DIPARTIMENTO DI AGRARIA

Ph.D. Thesis

DOTTORATO DI RICERCA IN FOOD SCIENCE

XXXIII CICLO

GUT MICROBIOTA CHARACTERIZATION AS A MARKER TO EVALUATE THE EFFECT OF DIFFERENT DIETARY REGIMES ON WATER BUFFALO CALVES DURING THE PREWEANING PERIOD

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Final Exam 2019 - 2020

UNIVERSITÀ DEGLI STUDI DI NAPOLI
FEDERICO II

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SUMMARY

INTRODUCTION

THE CALF'S DIGESTIVE SYSTEM

Rumen

The reticulum

The omasum

The abomasum

The Esophageal Groove

Milk digestion

WATER BUFFALOES CALVES FEEDING: FROM BIRTH TO WEANING

Colostrum and neonate immunity

Transition to formula milk

THE MICROBIOTA AND ITS ROLE

GUT MICROBIOTA IN RUMINANTES

State of art

Aim of study

MATHERIALS AND METHODS

Study population

Samples collection

DNA extraction and sequencing

Bioinformatic analysis

RESULTS

Experimental design

Body weight gain

Fecal microbiota composition in newborn water buffaloes calves: Alpha and beta diversity at T0

Fecal microbiota composition in water buffaloes calves at T0: taxonomic composition

Comparison of fecal microbiota in paired sample: α diversity longitudinal analyses

Comparison of fecal microbiota in paired sample: β-diversity longitudinal analyses

Fecal microbiota composition in water buffaloes calves: Alpha and beta diversity at T1

Fecal microbiota composition in water buffaloes calves: Alpha and beta diversity at T1

Difference in microbial composition

DISCUSSION

SUMMARY

Growth and development of calves are highly influenced by the composition and activity of their associated gut microbiota.

A classic example of the importance of the microbiota in ruminants is the rumen, where fermentation of dietary substrates due to bacteria results in the formation of short-chain fatty acids. Short-chain fatty acids are a major energy source for the host, and an important substrate for the development of the rumen epithelium. However, in newborn calves, milk is primarily digested in the small intestine, and microbes colonizing the small intestine can contribute to intestinal homeostasis, stimulation of the immune system, and enhancement of intestinal epithelium wellness and development.

In particular the first three months of life result to be the most sensitive rearing period for the young calf. Calves are challenged by a series of stress factors after birth, including changes in their farming environment. Indeed, after birth, the living environment changes from the sterile uterus to natural outside conditions and, in addition, changes occur in nutrition, digestion and absorption from natural milk provided by the mother to feed that calves gain by themselves.

Optimum level of nutrition in early life favors faster growth, earlier onset of puberty, enhanced productivity and colonization of gut microbiota, which can influence healthy status.

Rearing healthy calves is very important as it can have a significant impact on their growth and milk production performance in adult life. Adequate calf development is

therefore crucially important for the entire dairy industry. Pre-weaning calves makes them particularly vulnerable to specific diseases, such as enteritis, which, in this age group, is among the diseases causing the highest mortality rates. Colostrum quality, diet with formula milk and management, as well as calf-related hygiene practices (e.g., cleaning routine for feeding equipment, calving pens, group calf pens), can have a pronounced effect on calf health and mortality. One of the most important aspects is the establishing of a proper nutrition plan, which is fundamental for the development and health of calves. A correct and gradual transition from the neonatal phase to the subsequent phase of development ensures that the weaning phase is successfully overcome. In fact, during early life the diet may impact colonization of gut microbiota, which can influence health, leading to potential long-lasting consequences later in life.

Today, there are still too many Italian farms exhibiting mortality rates higher than the physiological averages or that cannot sufficiently anticipate the weaning of calves to bring them at the next phase of growth, in a good state of health and with a rumen ready to digest fodder and concentrates,; this has led to a clear slowdown of genetic improvement in most farms.

A functional diet chosen to promote the proper development of the gut microbiota is therefore a strategy to ensure animal welfare and productivity.

In view of the commercial value of water buffalo milk, in addition, it has become necessary to optimize and simplify the weaning of water buffaloes calves, identifying protocols to replace breast milk without compromising the development and health of

the animals themselves. This has led to use different types of feeding that consequently impacted on the development of the gastrointestinal microbiota and consequently on the health of animals, especially in calves, because in the first period of life the microbial colonization is not yet stable and therefore can be easily affected. Many studies have been conducted both in humans and in different animal species about the role of microbiota and how it changes in relation to diet. In particular, in the ruminants species, the attention was focused on the rumen microbiota, since rumen plays the main role in the digestion of the adult animal. In calves, instead, when the animal is monogastric, intestine is the site playing the most important role in the digestion so, it is important to focus attention on this organ and its microbial flora organization.

Information is extremely limited on ruminant gut colonization, especially when focusing on the role of the microbiota in the early development of the gastro-intestinal (GIT) during the pre-ruminant period.

Water buffalo farming is the main economic source for many families in many areas of the world: Europe, Australia, North America, South America and some African countries. For this reason it is important to invest in research in this area with the aim to integrated knowledge and to promote quality and innovation in primary productions.

For these reasons the purpose of this study was to:

- characterize the fecal microbiota in calves during the pre-weaning phase

- determine which diet (water buffalo milk, formula milk or mixed diet) can contribute to the proper development of the gut microbiota
- identify the most useful diet to promote the growth and development of calves.

The results demonstrated the homogeneity in gut microbiota composition of newborn calves belonging to the same farm. In particular, consistent with what is described in dairy calves, the predominant phyla in newborn calves are: *Proteobacteria*, *Firmicutes* and *Bacteroidetes*.

Differences are evident between samples collected at two time points and displaying maturation of the intestinal microflora in the first weeks of life.

Difference occurs in groups feed with mixed diet were the microbiota structure is different and change the abundance of six genera in feces samples: *Faecalibacterium, Clostridia_UCG-014, Bifidobacterium, Collinsella, Parabacteroides, Eubacterium_coprostanoligenes_group.*

The high-throughput sequencing demonstrated to be a suitable approach to the study of the gut microbiota of newborn calves allowing the characterization of the intestinal microbiota during the pre-weaning phase.

The characterization of the gut microbiota in calves during the pre-weaning phase and monitoring changes over time could represent a useful tool for monitoring the health status of calves.

INTRODUCTION

Water Buffalo breeding in Italy

In the Italian livestock scenario, the "Italian Mediterranean Buffalo" has recorded a constant increase from the post-war period to today.

Most of the animals bred in Italy are concentrated in the Campania region, although farms are now arising in all Italian regions. At present (data updated at December 31st, 2020,) the situation reported by the National Database of the Zootechnical Registry (https://www.anasb.it/bufala-mediterranea-italiana/specie-bufalina) is shown in Table 1.

REGION	N° FARMS	N° ANIMAL
Abruzzo	16	153
Basilicata	25	4.275
Calabria	21	1.705
Campania	1.291	296.230
Emilia-Romagna	22	306
Friuli-Venezia Giulia	28	1.035
Lazio	702	79.716
Liguria	4	298
Lombardia	82	6.607
Marche	94	734
Molise	12	629
Piemonte	25	3.432
Puglia	71	11.942
Sardegna	4	50
Sicilia	23	2.055
Toscana	19	943
Trentino-Alto Adige	2	7
Umbria	33	626
Valle D' Aosta	1	7
Veneto	125	2.474
Total	**2.600**	**413.224**

Table 1. Italian distribution of water buffalo farms.

About 75% of the animals raised and more than 50% of the farms are in Campania region, in particular in the provinces of Caserta and Salerno.

These areas with the provinces of Latina and Frosinone in Lazio region, the province of Foggia in Puglia region and province of Isernia in Molise region constitute the

area of "DOP water buffalo mozzarella Chees" (“Mozzarella di Bufala Campana DOP”).

The progress in livestock technology is the result of research and innovations developed over the years thanks to the introduction of new strategies based on the increase in animal productivity and on wellness with particular attention to newborns. In fact among all animals present in farm, the highest morbidity and mortality rates generally occur in newborn calves prior to weaning.

Raising calves seems to be very easy, but actually raising calves takes a lot of time, money and efforts.

Calves need good management and breeding practices to obtain optimum gain in body weight, so that they attain about 75-80% of mature body weight at puberty.

With biological, environmental, and nutritional stressors, the success of this first rearing phase and the subsequent development of the animal, from which its well-being and productivity derive, depend on how carefully and properly calves are managed (source: https://dairy-cattle.extension.org/wp-content/uploads/2019/08/RDR-Calf_Nutrition.pdf).

Due to the low immune defenses and the incomplete development of the digestive system in young calves, any interference from the external environment or changes to the nutritional intake can drastically affect their development. Some of the problems include diarrhea and slow weight gain, as well as respiratory tract disease, which can lead to high levels of morbidity and mortality, and pose significant challenges to breeding (Turnbaugh et al., 2008).

A very important aspect in the management of calves is the diet chosen as a substitute for breast milk.

Poor feeding of young calves, in fact, leads to higher age at first calving and overall loss of productive performance in adult age. Malnutrition also results in reduced vigor, poor immune system, suppressed vitality and more prone to disease, ultimately leading to death of calves (source: https://www.dairyknowledge.in/sites/default/files/calf_nutrition-eng.pdf).

The main objective is establishing a nutritional program to improve the health of the intestines of calves during the pre-weaning period necessary to minimize the susceptibility of calves to diseases.

This requirement is necessary also because the new regulations limiting the prophylactic use of antimicrobials (Ring et al., 2018), determine an urgent need to find new approaches to minimize diarrhea incidence in neonatal calves is urgent.

For these reasons, in recent years, in the livestock farming and primary production, the study based on the search for functional foods and the evaluation of their effects on gut microbiota, as for humans, has gained increasing interest with the purpose to understand how diet impacts health and the physiological functions.

Indeed food has a big impact on gastrointestinal system and in particular on the gastro-intestinal microbiota, which is not just a passive bystander, but actively impacts multiple host functions, including circadian rhythmicity, nutritional responses, metabolism and immunity.

The aim is therefore to establish the correlation between the food and body with the purpose to promote wellness.

THE CALF DIGESTIVE SYSTEM

The digestive tract of ruminants consists of four compartments which include: rumen, reticulum, omasum and abomasum. The rumen, reticulum and omasum remain undeveloped at birth and during the first few weeks of life (figure 1).

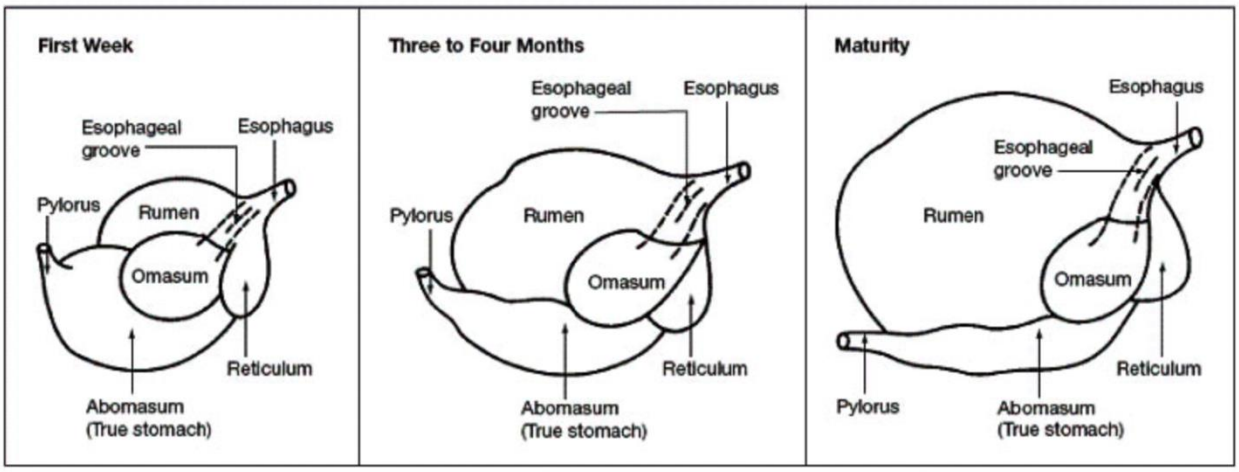


Figure 1. Development of ruminants stomach compartments from birth to maturity (source: https://calfcare.ca/management/the-calfs-digestive-system)

Rumen

The rumen is made up of two layers: the epithelial layer and the muscular layer. The muscular layer is responsible for rumen contractions and gives support to the epithelial layer, which in turn provides absorption. The end products of rumen fermentation, particularly propionate and butyrate acid, provide the stimulus needed for development of the epithelial layer.

Prior to weaning rumen must develop to be able to absorb and metabolize volatile fatty acids (VFA). Calves that do not eat dry feed – like milk fed veal – will not

develop a functional rumen. It is the grain that develops the rumen and allows calf transition from a milk–based to a feed–based diet.

In a newborn calf, the rumen makes up to 25%of the calf's stomach capacity. The rumen is constantly growing and changing, as the calf becomes a ruminant, and by three to four months of age, the rumen makes up 65 per cent of capacity. It is the most important part of the digestive system in a grain fed calf.

The reticulum

The reticulum is a pouch-like structure in the forward area of the body, close to the heart. The tissues in the reticulum form a network similar to a honeycomb. A small tissue fold lies between the reticulum and rumen, but the two aren't separate compartments. Together they're called the rumino-reticulum.

Heavy or dense feed and metal objects eaten by the cow drop into this compartment. Nails and other sharp objects may work into the tissue and cause "hardware disease." You can use magnets to prevent disease or correct the problem through surgery. Leaving it untreated may lead to infection and possibly death.

The omasum

The omasum is a globe-shaped structure containing leaves of tissue (like pages in a book). It absorbs water and other substances from digestive contents. Feed

material (ingesta) between the leaves will be drier than ingesta found in the other compartments.

The abomasum

For the first two weeks of a calf's life it is a monogastric – or simple–stomached – animal, using only the abomasum to digest the milk or milk replacer. The abomasum releases digestive enzymes to break down fats, carbohydrates and protein. The energy requirements are met from the absorption of glucose from the abomasum.

