences that match bacterial genera associated with water and soil. The importance of this problem when analyzing low biomass samples, despite these high-profile reports of reagent contamination, appears to still be underestimated in the microbiota research community (SALTER et al, 2014).

The material harvested from the compost-barn bed had a similar profile to the fecal samples. It was possible to detect 41,659 reads with greater abundance of the phylum Firmicutes. It seems to be logical, because the material of the bed are manure and organic compounds.

Some of the observed core genera have been isolated from environmental sources and could thus represent persistent contamination, previously uncharacterized animal-associated species or transient colonizers of the cow originated from its feed.

The control analysis data allow the claim that it is not possible to exclude all potential contaminants with absolute certainty, the most prevalent and abundant bacterial genera observed in cow were mostly known commensals as expected.

The microbiota profile determined in our study are each consistent with those previously reported. Colostrum samples showed most abundance of phylum Proteobacteria (72.91%), followed by Firmicutes (10.76%), Bacteroides (7.22%) and Actinobacteria (7.05%), the greatest abundance of Proteobacteria was similar to the one found by Klein-Jobstil et al, (2018) and Yeoman et al, (2018). However, these

results differ from those described by Lima et al, (2017), which indicated most abundance of the phylum Firmicutes. These differences can be justified by the design of each study, because the latter authors evaluated the colostrum of cows with mastitis and healthy, primiparas and multiparas. At the family and genus level, the results of this study showed a predominance of *Pseudomonaceae*, *Staphylococcaceae* and *Enterobacteriaceae*; *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Streptococcus*, *Bifidobacterium*, *Lactobacillus*. This profile was also described in other studies of bovine colostrum from udder healthy (LIMA et al, 2017; LIMA et al, 2018; DERAKHSHANI et al, 2018; YEOMAN et al, 2018; KLEIN-JÖBSTL et al, 2019). Although some of the bacterial genera found are considered pathogens of mastitis, because that some authors hypothesize that, in small quantities, these bacteria can be part of the normal milk microbiota, and therefore clinical mastitis may be developed only as mammary gland dysbiosis, rather than a simple primary infection (OIKONOMOU et al, 2014).

Other genera were also found *Ochrobactrum*, *Thauera* and *Paracoccus* with 10%, 30% and 20 % prevalence respectively. There is no literature related to these bacteria genera (p_Proteobacteria) in the health of the mammary gland in cattle, however, these genera are associated with the environment (water and soil) and are denitrifying communities (SONG et al, 2000). Hence, the finding of these microorganisms may be related to the type of housing (compost barn) of the cows in the last month of gestation.

In relation to the vaginal microbiome, the abundances of the phylum Firmicutes (54.35%), Bacteroidetes (25.41%), Proteobacteria (14.72%) and Actinobacteria (3%) were observed. In addition, the most abundant families and genus identified were *Ruminococcaceae*, *Bacteroidaceae*, *Clostridia* and *Lachnospiraceae*, *Ruminococcus*, *Streptococcus*, *Prevotella*, *Ruminobacter* and *Oscillospira*. These results were similar to other studies in Holstein cows at the time of calving. (LAGUARDIA-NASCIMENTO et al, 2015; ALIPOUR et al, 2018; YEOMAN et al, 2018; KLEIN-JÖBSTL et al, 2019). In genus identification, *Acinetobacter* and 5-7N15 was the most abundant in vaginal samples. This genus was previously found in fecal and bulk tank milk samples (LIMA et al, 2015; RODRIGUES et al, 2017; ALIPOUR et al, 2018). The 5-7N15 has been identified in fecal samples from dairy cows, however, information regarding this genus is limited (YOUNG et al., 2015). The identification of

these bacterial genera in vaginal samples may be associated with the anatomical proximity of the anus to the vulva.