When a calf drinks milk, it passes over the rumen to the abomasum via the esophageal groove. The abomasum makes up to 60% of a newborn calf's stomach capacity. By the time the calf is three to four months old, the abomasum makes up 20 per cent of the capacity, and as the animal matures, that shrinks to only 8% of the stomach capacity.

The esophageal groove

Soon after birth, calves present an esophageal groove, which consists of muscular folds from the reticulo-rumen that come together to bypass the rumen, reticulum and omasum through to the abomasum when the calf drinks milk. The suckling reflex and milk protein stimulates the groove to open.

Calves should avoid to drink water right after the milk feeding. If it happens, the groove will still be open, letting the water into the abomasum. This weakens the clot

that is formed and the calf will not digest the milk as well as it should. This occurs until a calf is weaned (figure 2).

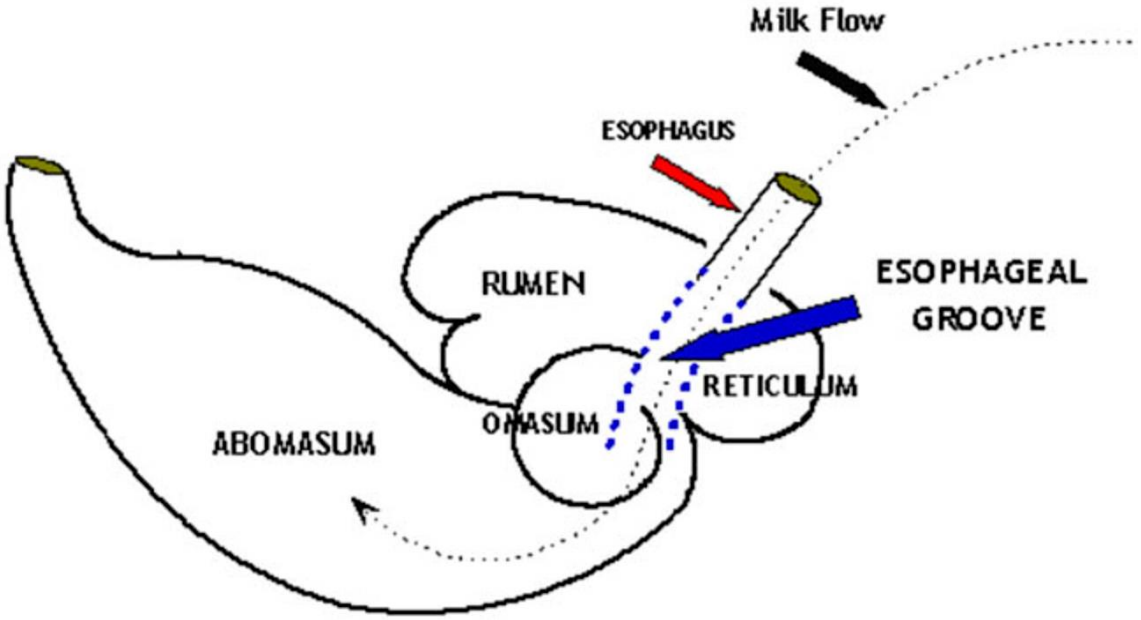


Figure 2. The Esophageal Groove in newborn calves (source: https://calfcare.ca/management/the-calfs-digestive-system)

Milk digestion

When a calf drinks milk or a milk replacer based from skim milk, it goes into the abomasum. Within ten minutes, the milk forms a clot in the abomasum from the coagulation of milk protein or casein, the enzymes rennin and pepsin, and the hydrochloric acid in the abomasum. Other milk components, primarily whey proteins, lactose and most minerals separate from the curd and rapidly pass into the small intestine (as much as 200 ml per hour). The lactose is digested quickly and, in contrast to casein and fat, provides immediate energy to the calf. The clot is then slowly absorbed by the blood stream over the next 12–18 hours.

WATER BUFFALO CALVES FEEDING: FROM BIRTH TO WEANING

Colostrum and neonate immunity

The in utero sterile mammalian gastrointestinal tract (GIT) is rapidly colonized by an array of microbiota during and after birth. This process of colonization has been described as a co-evolution due to the two-way interaction between host and microbes (Van den Abbeele et al., 2011). Host (luminal pH, food retention time in the gut, and immune defense mechanisms), microbial factors (adhesion, survival mechanisms under oxygen gradient, and mechanisms to obtain nutrients from the host), and external factors, such as maternal microbiota, delivery mode, diet, and antibiotic treatment during early life, all combine to influence gut colonization (Penders et al., 2006).

Despite of pregnancy in water buffalo being 308 to 318 days, the water buffalo calf can be considered immature at birth. The incisors of newborns, in fact, are almost all covered with the gingival mucosa. In view of the tropical origin of the species and its poor tolerance to low temperatures, newborns are very sensitive not only to neonatal diseases but also to environmental factors better tolerated by other domestic ruminants. Neonatal mortality is higher on farms that do not have a delivery room to avoid stress at birth.

Generally, the taking care of the buffalo calf begins 90 days before the calving of the female buffaloes, which may stay in areas of good feeding conditions receiving body reserves needed for the future lactation. This period involves the optimum

development of the mammal glands and colostrum production, with the proper quality and volume for the consumption of the newborn.

The immune system of the newborn is functionally immature because the placenta prevents transfer of maternal serum immunoglobulins to the calf before it is born, therefore, the neonatal calf is entirely dependent on colostral immunoglobulins for protection from disease. The calf's acquisition of colostral immunoglobulins through absorption in the intestine is called passive transfer or passive immunity (Quigley and Drewry, 1998).

The importance of colostrum to the health and survival of newborn calves is well established and its protective role against infectious disease in calves has long been recognized and associated with the transfer of colostral immunoglobulins (Matte et al., 1982).

Feeding calves the correct amount of high-quality colostrum immediately after birth is the single most important management practice in calf nutrition. Colostrum, defined as milk extracted from the mammary gland in the first 24 hours after birth, contains immunoglobulins that, when absorbed by gut, allow calf to defend himself against possible diseases.

The immunoglobulins found in colostrum are large proteins. Calves have openings in the small intestines to accommodate the protein’s absorption, but these opening close shortly after birth. Three types of immunoglobulins (Ig) can be found in the colostrum. Immunoglobulin G (IgG) makes up to 70% to 80% of the immunoglobulins and helps identify and destroy invading pathogens.

Immunoglobulin M (IgM) comprises 10% to 15 % of immunoglobulins and serves as the first line of defense against septicemia. Immunoglobulin A (IgA) comprises the remaining 15 %of immunoglobulins in colostrum and protects the mucosal surfaces, such as the intestine, from invasive pathogenic bacteria.

Colostrum also contains vitamins, minerals, energy (carbohydrate, fat) and proteins needed for calf metabolism, growth and for additional stimulation of the calf's immune system. Hormones (insulin) and growth factors (IGF-1) in colostrum also aid metabolism.

Spent the first 24 hours, water buffalo produces, between 24 and 72 hours, transition milk with a different composition from colostrum and finally milk composition change again when it is harvested 72 hours after calving and it is considered whole sellable milk.

Research has clearly shown that calves with adequate passive transfer grow better and have lower mortality and health cost when compared to calves with failed passive transfer.

For the reasons explained above in our study all calves were fed with colostrum before being recruited and fed according to the established diet according to their experimental group.

Transition to formula milk

After the period of colostrum feeding, the traditional nutritional strategy for calves has been to minimize liquid feed consumption, maximize solid feed consumption,

stimulate early rumen development, and wean calves at a relatively young age (usually 4-8 week).

During the first period of artificial feeding, calves are placed in individual or multiple boxes (De Franciscis and Zicarelli, 1974) and for at least five days colostrum is administered at 37 °C in two daily administrations and 2 or 3 liters per feeding.

After 6-7 days about 5-6 liters of formula milk daily should be administered, divided into two feeding. Formula milk should be prepared by mixing 160 to 180 g of powder with 840-820 g of water at a temperature of 40-44 °C. This quantity remains fixed until the age of 40 days. The reason why calves are separated from their mothers and fed with formula milk is linked to economic factors since milk produced by dams is intended for sale. (Hulbert et al., 2016).

THE MICROBIOTA AND ITS ROLE

The term microbiota was coined for the first time by Joshua Lederberg in 2001 to signify “the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and can be determinants of health and disease”. Specifically, the term “microbiota” represents the microbial taxa associated with body and the term “microbiome” is the collection of these microbes and their genes. The Human Microbiome Project and Metagenomics of the Human Intestinal Tract (MetaHIT) (www.metahit.eu/) project clearly demonstrated that gut microorganisms are not just passive residents, carrying out a range of biological functions that are important in nutrition and well-being of the individual. (Dewhirst et al., 2010). Approximately 100 trillion micro-organisms exist in the gastrointestinal tract, therefore the microbiome is now best thought of as a virtual organ of the body.

The first colonization of the intestine is one of the most profound immunological exposures faced by the newborn and it is influenced by external and internal factors. The early composition of microbiota could have long-lasting metabolic effects and the initial composition of intestinal bacteria is also known to affect postnatal immune system development. Both animal and human studies have demonstrated that diet can influence the composition and function of the gut microbiome. Other factors, including genetics; the mode of delivery at birth; the method of infant feeding; and the use of medications, especially antibiotics, also contribute to the composition and function of the gut microbiome (Wen et al., 2017).

Mammals, indeed, have complex microbial communities associated with them, composed primarily of bacteria, but also including archaea, fungi, and viruses (Smith et al., 2015). These microorganisms essentially coat all the skin and mucosal surfaces of the host, with the largest populations residing within the gastrointestinal tract. The interrelationship between diets, the gut flora (often called microbiota) and health has been appreciated for over a century.

In the 1908 Metchnikoff proposed that the putrefaction by microbes in the colon was responsible for aging and senility and suggested supplementation of bacteria rich fermented milk products as a method to avoid putrefaction.

Research over the past century has concentrated primarily on the role of microbes in human health and animal disease, through the fermentative role of the intestinal microbiota in the digestion of dietary fiber (Macfarlane et al., 2012). More recently however, through advances in molecular biology and DNA sequencing technologies to investigate diverse microbial communities, research into the microbiota has rapidly expanded uncovering evidence of wide-ranging impacts on host physiology (Walker, 2016). Over the past decade, accumulating evidence also suggests that modern diets, lifestyles, and medical care are shaping the human microbiota in novel and potentially detrimental ways to health.

It is possible to hypothesize, based on comparative studies, that the same principles can be applied across the animal world, since bad farming practices, especially as regards nutrition, can affect the gut microbiota development that could result in a condition of dysbiosis harmful to health.

This would have effects on primary production, as unhealthy animals would be poorly productive and, above all to human health, if we consider that the percentage of the population that consumes animal or products of animal origin is by far greater than the vegetarian population.

To date, the molecular basis of dysbiosis and the key bacterial groups involved remain poorly defined. It is clear however that if the gut microbiota is disrupted (eg, in case of antibiotic treatment or gut inflammation) the risk of disease can substantially increase (Antharam et al., 2013) and reestablishment of the normal microbiota can result in recovery from disease.

For all these reasons a functional diet chosen to promote the proper development of the gut microbiota is therefore a strategy to ensure animal welfare and productivity.

GUT MICROBIOTA IN RUMINANTES

State of the art

Young ruminants present at birth an undeveloped reticulo-rumen, therefore, until the system is fully matured they function as monogastric fed on milk-based diets that are not digested in the rumen but in the abomasum (Davis et al., 1998) A smooth transition from a monogastric to ruminant animal, with minimal loss in growth, requires the development of the reticulo-rumen and its associated microbial population for efficient utilization of dry and forage-based diets (Heinrichs et al., 2005).

Several molecular studies can provide accurate information about cultivable and non-cultivable species present in the animal intestinal microbiota; especially in the ruminant microbiota. Culture dependent studies have shown a bacterial load of 10^4-10^6 per gram of content, in the large intestine and in the rumen (Piccione et al., 2002). The four stomachs of ruminant animals contain a great diversity of prokaryotic (bacteria, archaea, virus) and eukaryotic (protozoa and fungi) micro-organisms that together breakdown and ferment the feed ingested by the host animal (Yáñez et al., 2015).

The rumen microbiota in adult animals has been demonstrated to be highly redundant, resilient and host-specific (Weimer et al., 2015). As a result, when any nutritional intervention ceases, the rumen microbiota and its function return to the original state making it difficult to permanently modify a fully mature rumen microbiome in adult animals. For this reason, the developing rumen of the newborn animals may represent an opportunity for microbial programming by modifying the type of microbial groups that first occupy the ecological niches in the rumen of young ruminants (Yáñez et al., 2015).

Animal microbiota studies have examined primarily cats, poultry and cattle. *Furet et al.* (Furet et al., 2009) conducted a comparative molecular study on the human fecal microbiota and that of domestic and farm animals. The results show that a low water content in the intestinal lumen can contribute to the presence of a high bacterial count in feces, both in sheep and goats. In addition, the study confirmed that in the human fecal microbiota there are species belonging to the *Clostridium* coccoides group and

the *Bacteroidetes*/*Prevotella* group, both dominant also in the feces of ruminants such as cattle, sheep and goats, but with a higher charge.

For the genus *Lactobacillus* the study did not show a significant difference between human and animal gut microbiota. The results, finally, showed a higher charge of Bifidobacteria in the fecal microbiota of cattle compared to human, sheep or goats.

Studies on fecal microbiota in ruminants, furthermore, clearly indicate that the most abundant Phyla in calves are *Firmicutes*, *Bacteroidota*, *Proteobacteria* (Malmuthuge et al., 2014).

Commonly identified enteric commensal bacteria include the Phyla of *Firmicutes* (including the genera *Lactobacillus*, *Clostridium*, *Enterococcus*), *Bacteroidetes* (including the genus *Bacteroides*), *Proteobacteria* (including the genus *Escherichia coli*) and *Actinobacteria* (including the genus *Bifidobacterium*).

The relative frequencies of this taxa depend on both the physiological state and the age of the animal development because the fecal bacterial composition of dairy calves undergoes dynamic changes during the first 12 weeks of life (Uyeno et al., 2010).

In addition, another study, showed that the type of feed offered to calves impacts the structure of the gut microbiota by providing different dietary substrates to bacterial communities (Maslowski et al., 2011).

A study based on the use of milk replacer (MR) and pasteurized waste milk (pWM), showed that the fecal microbiota of calves in the MR treatment tended to have greater relative abundance of the phylum *Bacteroidetes* compared with the pWM calves,

whereas the relative abundance of *Firmicutes* tended to be lower in calves fed MR than in those fed pWM (Malmuthuge et al., 2014).

A pilot study with the purpose to examine the fecal microbiota of six Simmental dairy calves to investigate time-dependent dynamics of the microbial community highlighted a pronounced shift in the fecal bacterial composition from the beginning of life, when calves predominantly consume milk, to later time points, when calves ingest increasing amounts of solid feed and are weaned (Klein-Jöbstl et al., 2014). This study assumed that during early development these changes go hand in hand, whereas when the gastrointestinal tract stabilizes, the microbiota in the different sites of the digestive tract differs.

Finally, a study of characterization of the fecal bacterial microbiota of healthy and diarrheic dairy calves highlighted significant differences in community membership and structure among healthy calves from different farms. Differences in community membership and structure also were identified between healthy and diarrheic calves within each farm.

As discussed above there are many factors that can influence the different development of the gut microbiota.

Studies conducted to date suggest the possibility of intervening in the very early stages of development, i.e. those preceding weaning. At this stage, in fact, the microbiota is not yet developed but it can be affected from both internal and external factors.

Aim of the study

In the water buffalo farms, milk represent a fundamental economic resource and for that the newborn calves are fed with formula milk until the weaning.

The aim of the present study was to characterize the gut microbiota in newborn calves and to assess any differences due to different food regimes.

Weight gain was also considered and differences in fecal microbiota composition was evaluated with statistical tests.

The final goal is to provide new knowledge, because actually there are few studies on the water buffalo calves and many of these concern the rumen microbiota, while intestine is mainly responsible for digestion in this age.

MATHERIALS AND METHODS

Population under study

For these study we selected a total of 60 newborn calves. To avoid differences in farming practices, environmental conditions, climatic factors and other variables able to affect results, all animals were recruited from the same dairy farm. Finally, all the calves included in the study were vaginally delivered and were born during the winter period (from November 2018 to March 2019).

Within a few hours after birth calves were weighed and fed with maternal colostrum for 3 to 5 days. Animals were then divided into three groups, each one including twenty animals kept in individual boxes (1×2.30 m2) and homogeneous for live weight and month of birth, as described below:

- calves fed twice a day with formula milk (Group A);
- calves fed twice a day with water buffalo milk (Group B);
- calves fed twice a day, once with formula milk and once with water buffalo milk (Group C);

Animals included in the study were both males and females equally distributed and fed with 2l of water buffalo milk or milk replacer at 18% of dry matter (DM) for each meal (twice daily) during the experimental period.

For all calves included in the present study, the animal ID, sex, date of birth and the reference to the experimental group were reported in the Table 2.

Animal_ID	Date of birth	Sex	SampleID	Time	Diet	Experimentalgroup
4610	01/11/2018	Male	1	0	formula_milk	A
			2	1	formula_milk	A
4612	07/11/2018	Female	3	0	formula_milk	A
			4	1	formula_milk	A
4615	17/11/2018	Female	5	0	formula_milk	A
			6	1	formula_milk	A
4617	19/11/2018	Male	7	0	formula_milk	A
			8	1	formula_milk	A
4618	20/11/2018	Female	9	0	formula_milk	A
			10	1	formula_milk	A
4619	26/11/2018	Female	11	0	water_buffalo_milk	B
			12	1	water_buffalo_milk	B
4620	28/11/2018	Female	13	0	water_buffalo_milk	B
			14	1	water_buffalo_milk	B
4621	28/11/2018	Female	15	0	water_buffalo_milk	B
			16	1	water_buffalo_milk	B
4622	29/11/2018	Female	17	0	water_buffalo_milk	B
			18	1	water_buffalo_milk	B
4624	01/12/2018	Male	19	0	water_buffalo_milk	B
			20	1	water_buffalo_milk	B
4625	04/12/2018	Female	21	0	mixed	C
			22	1	mixed	C
4626	05/12/2018	Male	23	0	mixed	C
			24	1	mixed	C
4627	05/12/2018	Male	25	0	mixed	C
			26	1	mixed	C
4628	11/12/2018	Male	27	0	mixed	C
			28	1	mixed	C
4630	22/12/2018	Male	29	0	mixed	C
			30	1	mixed	C
4631	22/12/2018	Female	31	0	formula_milk	A
			32	1	formula_milk	A
4633	31/12/2018	Male	33	0	formula_milk	A
			34	1	formula_milk	A
4634	01/01/2019	Male	35	0	formula_milk	A
			36	1	formula_milk	A
4635	02/01/2019	Male	37	0	formula_milk	A
			38	1	formula_milk	A
4636	06/01/2019	Female	39	0	formula_milk	A
			40	1	formula_milk	A
4637	06/01/2019	Male	41	0	water_buffalo_milk	B
			42	1	water_buffalo_milk	B
4638	07/01/2019	Male	43	0	water_buffalo_milk	B
			44	1	water_buffalo_milk	B
4639	08/01/2019	Male	45	0	water_buffalo_milk	B
			46	1	water_buffalo_milk	B

4641	13/01/2019	Female	47	0	water_buffalo_milk	B
			48	1	water_buffalo_milk	B
4642	14/01/2019	Female	49	0	water_buffalo_milk	B
			50	1	water_buffalo_milk	B
4643	17/01/2019	Female	51	0	mixed	C
			52	1	mixed	C
4644	18/01/2019	Female	53	0	mixed	C
			54	1	mixed	C
4645	18/01/2019	Female	55	0	mixed	C
			56	1	mixed	C
4646	20/01/2019	Female	57	0	mixed	C
			58	1	mixed	C
4647	22/01/2019	Male	59	0	mixed	C
			60	1	mixed	C
4649	23/01/2019	Female	61	0	formula_milk	A
			62	1	formula_milk	A
4652	24/01/2019	Male	63	0	formula_milk	A
			64	1	formula_milk	A
4653	27/01/2019	Female	65	0	formula_milk	A
			66	1	formula_milk	A
4654	28/01/2019	Male	67	0	formula_milk	A
			68	1	formula_milk	A
4659	04/02/2019	Female	69	0	formula_milk	A
			70	1	formula_milk	A
4665	07/02/2019	Male	71	0	water_buffalo_milk	B
			72	1	water_buffalo_milk	B
4667	08/02/2019	Female	73	0	water_buffalo_milk	B
			74	1	water_buffalo_milk	B
4668	08/02/2019	Male	75	0	water_buffalo_milk	B
			76	1	water_buffalo_milk	B
4669	10/02/2019	Female	77	0	water_buffalo_milk	B
			78	1	water_buffalo_milk	B
4670	11/02/2019	Female	79	0	water_buffalo_milk	B
			80	1	water_buffalo_milk	B
4671	11/02/2019	Female	81	0	mixed	C
			82	1	mixed	C
4672	12/02/2019	Female	83	0	mixed	C
			84	1	mixed	C
4675	15/02/2019	Male	85	0	mixed	C
			86	1	mixed	C
4676	16/02/2019	Male	87	0	mixed	C
			88	1	mixed	C
4677	18/02/2019	Female	89	0	mixed	C
			90	1	mixed	C
4678	18/02/2019	Female	91	0	formula_milk	A
			92	1	formula_milk	A
4682	28/02/2019	Female	93	0	formula_milk	A

			94	1	formula_milk	A
4683	28/02/2019	Female	95	0	water_buffalo_milk	B
			96	1	water_buffalo_milk	B
4688	12/03/2019	Male	97	0	formula_milk	A
			98	1	formula_milk	A
4689	12/03/2019	Male	99	0	formula_milk	A
			100	1	formula_milk	A
4691	15/03/2019	Female	101	0	mixed	C
			102	1	mixed	C
4692	16/03/2019	Male	103	0	formula_milk	A
			104	1	formula_milk	A
4693	16/03/2019	Male	105	0	water_buffalo_milk	B
			106	1	water_buffalo_milk	B
4694	17/03/2019	Male	107	0	water_buffalo_milk	B
			108	1	water_buffalo_milk	B
4695	17/03/2019	Female	109	0	mixed	C
			110	1	mixed	C
4696	18/03/2019	Male	111	0	water_buffalo_milk	B
			112	1	water_buffalo_milk	B
4697	19/03/2019	Male	113	0	water_buffalo_milk	B
			114	1	water_buffalo_milk	B
4699	19/03/2019	Male	115	0	mixed	C
			116	1	mixed	C
4705	22/03/2019	Male	117	0	mixed	C
			118	1	mixed	C
4706	22/03/2019	Male	119	0	mixed	C
			120	1	mixed	C

Table 2. Table metadata of the subjects included in the study

Sample collection

Freshly voided fecal samples were collected from all calves at two different time points: at the end of administration of the colostrum (T0) and 21 days (T1) after the start of the specific diet.

All the samples were kept in a sterile container, transported in dry ice and immediately stored at -80 °C until DNA extraction.

Anamnestic information was collected for each animal including mother's conditions, such as health problems during pregnancy and eventual antibiotics use,

and calves' conditions such as weight at birth and at the end of the trial, and eventual gastro enteric diseases during trial.

Milk replacer (MR) was prepared according to the manufacture's procedure and its chemical composition is shown below (Table 3).

	MILK REPLACER
Dry Matter (%)	96.0
Ash (% Dm)	7.45
Crude Protein (% Dm)	23.96
Fat (% Dm)	20.83
Milk Forage Unit (% Dm)	1.45

Table 3. Chemical composition of formula milk.

DNA extraction and sequencing

DNA was extracted from feces using the standard operating protocol for fecal samples IHMS_SOP 07 V2 Version: 2 (Dore, J. et al. 2015) with some modifications related to use of the QIASimphony automatic DNA extractor and the kit QIAamp DSP DNA Mini Kit.

Briefly, samples were processed as described below:

1. Add 250µL Guanidine Thiocyanate to each tube containing frozen feces (~200mg)
2. Add 40µL of N-lauroyl sarcosine 10% and thaw
3. Then 500µL of N-lauroyl sarcosine 5% and vortex to mix well
4. Incubate 70 °C in dry bath for 1 hour
5. At the end of incubation, add 750mg of glass beads (0.1 mm) in each tube and vortex vigorously
6. Shake for mechanical disruption: - with Bead BeaterTM :

   Turned on (medium speed) for 5min. Stopped for 10min, and again Turned on (medium speed) for 5min
7. Add 15mg of PVPP (powder) per sample and vortex vigorously
8. Centrifuge at 14000rpm (18000 g) for 5min, 4°C
9. Recover the supernatant in a sterile tube
10. The supernatant obtained was used to extract DNA with the DSP DNA Mini Kit with QIAsymphony instrument with the protocol Complex 200_OBL_V4 DSP. DNA was eluted in a final volume of 60 µl.

After extraction DNA concentration was quantified using a high-sensitivity QubitTM fluorometer in order to have a concentration of 10 ng/µl for samples.

To characterize fecal microbiota in this study we amplified and performed high-throughput sequencing of the two hypervariable regions V3 and V4 of the 16S rRNA gene.

The PCR amplification was performed using specific primers targeting the regions of interest CCTACGGGNGGCWGCAG-3′ (forward) and 5′-GACTACHVGGGTATCTAATCC-3′ (reverse) with overhang adapters attached and the KAPA HiFi HotStartReadyMix DNA Polymerase (Roche Diagnostics, Mannheim, Germany) as described below (Table 4):

	Volume
Microbial DNA (5 ng/µl)	2.5 µl
Amplicon PCR Forward Primer 1 µM	5 µl
Amplicon PCR Reverse Primer 1 µM	5 µl
2x KAPA HiFi HotStartReadyMix	12.5 µl
Total	25 µl

Table 4. Set up PCR for 16S rRNA amplification

The PCR reaction was carried as follows:

- 95°C for 3 minutes
- 35 cycles of amplification:
    95°C for 30 seconds,
    55°C for 30 seconds
    72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

At the end of the amplification, 1 µl of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the size. Using the V3 and V4 primer pairs in the protocol, the expected size on a Bioanalyzer trace after the Amplicon PCR step is ~550 bp (figure 3)

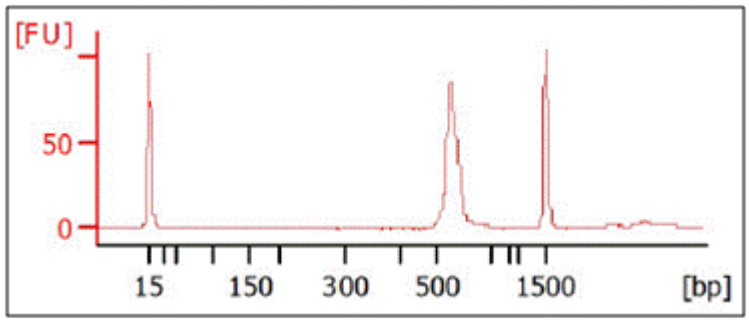


Figure 3.Bioanalyzer Trace after Amplicon PCR Step

Amplicons were then purified from free primers and primer-dimer species using the magnetic beads Agencourt AMPure XP-PCR Purification (Beckman Coulter, Brea, CA, USA) according to the 16S Metagenomic Sequencing Library Preparation protocol (Illumina,San Diego, CA, USA) and libraries were pooled to an equimolar amounts.

The performed steps are described below:

- Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute to collect condensation, carefully remove seal.
- Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough depending on the number of sample under processing.