The analysis of the fecal samples of the cow immediately after calving revealed a profile similar to phylum level with vaginal samples, with a domain of the phylum Firmicutes (67.02%), followed by Bacteroidetes (29.98%) and in less abundance Proteobacteria (0.66%) and Actinobacteria (0.68%). At the family level, the fecal microbiome profile was also similar to the vaginal one. The most abundant bacterial genera were *5-7N15*, *Oscillospira*, *CF231*, *Ruminococcus*, *Bifidobacterium* and *Treponema*. These findings were similar to the gastrointestinal microbiota of cattle reported by Jin et al, (2018) and Holman; Gzyl (2019). The unknown genus CF231 (Bacteroidetes, *Paraprevotellaceae*) has been reported in ruminal and fecal samples of cattle, however, there is no information on its role in the digestion of ruminants (ZHAO et al, 2015; JIN et al, 2018; PETRI et al, 2019).

In the literature, there are few articles that focus on the evaluation of the cow's microbiome profile at the time of calving, however, in the literature review, authors reported similar results in relation to the study of Beta diversity (YEOMAN et al, 2018; KLEIN-JÖBSTL et al, 2019).

Beta-diversity it demonstrates that there are similarities or differences between microbial communities (MORGAN; HUTTENHOWER, 2012), that is, what species and the extent to which they are shared (LOZUPONE; KNIGHT, 2009). The purpose of this analysis estimates the degree of overlap between the composition and structure of microbial populations. The Bray Curtis index evaluated the similarity/difference (distance) between the samples based on the presence or absence of the OTU, this analysis showed that the fecal, vaginal samples and compost barn, presented a similar profile in terms of abundance and shared OTUs, while the colostrum samples had a different profile. However, colostrum samples exhibited a smaller distance with some vaginal samples and controls (vaginal swab and colostrum). A similar result showed the Jaccard index, which evaluated the samples based on the presence and absence of OTUs. These results are justified by the difference in the number of reads that each type of sample presented, which was higher in the fecal samples, followed by vaginal ones, and finally the colostrum. And also because of the taxonomic profile that these samples presented, showing that fecal and vaginal samples had a greater abundance of Firmicutes, Bacteroides and Proteobacteria, while colostrum samples were dominated by the phylum Proteobacteria.

In addition, the presence of contaminating sequences is greater in low-biomass samples (such as from colostrum) than in high-biomass samples (such as from faeces), suggesting that there is a critical tipping point where contaminating DNA becomes dominant in sequence (SALTER et al, 2014). Many recent publications describe important or core microbiota members in humans and animals, often members that are biologically unexpected, which overlap with previously-described contaminant genera.

6.4 Correlation between metabolic profile, mammary gland health and cow's microbiome

In our concern, it is the first research presenting the correlation between the mammary gland health, metabolic profile with the microbiome of dairy cows immediately after calving.

Mammary gland secretion is known to be a dynamic biological sample which changes its nutritional and microbiological composition over time. Colostrum microbiota composition is influenced by diet, management and the host's physiological status (DRAGO et al, 2017).

In ruminants, nutritional challenges during the periparturient period can modulate composition and functionality of GIT microbiota, resulting in altered microbial-driven metabolites along the GIT (WANG et al, 2012; LIMA et al, 2015). An altered GIT microbiome can in turn affect systemic metabolic profiles (SHABAT et al, 2016). As such, changes in the GIT and circulating metabolite profiles may extend far beyond the GIT and affect overall physiology and immune homeostasis, resulting in modulation of microbiota profiles at several body areas.

The analysis of the correlation between metabolic parameters and mammary gland health status with microbiome disclosed interesting findings. In this discussion, the main genus or families of bacterial species was set independently of the samples type, considering that the correlation between the variables and microbioma had similar profile for colostrum, feces and vaginal secretion

It was observed that the *Lachnospiraceae* family presented a negative correlation with parameters associated with milk production in the previous lactation, number of lactations and IgG concentration in colostrum samples. Thus, this family bacteria was more abundant in animals with a low number of previous lactation that

can explain the less concentration of IgG negatively correlated with *Lachnospiraceae* family.

A study that associate the gut bacteria typical characteristic and metabolites profiling of high production dairy cows showed a similar result for this family. *Coprococcus* (*Lachnospiraceae* family). The authors also found a negative correlation between the abundance for this bacterial genus and with milk production (JAMI et al., 2014).