- Using a multichannel pipette, add 20 µl of AMPure XP beads to each well of the amplicon PCR plate. Change tips between columns.
- Gently pipette entire volume up and down 10 times if using a 96-well PCR plate or seal plate and shake at 1800 rpm for 2 minutes if using a MIDI plate.
- Incubate at room temperature without shaking for 5 minutes.
- Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
- With the amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.
- With the amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follow:
    - Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    - Incubate the plate on the magnetic stand for 30 seconds.
    - Carefully remove and discard the supernatant.
    - Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.
- With the amplicon PCR plate still on the magnetic stand, allow the beads to air dry for 10 minutes.
- Remove the amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 µl of 10 mM Tris pH 8.5 to each well of the amplicon PCR plate.

- Gently pipette mix up and down 10 times, changing tips after each column (or seal plate and shake at 1800 rpm for 2 minutes). Make sure that beads are fully resuspended.
- Incubate at room temperature for 2 minutes.
- Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
- Using a multichannel pipette, carefully transfer 50 µl of the supernatant from the amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

After quality and quantiy control, libraries were normalized, pooled and sequenced on MiSeq platform (Illumina, San Diego, CA) in a 2 x 300 bp paired-end format. (Figure 4). Negative controls for DNA extraction and PCR amplification were included in each MiSeq run for quality control.

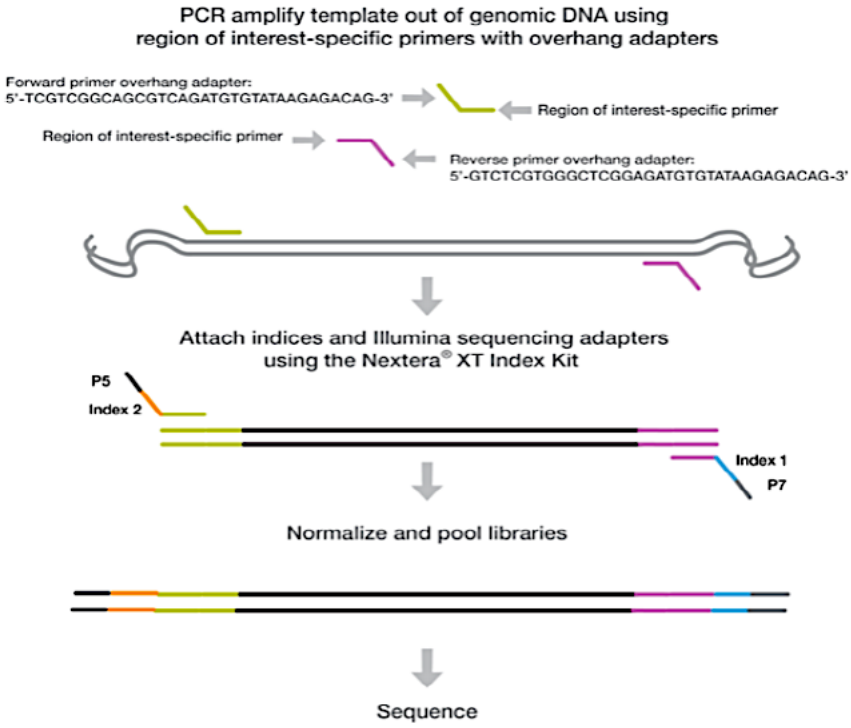


Figure 4. 16S V3 and V4 Amplicon Workflow

Bioinformatic analysis

The raw sequences were checked for quality control with the FastQC tool in order to estimate sequence quality and subsequently remove low quality ones (phred score < 20) which might cause bias in downstream analyses due to eventual incorrectly called nucleotides.

Sequence were then imported in Qiime2 software (v. 2020.8) for microbiota data analysis.

The first step of the analysis pipeline allowed the demultiplexing of the files in fastq format in order to use them as input for dada2 package to denoise, remove primers, remove chimaeras and exclude low quality reads. Based on the quality control check, forward reads were filtered and trimmed at 300 bp, while reverse reads were trimmed at 180 bp and resolved to high-resolution Amplicon Sequence Variants (ASVs),

which represent, as closely as possible, the original biological sequence of the sequenced amplicon.

ASVs were clustered at 99% similarity and clustered sequences were aligned to Silva 138 database (Quast et al., 2013) using feature-classifier classify-sklearn plug-in for taxonomy classification.

The sequences were then used as input to creating a sequence alignment using MAFFT and to build a phylogenetic rooted tree to be used for statistical diversity metrics.

In addition, in order to eliminate sampling depth heterogeneity, alpha and beta diversity were computed after standardization of sample size to 4800 sequences and after removing the features with a frequency lower than 150.

Bioinformatics analysis was carried out to evaluate taxonomic composition of the analyzed samples and the presence of any statistical differences between groups.

RESULTS

Body weight gain

Since this study was based on the administration of different dietary regimes to water buffalo calves, the first purpose was to evaluate if diet could have an effect on weight gain in animals. This is essential because an appropriate nutrition plan in early life promotes faster calves growth, earlier onset of puberty and enhanced productivity.

Body weight of calves in the three groups was recorded at the beginning of the trial and later, at the completion of the experimentation, which lasted for 21 days. Growth rate of the animals was estimated by the differences of these two measures.

Considering all the animals included in the study, mean body weight at birth was 42,29 kg ± standard deviation of 5,38 kg for females and 45,97 kg ± 4,30 kg for males. At the end of the trial the mean body weight was 62,69 kg ± 6,02 kg for female calves and 66,41 kg ± 6,55 kg for males. Changes in body weight over a 21-day period in the three groups were reported in table 5. The results were divided by experimental group.

Group	***Weight T0 (kg)***	***Weight T1 (kg)***	**Weight increase (kg)**	e_s
Water buffalo milk	43,99	64,79	20,8	0,81
Mixed	44,55	66,39	21,84	0,83
Formula milk	43,96	62,57	18,61	0,82

Table 5. Change in the body weight over the experimental period.

The average daily weight gain (ADG) for the male calves was 1.01 kg ±0.14 in the natural water buffalo milk group, 0.96 kg ± 0.2 in the formula milk group and 1.00 kg ± 0.15 for the mixed milk diet group.

Consistently, in the female calves, the observed ADG was 0.95 kg ± 0.15, 0.96 kg ± 0.16 and 0.94 kg ± 0.15 in the natural milk, formula milk and mixed milk groups, respectively. Table 6 reports the data collected from each animal.

Animal ID	Experimental group	Sex	Weight kg T0	Weight kg T1	DG
1	formula milk	m	39,8	47,8	0,38
2	formula milk	f	32,2	59,5	1,30
3	formula milk	f	37,6	57	0,92
4	formula milk	m	50	65,5	0,74
5	formula milk	f	46,8	65,5	0,89
6	buffalo milk	f	41,6	63	1,02
7	buffalo milk	f	43,6	62	0,88
8	buffalo milk	f	45	72,5	1,31
9	buffalo milk	f	48,2	72	1,13
10	buffalo milk	m	48	71	1,05
11	mixed	f	44	64,5	0,98
12	mixed	m	50,5	74,5	1,14
13	mixed	m	42,8	65,5	1,08
14	mixed	m	49,8	73,5	1,13
15	mixed	m	44,8	71	1,25
16	buffalo milk	m	44,6	63	0,88
17	buffalo milk	m	49,2	65,5	0,78
18	buffalo milk	m	47,8	65	0,82
19	buffalo milk	f	45	68	1,10
20	buffalo milk	f	39,8	55	0,72
21	mixed	f	40,8	59	0,87
22	mixed	f	41,4	60,8	0,92
23	mixed	f	49,6	68,5	0,90
24	mixed	f	27	47,6	0,98
25	mixed	m	52,5	75,5	1,10
26	formula milk	f	49,6	69	0,92
27	formula milk	m	48,2	71,2	1,15
28	formula milk	m	42,6	59,6	0,81
29	formula milk	m	47,6	66	0,88
30	formula milk	f	40,6	57	0,78
31	formula milk	f	45,4	64	0,89
32	formula milk	m	51,5	73,5	1,05
33	formula milk	f	48,6	67	0,88
34	formula milk	m	52,5	70,5	0,86
35	formula milk	f	42	60,2	0,87
36	mixed	f	39,8	58	0,87
37	mixed	f	46,4	68	1,03

38	mixed	m	43,6	66,5	1,09
39	mixed	m	47,8	74	1,25
40	mixed	f	41,2	63,5	1,06
41	buffalo milk	m	50	71	1,00
42	buffalo milk	f	32,4	55,5	1,10
43	buffalo milk	m	43,8	68	1,15
44	buffalo milk	f	40,4	51,5	0,53
45	buffalo milk	f	45,8	67,5	1,03
46	formula milk	f	45	67,5	1,07
47	formula milk	f	45,2	63,2	0,86
48	formula milk	m	51	67,5	0,79
49	formula milk	m	38,2	55,5	0,82
50	formula milk	m	42,4	62	0,93
51	buffalo milk	f	48,2	71	1,09
52	buffalo milk	m	37,6	57	0,92
53	buffalo milk	m	43	64	1,00
54	buffalo milk	m	45,6	68	1,07
55	buffalo milk	m	48,4	73,5	1,20
56	mixed	f	40	60,5	0,98
57	mixed	f	35,6	62,5	1,28
58	mixed	m	40,8	56,2	0,73
59	mixed	m	39,6	61,5	1,04
60	mixed	m	45,2	69	1,13

Table 6. Weight at different time points and daily weight gain.

Statistical analysis to compare body weight gain for three different groups was performed first considering the normal distribution of samples by Kolmogorov-Smirnov tests and then calculating the means of the values within the groups.

Finally a comparison of means was performed using the ANOVA test, considering significant values lower than < 0.05.

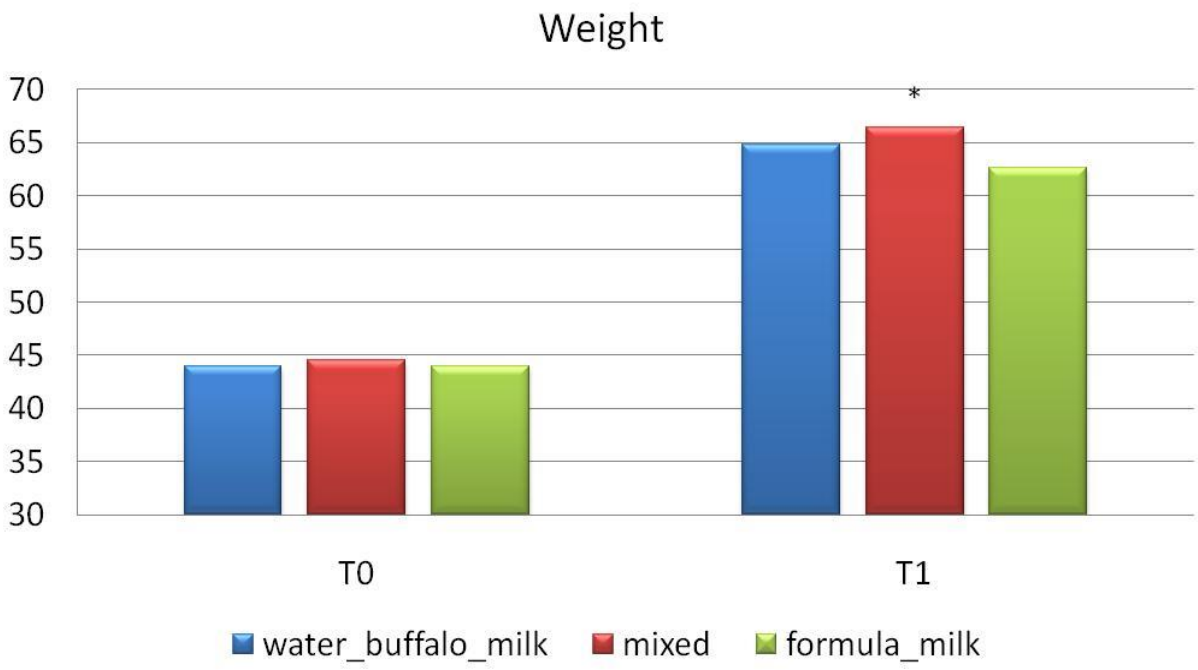


Figure 4. Comparison of body weight changes in 21-day period (the values are expressed in Kg). The effect on weight gain is evident specially in mixed diet group where the value is significantly increased *p-value < 0.005.

The results in figure 4 showed that the weight of animals at T0 time was equally distributed in the three groups.

The comparison with T1 time, instead, showed that the highest increase in body weight occurred in calves fed with mixed diet compared with formula milk ones (p-value <0.005).

Fecal microbiota composition in newborn water buffalo calves: Alpha and beta diversity at T0

Fecal samples collected from newborn calves were analyzed to characterize the composition of the microbiota in newborn water buffalo calves.

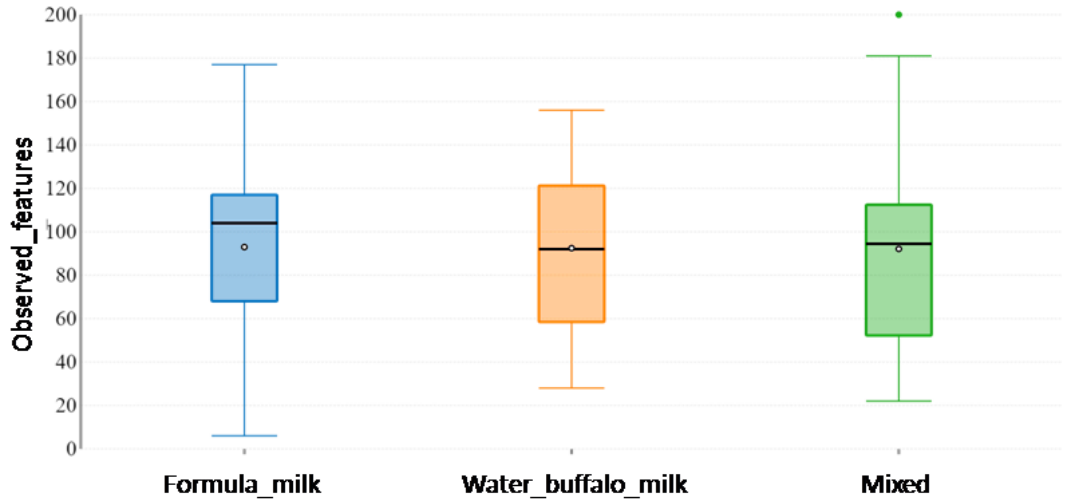
Samples were collected at T0 and were analyzed to define alpha and beta diversity and the microbial composition based on the relative frequencies of the taxa present in the analyzed samples.

Alpha diversity analysis was performed through QIIME2 pipeline to measure the diversity within samples.

The rarefaction curves of all samples almost reached a plateau, suggesting that the sequencing depth was sufficient (data not shown).

Differences in species representation (richness) and diversity (richness and abundance) of fecal microbial species within groups were assessed for all of the 60 subjects using two indices: Observed features index, used as a qualitative measure of species richness (Shannon and Weaver, 1949), and Shannon entropy, used as a quantitative measure of community richness. (figure 5).

A. *Observed features*



B. *Shannon entropy*

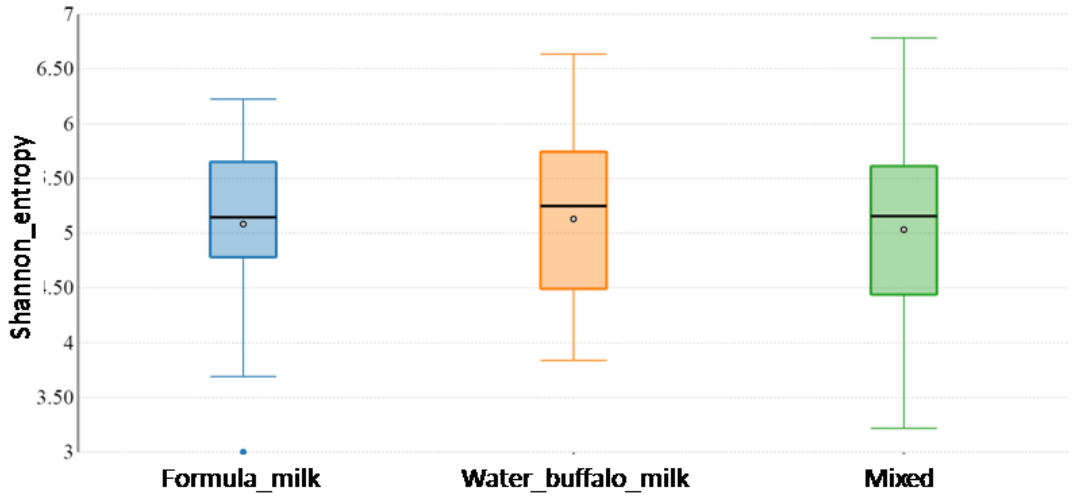


Figure 5. Box plots showing alpha diversity at T0 time in formula milk (blu), water buffalo milk (orange), and mixed milk (green) based on the number of observed features and Shannon entropy.

The alpha analysis was performed using the nonparametric Kruskal-Wallis statistical test for unpaired samples that compares two or more unpaired groups.

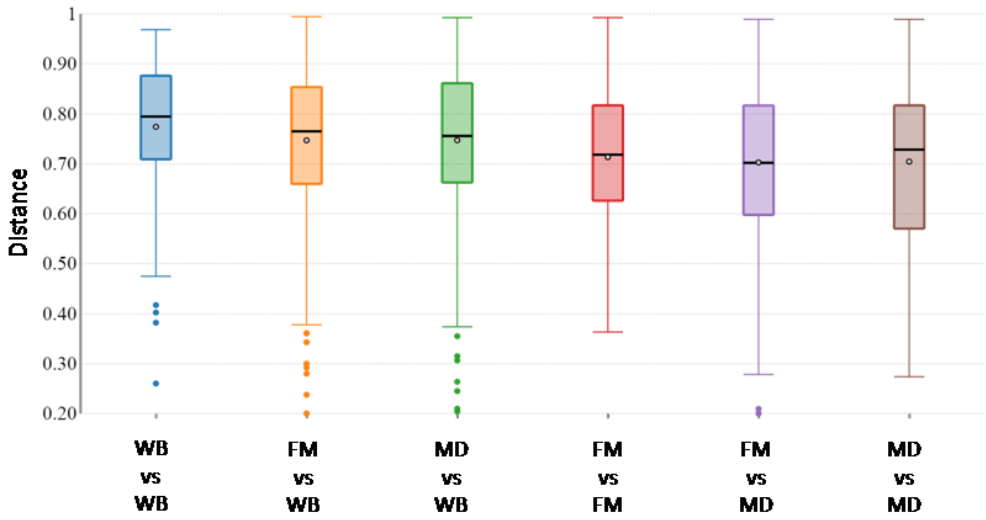
Results indicated that the microbiota of the animals at T0 did not reveal significant differences in diversity within groups neither in microbial richness (Kruskal-Wallis test; Observed features index; H= 0.246; P-value=0.884) not evenness (Kruskal-Wallis test; Shannon index; H= 0.179; P-value=0.913).

Beta diversity analysis was performed to verify the absence of possible differences in the composition of the fecal microbiota of the animals that constituted the three groups and therefore to assess their starting uniformity.

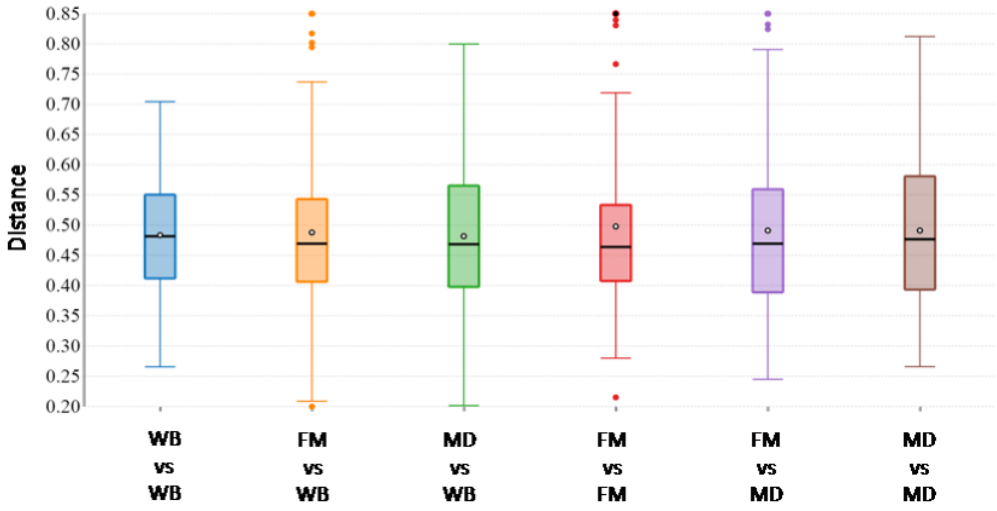
Samples (fecal microbiota T0) were analyzed using PERMANOVA test which allows to analyze multivariate data based on distance matrices.

Beta diversity was evaluated using Bray Curtis (a quantitative measure of community dissimilarity), unweighted (a qualitative measure of community dissimilarity incorporating phylogenetic relationships between the features) and weighted (a quantitative measure of community dissimilarity incorporating phylogenetic relationships between the features) UniFrac distances matrices. (Figure 6)

A. *Bray Curtis*



B. *Unweighted Unifrac*



C. *Weighted Unifrac*

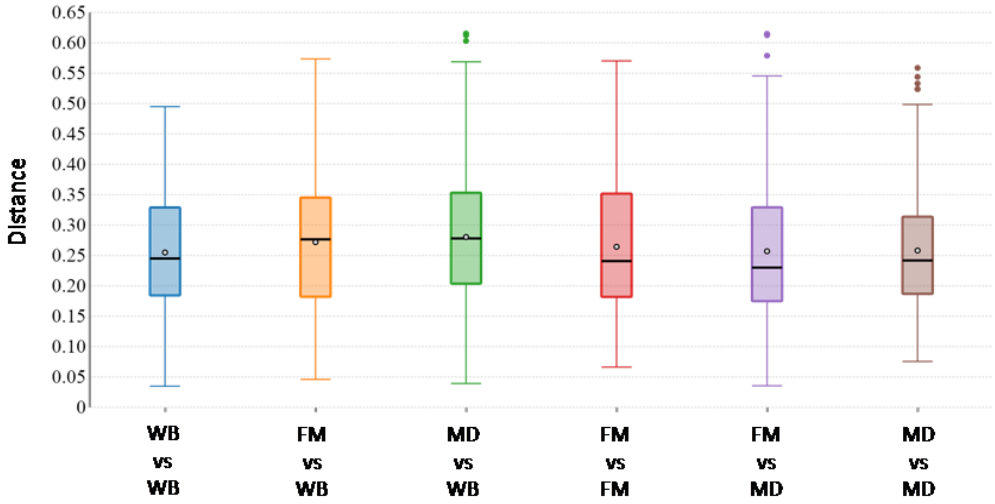


Figure 6. Box plots showing beta diversity distance amongst different groups at T0 time. (A) Bray Curtis dissimilarity; (B) Unweighted Unifrac distance and (C) Weighted Unifrac distance. WB represents water buffalo milk fed group; FM represents formula milk group and MD represents group feed with mixed diet.

Measures of species abundance (PERMANOVA; Bray-Curtis; pseudo-F =1.113; P = 0.27), and presence (PERMANOVA; Unweighted UniFrac; pseudo-F =0.709; P = 0.904) showed that the diversity among three groups at T0 time were not significant, while considering both presence and abundance (PERMANOVA; Weighted UniFrac; pseudo-F =2.638; P = 0.014) within the three groups there were statistically significant differences. These data were subsequently compared with those obtained from samples collected after treatment.

Fecal microbiota composition in water buffalo calves at T0: taxonomic composition

The relative abundance of amplicon sequence variants (ASVs) in all samples collected at T0 was evaluated at the phylum, family and genus level.

To assess the taxonomic composition, the phylogenetic analysis of 60 fecal samples from calves at T0 time exhibited the presence of four phyla: *Proteobacteria* (mean frequency value of 40.02% ± standard deviation of 24.08%), *Firmicutes* (30.77% ± standard deviation of 17.65%), *Bacteroidota* (23.79%±20.22%) and *Actinobacteriota* (3.09% ± 5.02%), *Verrucomicrobiota* (1.52%±4.43%), *Fusobacteriota* (0.6% ± 1.54%), *Cyanobacteria* (0.12%± 0.81%), *Euryarcheota* (0.02% ± 0.21%).

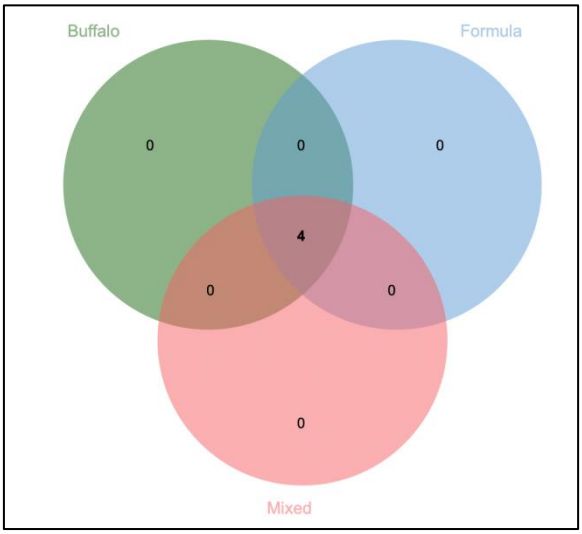
Considering the family level taxonomic assignment we identified 39 taxa and the most abundant (mean relative frequencies >2%) were *Enterobacteriaceae* (39.07% ± 24.12%), *Bacteroidaceae* (21.94% ± 19%), *Lachnospiraceae* (8.72%±7.21%), *Clostridiaceae* (4,18%±3.30%), *Butyricicoccaceae* (3.9%± 4.36%), *Lactobacillaceae* (3.24%± 5.55%), *Ruminococcaccaceae* (2.18% ± 5.29%).

Finally, at genus level, 82 taxa were identified in the fecal microbiota of newborn water buffalo calves. Most of these had low frequency (mean relative frequencies < 1%), while seven genera exhibited a mean relative frequency >2%: *Escherichia-Shigella* (38.27%±23.82%), *Bacteroides* (21.94± 19%), *Butyricicoccus* (3.9%±4.36%), *Lactobacillus* (3.24% ±5.55%), *Ruminococcus*_gnavus_group (2.43% ± 2.87%), *Clostridium*(2.40% ± 2.47%), an unclassified genus of the family *Lachnospiraceae* (2.30% ± 2.58%).

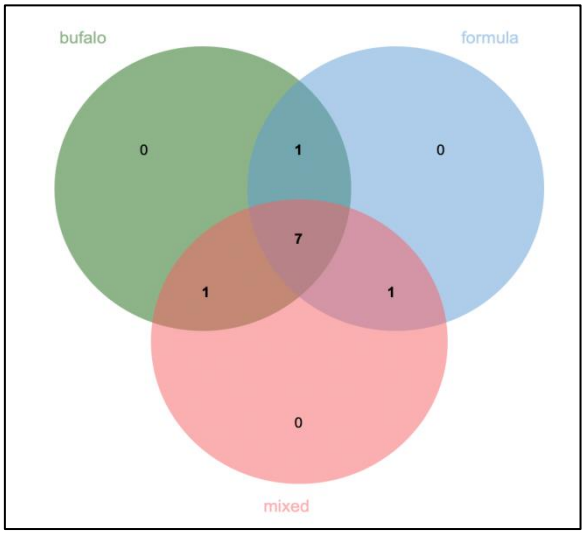
After taxonomic identification, the core microbiota, which defines the taxonomic profile of the gut microbiota, was determined by including all the taxa present in at least the 80% of samples in each group.

Venn analysis showed core microbiota taxa distributions referred to three taxonomic levels: phylum, family and genus (Figure 7).