The genus *Coprococcus* has been widely reported in ruminal samples and in minor proportion in vaginal and milk samples.

This genus contributes to the production of butyrate, which is used as a source of energy by the gastrointestinal mucosa, and is positively associated with greater efficiency of food conversion in ruminants (KIM et al, 2014; JEWEL et al, 2015). A study in adult mice showed that bacterial genera *Eubacterium*, *Coprococcus* and *Faecalibacterium* detected on fecal samples had a positive relationship with metabolism markers such as NEFA and lipids (ZHANG et al, 2017). In contrast, our study found a negative correlation among the genus *Coprococcus* and metabolism biomarkers such as NEFA, BHB, triglycerides and cholesterol in colostrum and vaginal samples. *Coprococcus* genus source is the cow feces, and it probably had a contamination effect on the vagina and colostrum samples. It is important to point out that cows with low concentration of energetic biomarkers had more chances of the contamination of the different sites of the body.

The *Treponema* genus was identified in colostrum, vaginal and fecal samples and presented negative association mainly with biomarkers of energetic metabolism. Species of the genus *Treponema* are typically anaerobic, fastidious and highly motile microorganisms with a spiral morphology that are capable of occupying a diverse range of hosts and tissues, including the oral cavity and genital tract of humans, the gastrointestinal (GI) tract and feet of ruminants. Whereas GI colonisation has been associated with commensalism, several taxa have been shown to play a pathogenic role in a number of diseases, including bovine digital dermatitis and periodontal disease (EVANS et al, 2009; BORSANELLI et al., 2015). *Treponema* species (*T. denticola*, *T. maltophilum*, *T. medium*, *T. putidum*, *T. phagedenis* and *T. paraluisuniculi*) are nearly ubiquitously found in rumen and fecal microbiomes, suggesting that the gut is an important reservoir of this microbes (ZINICOLA et al, 2015). There is any information in the literature associating the metabolic profile with

Treponema abundance. It is possible that the cholesterol is a nutritional requirement for this spirochete (STANTON, 1987).

Other interesting finding was that two different OTUs of *Treponema* genus in fecal samples presented both negative and positive association with NEFA, but this may be justified due to the wide diversity of species of the *Treponema* genus in cattle (KLITGAARD et al, 2014). *Treponema denticula* were found in subgingival microflora and represent a potential microorganism involved in bovine periodontitis. This disease has a great economic and health importance in Brazilian cattle herd (BORSANELLI et al., 2015). Our hypothesis is that some species from *Treponema* genus could be associated with periodontitis that results in a reduced dry-matter intake and high lipolysis.

The genus *Mogibacterium* presented a negative correlation with the body weight, BCS, NEFA and total protein. On the other hand, it presented a positive correlation with the colostrum volume and plasma glucoses. This genus is common in bovines, and it is associated with ammonia assimilation through the ruminal epithelial wall for the biosynthesis of phenylacetate and the phenylacetate that bind to the glutamine to form phenylacetyl glutamine by rumen bacteria. This mechanism is very important to the ammonia metabolism and its absorption by rumen mucosa (NAKAZAWA et al, 2000). It is possible that the cows of our experiment with more mammary gland demand of glucoses for milk production need to metabolize more fat acids. In addition, this energetic demand can be modulated in the rumen increasing the population of the genus *Mogibacterium*, which has the capacity to increase the ammonia that is the source of nitrogen to form proteins.

Archaeas microorganisms, *Methanocorpusculum* genus were found only in fecal and vaginal samples, and showed a positive association with the biomarkers NEFA, BHB, triglycerides and cholesterol. In ruminants, the methanogens are extremely halophilic, thermophilic, and anaerobic, due to their unique modes of energy metabolism. Methanogens use a limited range of one-carbon compounds as substrates and convert them to methane (DAQUIADO et al, 2014).

Ruminococcaceae play a predominant role in ruminal biohydrogenation and is one of the most abundant families of all the types of samples in this research. Some authors correlated negatively the genus such as *Ruminococcus* and *Oscillospira* genus with milk yield (JAMES et al, 2014). However, in this study it was not possible to

identify the genera belonging to this family, making it difficult to interpret the data generated in the correlations.