A



B



C

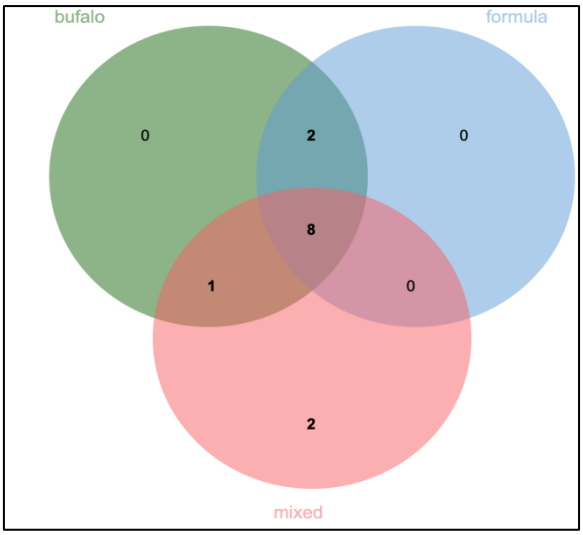


Figure 7.[a,b,c] VENN analyses among the three calves groups at T0 time at phylum (A), family (B) and genus (C) level.

Consistently with the uniformity revealed by alpha and beta diversity analysis determination of core microbiota indicated that all the groups shared mostly the same taxa. In particular, in the three groups a total of four phyla was shared by all calves: *Proteobacteria*, *Firmicutes*, *Bacteroidota* and *Actinobacteriota.*

At family level we found a total of seven taxa that constituted the core microbiota including: *Bacteroidaceae, Erysipelatoclostridiaceae, Clostridiaceae, Lachnospiraceae, Butyricicoccaceae, Oscillospiraceae, Enterobacteriaceae.* Finally, at genus level there were eight taxa shared by all: *Bacteroides*, *Erysipelatoclostridium*, *Clostridium_sensu_stricto_1*, *Clostridium_sensu_stricto_2*, *Ruminococcus gnavus_group*, *Butyricicoccus*, *Escherichia-Shigella* and an unclassified genus of the family *Lachnospiraceae*.

Comparison of fecal microbiota in paired sample: α diversity longitudinal analyses

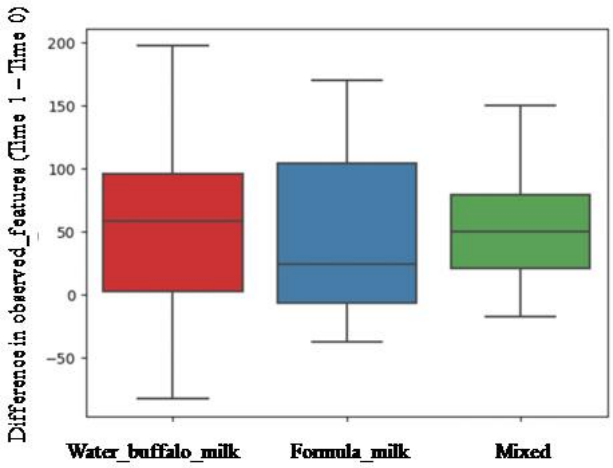
The progressive changes in stability and diversity of microbiota over the course of the two sampling times within subjects were evaluated using statistical longitudinal analyses on paired samples.

The alpha diversity analysis was performed using the q2-longitudinal plugin which is specifically optimized for the analysis of paired samples.

Observed features and Shannon entropy, indeed, were used to evaluate differences between longitudinal samples within each groups. The first one was used as a measure of abundance of features in the fecal microbiota, while the second one as a quantitative measure of diversity of the microbial community. (figure 8). Statistical

significance was evaluated by Wilcoxon signed-rank test (W), using $p \leq 0.05$ as a measure of significance.

A. Observed features



B. *Shannon_entropy*

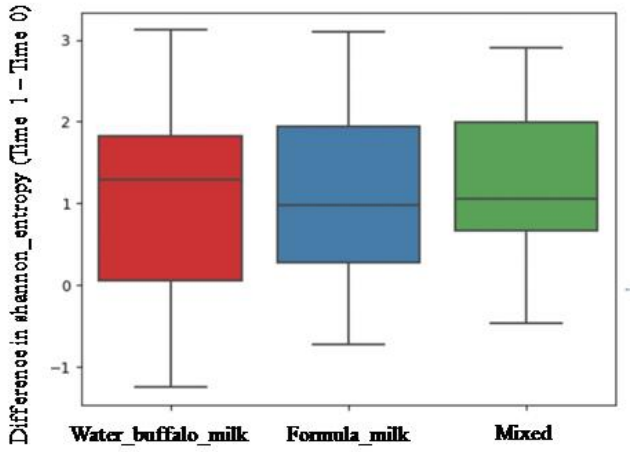


Figure 8. Boxplot for paired samples displaying Observed features (A) and Shannon entropy (B) between two time points in each group.

Results showed changes in the bacterial community structure over time. Observed features index was highly significantly affected by time of sampling in all the three experimental group: water buffalo milk group (p-value = <0.005) formula milk (p-value = 0.02) mixed milk (p-value = <0.005).Therefore the data indicated a

dissimilarity in terms of species abundance in the fecal microbiota of the animals fed with different diets.

In particular the results indicated that composition of gut microbiota in calves fed with mixed diet was dynamically less stable.

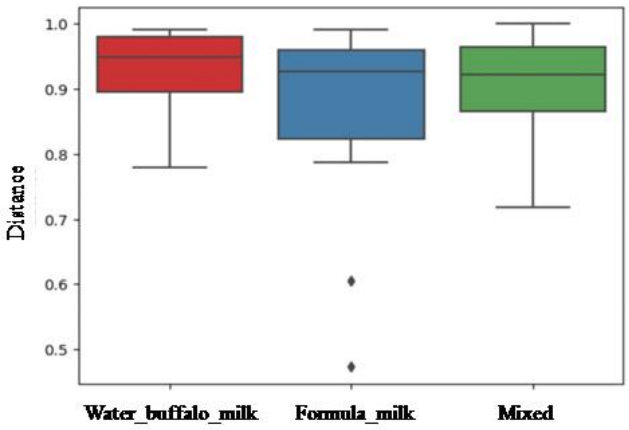
Significant difference in alpha diversity were observed also for Shannon diversity index, where great variability occurred between longitudinal samples.

The test showed an important impact of time in the differential develop of gut microbiota for all the treated groups. Indeed differences in Shannon entropy resulted significant within all the tested groups: water buffalo milk p-value < 0.005, formula milk p-value < 0.005 and mixed milk p-value < 0.005.

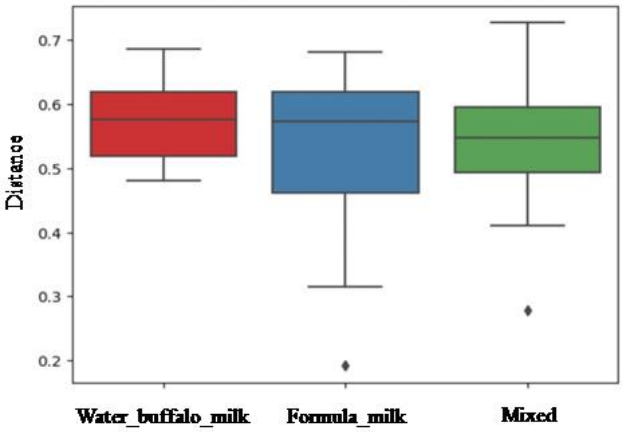
Comparison of fecal microbiota in paired sample: β-diversity longitudinal analyses

Beta diversity was also evaluated in order to capture changes between paired samples at different time, within each group. For this reason a longitudinal analysis was performed on differences related to Bray-Curtis Index, Unweighted and Weighted Unifrac distance matrix. The Kruskal Wallis test for Multiple groups were used. The results are shown below (figure 9).

A. Bray Curtis



B. Unweighted Unifrac



C. *Weighted Unifrac*

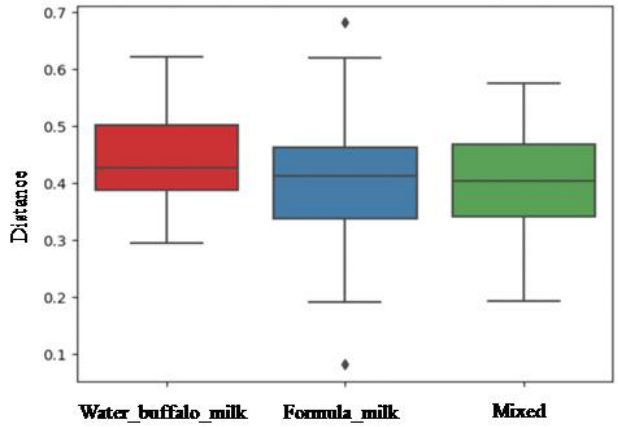


Figure 9. Beta diversity analysis for paired samples in each group. (A) show Bray Curtis distance matrix, (B) and (C) Unweighted and Weighted UniFrac distances respectively.

In particular, Bray-Curtis distance matrix (figure 9 A) did not reveal any significant influence of diet on community structure (p-value = 0.263) over time, demonstrating a decrease in dissimilarity in calves fed with different dietary regimes.

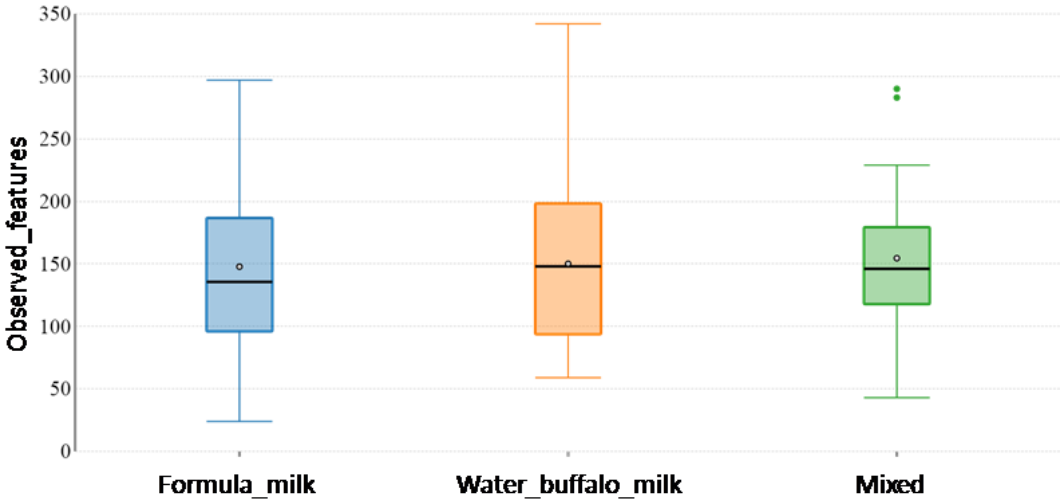
The same results could be observed for both the Weighted UniFrac distance matrix (p-value = 0.352), which takes into account the relative abundances of taxa, and the Unweighted UniFrac distance (p-value = 0.635) matrix which equally weights rare and abundant taxa (figure 9 B and 9 C).

Fecal microbiota composition in water buffalo calves: Alpha and beta diversity at T1

Kruskal Wallis statistical analysis of the fecal samples collected a T1 time was performed to evaluate the impact of different diets on the fecal microbiota composition and the presence of possible dissimilarities.

As shown below (Figure 10) the alpha diversity analysis based on the observed features index indicated that, the differences observed in species richness between the groups were not significant (Kruskal-Wallis test; Observed features index; H= 0.11; P-value=0.94). The same results were obtained for the Shannon entropy (Kruskal-Wallis test; Observed features index; H= 0.532; P-value=0.766).

A. *Observed features*



B. *Shannon entropy*

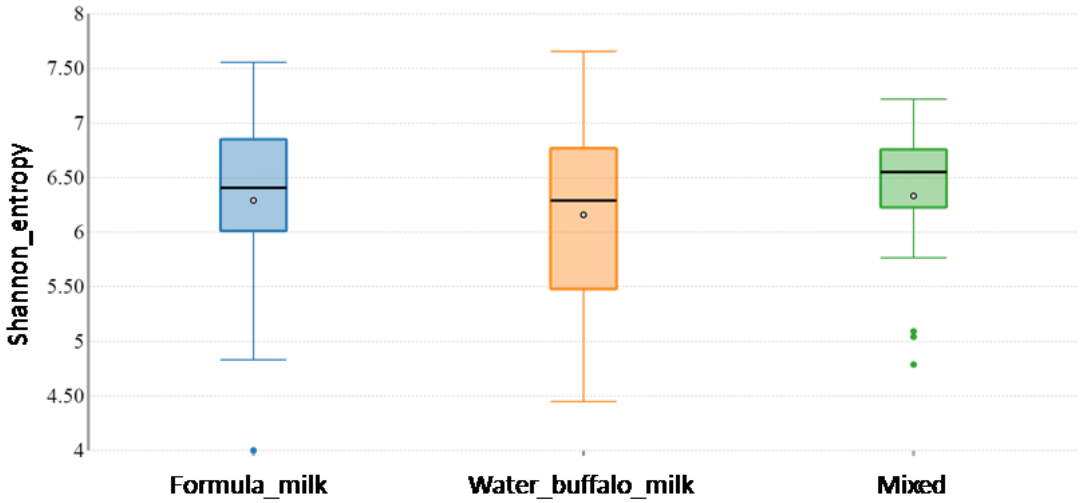
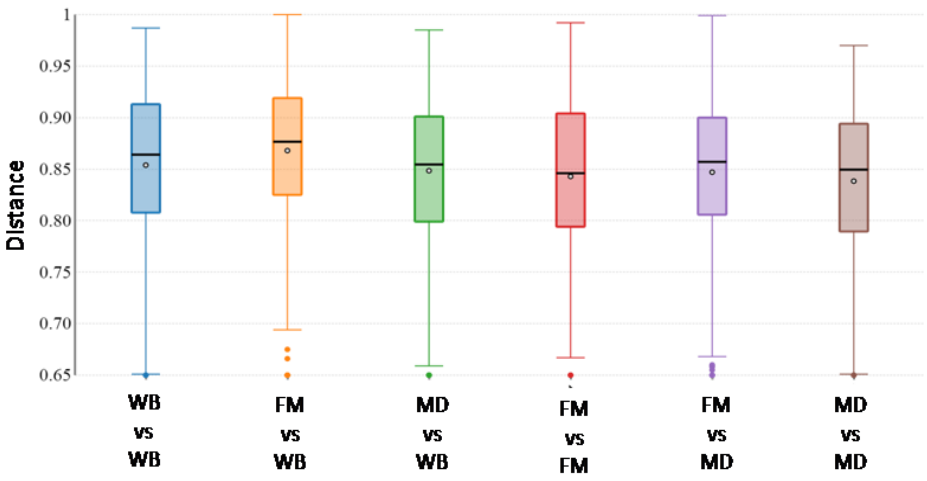


Figure 10. Box plots showing alpha diversity at T1 time in formula milk (blu), water buffalo milk (orange), and mixed milk (green) on the basis of the number of observed features and Shannon entropy.