This research describe the metabolic profile, mammary gland health status and its correlation with cow microbioma, by the study of samples from colostrum, feces and vaginal secretion harvested immediately after calving. It was significant to observe the high number of correlations found between the microbiome and metabolic profile presented by dairy cows immediately after calving. It proves that the microbiota composition is very important for the cow metabolism and their mammary gland health.

7 CONCLUSIONS

This research found a large number of correlations between metabolic profile, health status of the mammary gland, and colostrum microbiome, feces, and vaginal discharge harvested immediately after calving.

Fecal samples showed a higher abundance of Firmicutes, followed by Bacteroidetes and Actinobacteria. Vaginal samples showed a predominance of Firmicutes, Bacteroidetes and Proteobacteria. On the other hand, the colostrum microbiota showed a predominance of Proteobacteria, Firmicutes and Bacteroidetes. ~~Our Data obtained from the~~ different maternal sources allowed us to conclude that the fecal and vaginal microbiota are similar, while the maternal colostrum shows a different profile. ~~This fact allows us to elucidate that~~ Thus, the vaginal microbiota is strongly influenced by feces due to its anatomical proximity. ~~On the other hand, While~~ colostrum has its own microbial profile determined by the environment.

Most of the correlation found in this investigation was negative, when the microbiota was associated with metabolic biomarkers and the health status of the mammary gland. This association was detected mainly in the *Lachnospiraceae* and *Ruminococaceae* bacterial families correlated with the metabolic biomarkers NEFA, BHB and productive parameters of cows. Therefore, this research concludes that there is a relationship between microorganisms, mainly commensals, and the physiological or pathological state of the host at the time of calving.

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APENDIX - A

Chart 1. Score result of the assessment mammary gland in Holstein cows immediately after calving

Cow	Quarter	GV	LV	Color.	SE	T	MGC	Lymph.	SC	CMT	Total/quarter	Mean Cow
6936	RF	1	0	1	1	1	4	0	0	1	9	9.5
	LF	1	0	1	1	1	5	0	0	1	10	
	RR	1	0	1	1	1	4	0	0	1	9	
	LR	1	0	1	1	1	5	0	0	1	10	
6861	RF	1	0	0	1	0	4	0	0	1	7	7.25
	LF	1	1	0	1	0	4	0	0	1	8	
	RR	1	0	0	1	0	4	0	0	1	7	
	LR	1	0	0	1	0	4	0	0	1	7	
6414	RF	1	0	1	0	0	4	0	0	1	7	7
	LF	1	0	1	0	0	4	0	0	1	7	
	RR	1	0	1	0	0	4	0	0	1	7	
	LR	1	0	1	0	0	4	0	0	1	7	
7922	RF	1	0	0	1	0	4	0	0	1	7	7
	LF	1	0	0	1	0	4	0	0	1	7	
	RR	1	0	0	1	0	4	0	0	1	7	
	LR	1	0	0	1	0	4	0	0	1	7	
3014	RF	1	0	0	1	0	4	0	0	1	7	7
	LF	1	0	0	1	0	4	0	0	1	7	
	RR	1	0	0	1	0	4	0	0	1	7	
	LR	1	0	0	1	0	4	0	0	1	7	
7839	RF	0	0	0	0	0	0	0	0	1	1	1.5
	LF	0	0	0	0	0	0	0	0	2	2	
	RR	0	0	0	0	0	0	0	0	1	1	
	LR	0	1	0	0	0	0	0	0	1	2	
6593	RF	1	0	1	1	1	5	0	0	1	10	10
	LF	1	0	1	1	1	5	0	0	1	10	
	RR	1	0	1	1	1	5	0	0	1	10	
	LR	1	0	1	1	1	5	0	0	1	10	
7647	RF	1	0	1	1	1	5	0	0	2	11	10.25
	LF	1	0	1	1	1	5	0	0	1	10	
	RR	1	0	1	1	1	5	0	0	1	10	
	LR	1	0	1	1	1	5	0	0	1	10	
8371	RF	1	0	1	1	1	4	0	0	1	10	10
	LF	1	0	1	1	1	4	0	0	1	10	
	RR	1	0	1	1	1	4	0	0	1	10	
	LR	1	0	1	1	1	4	0	0	1	10	
7903	RF	1	0	1	1	0	5	0	0	1	9	9.5
	LF	1	0	1	1	0	5	0	0	2	10	
	RR	1	0	1	1	0	5	0	0	1	9	
	LR	1	0	1	1	0	5	0	0	2	10	

Cow	Quarter	GV	LV	Color.	SE	T	MGC	Lymph.	SC	CMT	Total/quarter	Mean Cow
7015	RF	1	0	1	1	1	5	0	0	1	10	10
	LF	1	0	1	1	1	5	0	0	1	10	
	RR	1	0	1	1	1	5	0	0	1	10	
	LR	1	0	1	1	1	5	0	0	1	10	
5239	RF	N	N	N	N	N	N	N	N	N	N	9
	LF	0	0	0	1	1	4	0	1	2	9	
	RR	0	1	1	1	1	4	0	0	1	9	
	LR	0	1	0	1	1	4	0	0	1	8	
5765	RF	0	0	0	1	1	4	0	0	1	7	7
	LF	0	1	0	1	1	4	0	0	1	8	
	RR	0	0	0	1	1	4	0	0	1	7	
	LR	0	0	0	1	1	4	0	0	1	7	
8356	RF	0	0	0	1	1	4	0	0	2	8	8
	LF	0	0	0	1	1	4	0	0	2	8	
	RR	0	0	0	1	1	4	0	0	1	7	
	LR	0	0	0	1	1	4	0	0	1	7	
8423	RF	0	1	1	1	0	4	0	0	2	9	8
	LF	0	0	1	1	0	4	0	0	1	7	
	RR	0	0	1	1	0	4	0	0	1	7	
	LR	0	0	1	1	0	4	0	0	1	7	
8306	RF	1	0	1	1	1	4	0	0	1	9	9
	LF	1	0	1	1	1	4	0	0	1	9	
	RR	1	0	1	1	1	4	0	0	2	10	
	LR	1	0	1	1	1	4	0	0	1	9	
8744	RF	1	0	1	1	1	4	0	0	1	9	9
	LF	1	0	1	1	1	4	0	0	1	9	
	RR	1	0	1	1	1	4	0	0	1	9	
	LR	1	0	1	1	1	4	0	0	1	9	
6979	RF	1	0	1	1	1	5	0	0	1	10	11
	LF	1	0	1	1	1	5	0	0	1	10	
	RR	1	0	1	1	1	5	0	0	2	11	
	LR	1	0	1	1	1	5	0	0	2	11	
8095	RF	0	0	1	1	1	5	0	0	1	9	9
	LF	0	0	1	1	1	5	0	0	1	9	
	RR	0	0	1	1	1	5	0	0	2	10	
	LR	0	0	1	1	1	5	0	0	1	9	
8812	RF	1	0	1	1	1	5	0	0	1	10	10
	LF	1	0	1	1	1	5	0	0	1	10	
	RR	1	0	1	1	1	5	0	0	2	11	
	LR	1	0	1	1	1	5	0	0	1	10	

GV – Generalized volume: 0 – absence / 1 – presence; LV – Localized volume: 0 – absence / 1 – presence; Color. – Coloration: 0 – Normal / 1 – Reddish / 2 – Purplish; SE – Skin elasticity: 0 – Normal / 1 – Decreased; Temp. – Temperature: 0 – Normal / 1 – Increased; MGC – Mammary Gland Consistency:

0 - Normal / 1 - Soft with small nodules / 2 - Soft with median nodules / 3 - Slightly hardened / 4 - Edemaciated / 5 - Hardened; Lymph. - Lymph nodes: 0 - Normal / 1 - Only one lymph node changed / 2 - Two altered lymph nodes; SC - Strip cup: 0 - No alterations; CMT - California mastitis test: 1 / 2