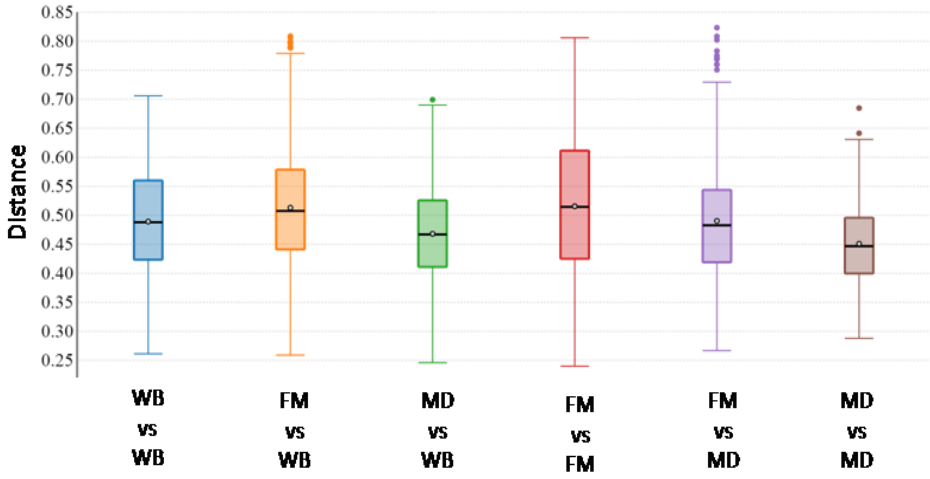
Beta diversity analysis was used to measure the differences in microbial community composition among groups.

In the present study the beta diversity statistics performed on the samples collected at the end of the trial and based on the Bray Curtis, Unweighted and Weighted distance matrix, displayed the results shown below (figure 11).

A. *Bray Curtis*



B. *Unweighted Unifrac*



C. *Weighted Unifrac*

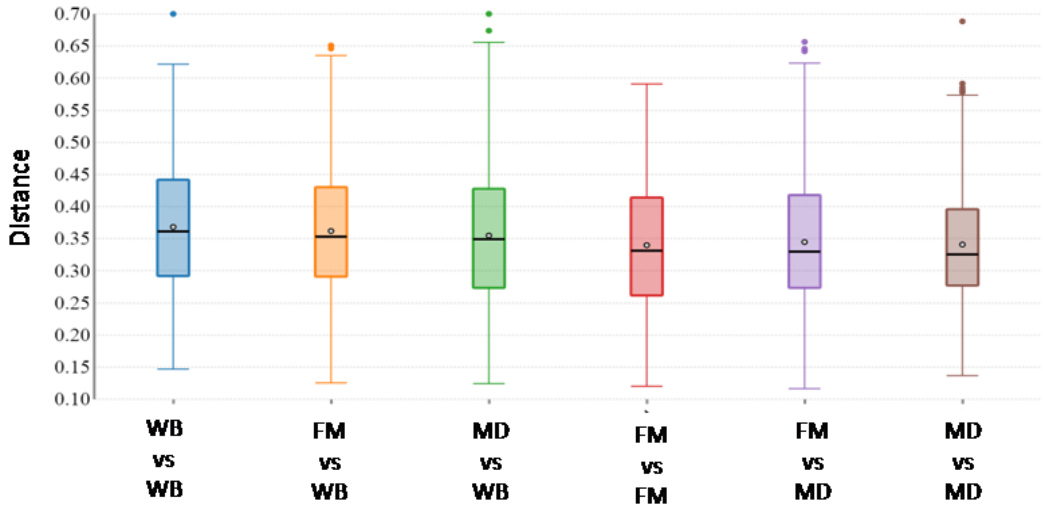


Figure 11. Box plots showing beta diversity distance amongst different groups in T1 time. (A) Bray Curtis dissimilarity; (B) Unweighted Unifrac distance and (C) Weighted Unifrac distance. WB represents water buffalo milk fed group; FM represents formula milk group and MD represents mixed milk group.

Differences in the Bray Curtis index based on the species abundance among the three groups (PERMANOVA; pseudo-F= 1.424; P = 0.01) were statistically significant, suggesting differences in the microbiota composition among groups.

In particular, the greatest difference was found in the species abundance between the group supplied with water buffalo milk and the one fed with formula milk (p-value =

0.002) suggesting the impact of different diets on the species presence in fecal microbiota.

Unweighted Unifrac distance matrix (PERMANOVA; pseudo-F= 1.413; P = 0.1) showed no significant differences between the formula milk and mixed milk groups, while a significant difference was observed between the group supplied with water buffalo milk and the one supplied with formula milk (p-value = 0.03) consistently with what was described above.

Finally, Weighted Unifrac distance matrix showed no significant statistical values(PERMANOVA; pseudo-F= 1.439; P = 0.139) highlighting no difference in the overall gut microbiota profiles among the three calf groups.

Fecal microbiota composition in water buffalo calves at T1: taxonomic composition

The taxonomic composition of 60 fecal samples collected at the end of the experimental period (T1) was based on the relative abundance of amplicon sequence variants (ASVs) and evaluated at the phylum, family and genus level.

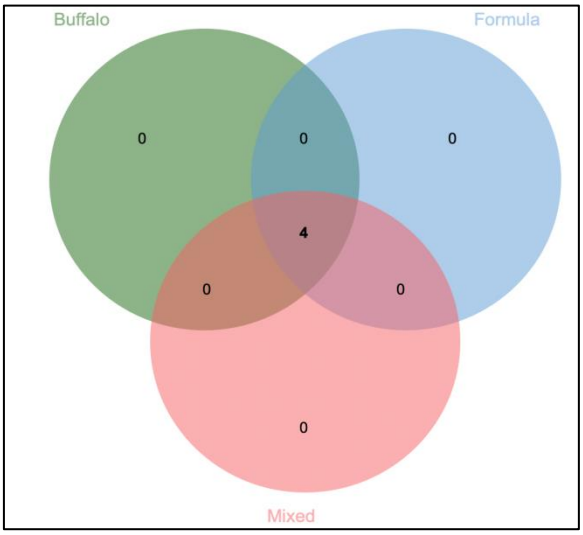
Phylogenetic analysis exhibited the presence of eight phyla: the *Euryarchaeota* belonging to the *Archaea,* was the most abundant in the samples (mean frequency value of 57.15% ± standard deviation of 16.21%), followed by *Actinobacteriota* (19.14% ± 15.10%), *Bacteroidota* (9.85% ± 12.30%), *Cyanobacteria* (7.85% ± 9.9%), Desulfobacterota (3.70% ± 10.72%), *Firmicutes* (1,65% ± 3.1%), *Proteobacteria* (0.34% ± 0.82%), *Verrucomicrobiota* (0.29% ± 0.51%).

At the family level 44 taxa were identified and the most abundant (mean relative frequency> 2%) were *Methanobacteriaceae* (23.58% ± 24.10%), *Actinomycetaceae* (17.22% ± 16.51%), *Bifidobacteriaceae* (8.09%±6.19%), *Atopobiaceae* (7.04% ± 9.92%), *Coriobacteriaceae* (5.83% ± 8.89%), *Eggerthellaceae* (3.29% ± 5.25%), *Bacteroidaceae* (2.84% ± 3.15%), *Barnesiellaceae* (2.78 ± 5.91), *Marinifilaceae* (2.67 ± 3.38), *Muribaculaceae* (2.6 ± 8.27), *Prevotellaceae* (2.5 ± 3.61).

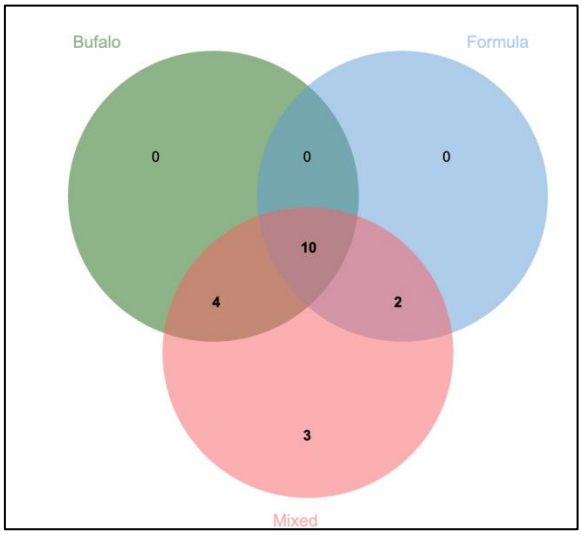
Finally at genus level 99 taxa were identified in the fecal microbiota of newborn water buffalo calves. Most of these had low frequency (mean relative frequency < 1%), while ten exhibited a mean relative frequency > 2%: *Methanobrevibacter* (23.17% ± 23.70%), *Actinomyces* (17.22 ± 16.51%), *Bifidobacterium* (5.83% ± 8.89%), *Olsenella* (3.62% ± 8.67%), *Collinsella* (3.29% ± 5.25%), *Eggerthella* (2.78% ± 5.91%), *Paraeggerthella* (2.6 ± 8.27), *Slackia* (2.5 ± 3.61), *Bacteroides* (2.42 ± 4.27), *Barnesiella* (2.24 ± 2.36).

In addition, the core microbiota composition was evaluated to underline possible differences related to specific diet intake and whether the observed homogeneity in gut microbiota composition at T0 time had changed. Obtained results are reported in the following figure (figure 12).

A



B



C

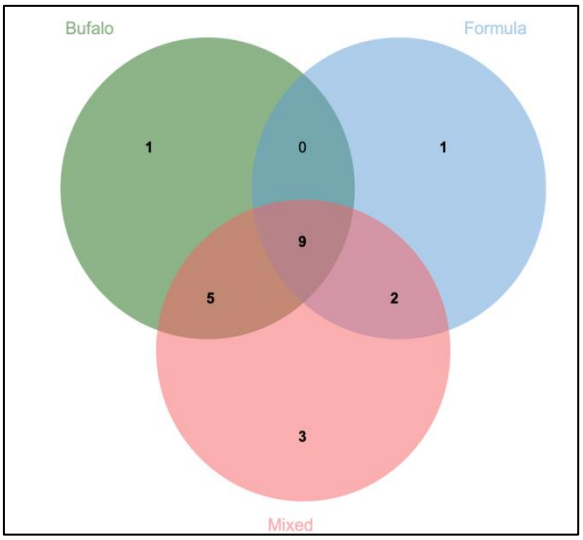


Figure 12.[a,b,c] VENN analyses among the three calves groups at T1 time at phylum (A), family (B) and genus (C) level.

Venn analyses showed a taxa distribution coherent with the development of gut microbiota during animal growth (figure 12).

At phylum level, the core microbiota was composed of four phyla, as for T0. At family level, the core microbiota of the three groups included ten families, while four

more families were shared by water buffalo milk and mixed milk groups, only. At genus level the core microbiota included nine genera, with five more genera shared by mixed milk and water buffalo groups.

At phylum level we observed a total of four taxa: *Firmicutes, Bacteroidota, Actinobacteriota,* and *Proteobacteria* which were present in at least 80% of samples from each group, as observed for samples collected at T0.

At family level we found a total of ten bacteria that constituted the core microbiota and were: *Bifidobacteriaceae, Coriobacteriaceae, Bacteroidaceae, Tannerellaceae, Lactobacillaceae, Lachnospiraceae, Oscillospiraceae, Ruminococcaceae, Acidaminococcaceae* and *Enterobacteriaceae.*

Three genera were present only in the group fed with mixed diet and were *Eggerthellaceae, Clostridiaceae* and *Butyricicoccaceae;* three families were shared between water buffalo and mixed diet groups and were *Erysipelatoclostridiaceae*, *Eubacterium* coprostanoligenes_group and *Peptostreptococcaceae* and two bacterial family *Muribaculaceae* and *Clostridia*_UCG-014 were shared between formula milk and mixed diet groups.

These results indicate that the two groups mixed milk and water buffalo milk are more similar to each other and are consistent with significant differences observed in beta diversity analyses between formula and water buffalo milk, as reported above.

T1: Taxonomic composition

The changes of fecal microbial communities from birth until the end of the experimental period were evaluated based on the variations occurring in the taxonomy composition. Here are reported the comparisons among the three groups in relation to the T0 (figure 13).

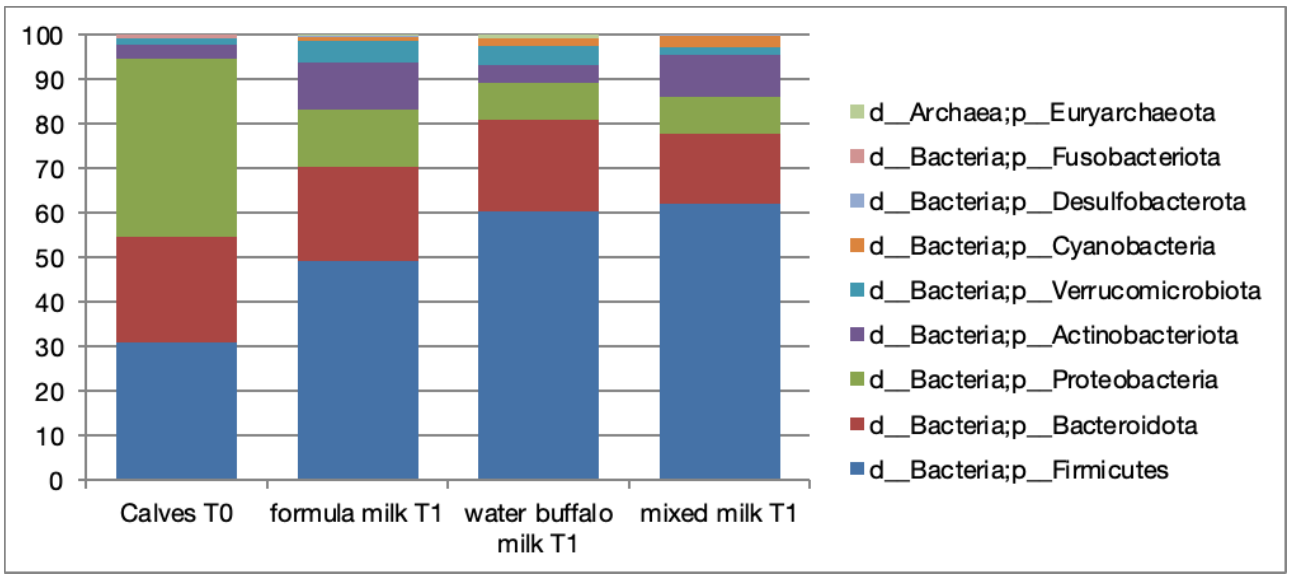


Figure 13. Comparison of relative frequencies in phyla between the two times.

As described above, all calves before the inclusion in the experimental group had a homogeneous gut microbiota, for this reason we unified all the samples from the three groups at T0 and compared them with samples from the three groups (formula milk, water buffalo milk and mixed diet, respectively) at the end of the trial (bar 2 , bar 3 and bar 4).

The table 7 describes the mean relative frequencies of the phyla present in the microbiota of calves at T1 for each experimental group.

	mean relative frequency %			
Phyla	All To	Formula milk T1	Water buffalo milk T1	Mixed diet T1
d__Bacteria;p__Proteobacteria	40,0	12,8	8,4	8,3
d__Bacteria;p__Firmicutes	30,8	49,2	60,2	62,0
d__Bacteria;p__Bacteroidota	23,8	21,1	20,5	15,8
d__Bacteria;p__Actinobacteriota	3,1	10,6	3,8	9,2
d__Bacteria;p__Verrucomicrobiota	1,5	5,0	4,3	1,8
d__Bacteria;p__Cyanobacteria	0,1	0,7	1,7	2,6
d__Bacteria;p__Desulfobacterota	0,0	0,4	0,3	0,2
d__Bacteria;p__Fusobacteriota	0,6	0,0	0,0	0,0
d__Archaea;p__Euryarchaeota	0,0	0,2	0,7	0,1

Table 7. Relative abundance of phyla in all sample a T0 time and in each group at T1 time.

Comparison of phyla abundance in the group showed difference in the microbiota composition, indeed, feces from the young calves in the present study were dominated by *Proteobacteria (40%), Firmicutes (30.8%)* and *Bacteroidota (23.8%)* representing almost all the taxa present. These phyla are indeed those included in the core microbiota at phylum level showed above.

In the formula milk group a T1 time, the percentage of *Proteobacteria* (12.8%) decrease, while percentage of *Firmicutes* were increase (49.2%), increase also the percentage of *Actinobacteriota* (10.6%) and *Verrucomicrobiota* (5%).

In calves fed with water buffalo milk the abundances of *Firmicutes* (60.2%), *Bacteroidota* (20.5%), *Verrucomicrobiota* (4.3%), *Cyanobacteria* (1.7%) increased, whereas that of *Proteobacteria* had dropped at 8.4%.

The relative frequency of *Actinobacteriota* (3.8%) was almost unchanged among the three groups.

Finally, when analyzing the gut microbiota of calves fed with mixed diet we found that the phylum *Firmicutes* (62%) was the most prevalent followed by *Bacteroidota* (

15.8%), *Actinobacteriota* (9.2%) and *Proteobacteria* (8.3%). We detected a lower abundance of *Verrucomicrobiota* (1.8%), and *Cyanobacteria* (2.6%) comparated with those of the other two groups.

The same analysis was performed on the fecal microbiota at the family level (figure 14), where we observed which families were expressed in relation to the type of administered diet.

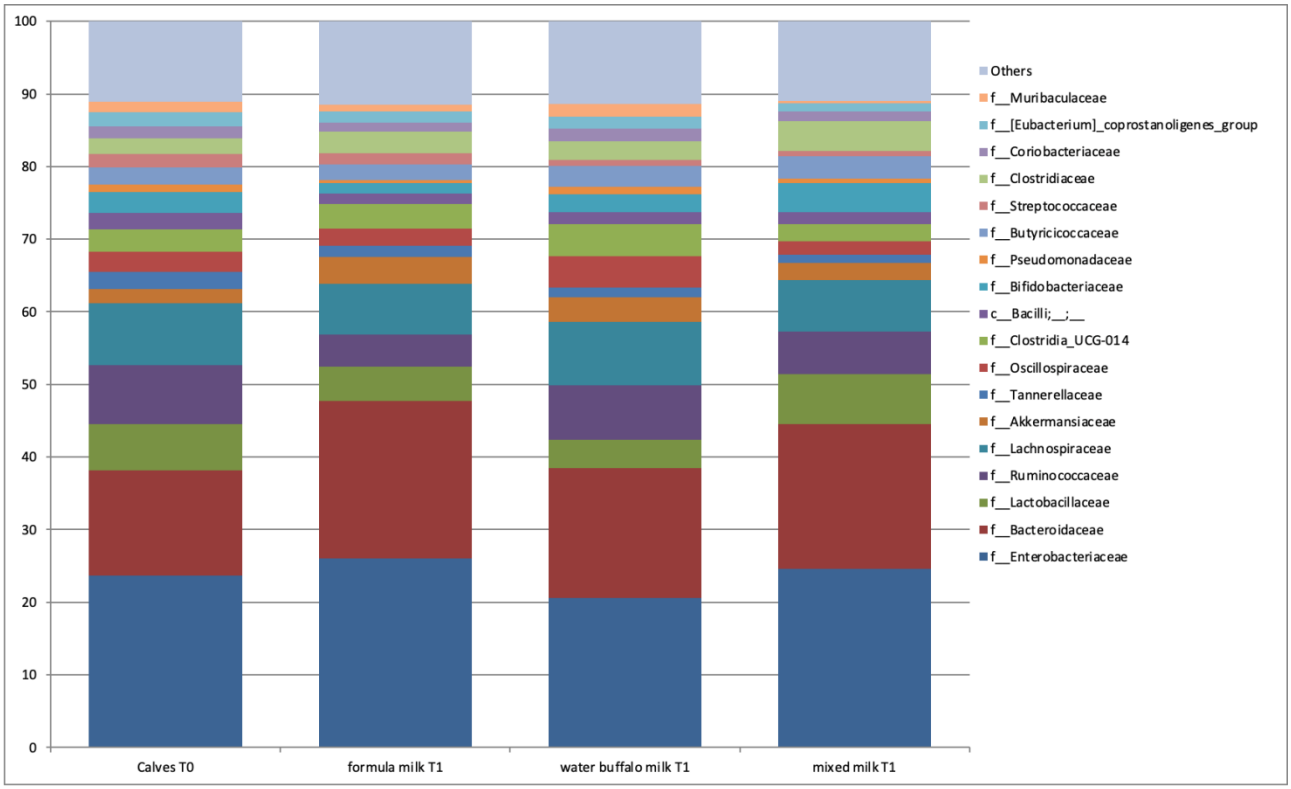


Figure 14. Comparison of relative frequencies at family level between samples at T0 and T1 for each diet based group.

The composition of gut microbiota at family level in calves showed the predominance of five taxa (table 8) including *Enterobacteriaceae, Bacteroidaceae, Lactobacillaceae, Ruminococcaceae, Lachnospiraceae*. If compared with the formula milk group, water buffalo and mixed milk groups at T1 showed that *Enterobacteriaceae, Bacteroidaceae, Lactobacillaceae* remained the most abundant

families as shown below. The family *Ruminococcaceae* was instead detected at lower abundances (4.3%) in claves fed with formula milk, while *Akkermansiaceae* resulted increased in formula milk (3,7%) and in water buffalo milk (3.5%) groups. In calves fed with water buffalo milk, a number of families also resulted increased, including *Oscillospiraceae* (4,4%), *Clostridia_UCG-014* (4.4%). In formula milk group *Bifidobacteriaceae* (1,4%) decreased and finally in the mixed diet group *Tannerellaceae (1,1%), Oscillospiraceae (1,8%), Streptococcaceae (0,8%), Eubacterium_coprostanoligenes group (1,8%)*and *Muribaculaceae (0.3%)*were present at lower abundance, while *Bifidobacteriaceae (4%)* and *Clostridiaceae (4,1%)* were increased.

	mean relative frequency %			
Family	**All To**	**Formula milk T1**	**Water buffalo milk T1**	**Mixed diet T1**
f__Enterobacteriaceae	23,7	26,1	20,6	24,6
f__Bacteroidaceae	14,4	21,6	17,8	19,9
f__Lactobacillaceae	6,4	4,8	3,9	6,9
f__Ruminococcaceae	8,1	4,3	7,5	5,9
f__Lachnospiraceae	8,5	7,0	8,7	7,0
f__Akkermansiaceae	2,0	3,7	3,5	2,4
f__Tannerellaceae	2,3	1,6	1,3	1,1
f__Oscillospiraceae	2,8	2,4	4,4	1,8
f__Clostridia_UCG-014	3,1	3,4	4,4	2,4

Table 8. Relative abundance of family in all sample a T0 time and in each group at T1 time.

Finally, the taxonomic composition at the genus level is shown in figure figure 15.

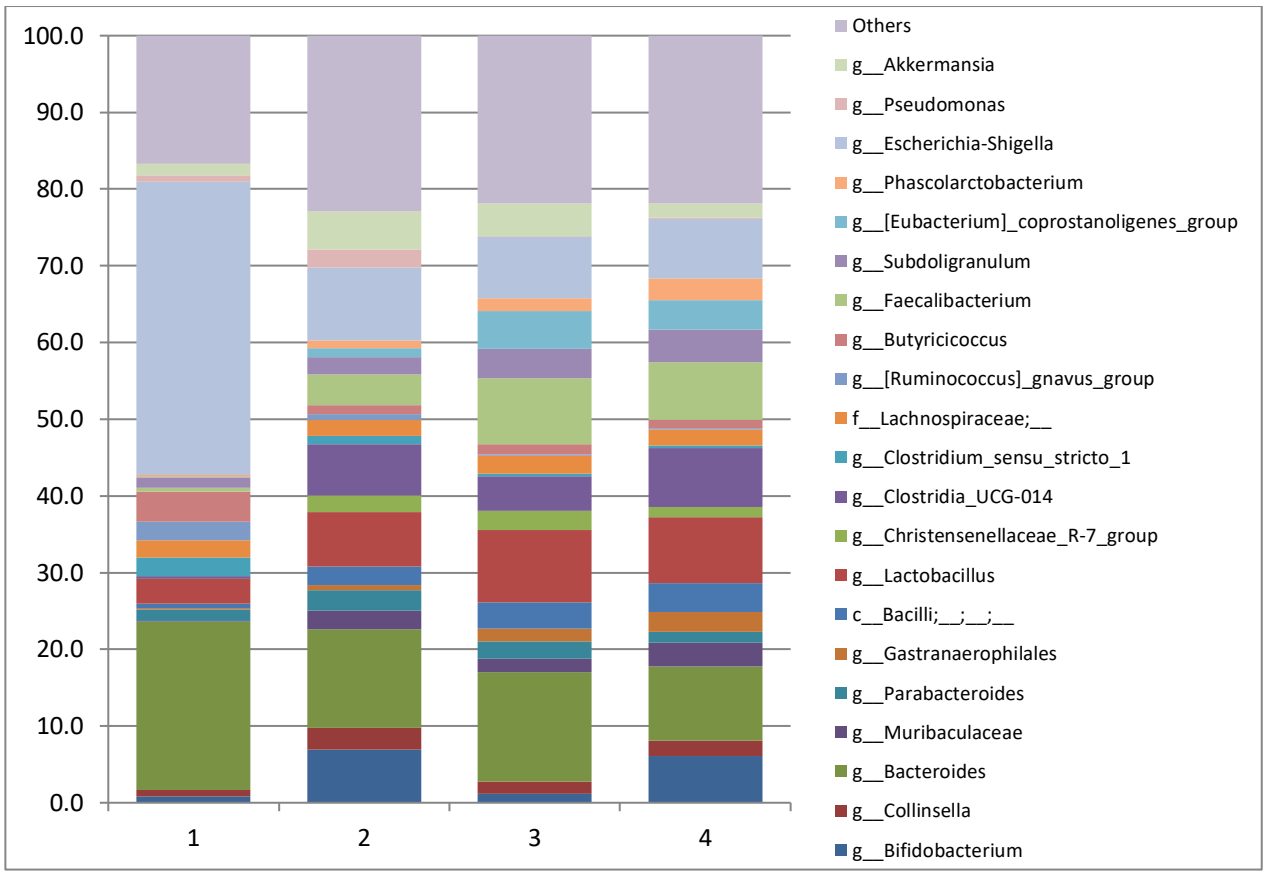


Figure 15. Comparison of relative frequencies at genus level between T0 and T1 for each diet based group.

As discussed above the genera most present in the gut microbiota of newborn calves were *Escherichia-Shigella* and *Bacteroides* followed by *Butyricicoccus, Lactobacillus, Clostridium and Ruminococcus* (table 9).

Data compared with those collected at the end of the trial highlighted a remarkable decrease in both *Escherichia-Shigella,* from an mean value of 38.3% at T0 to 9.5% in formula milk, 8% in water buffalo milk at T1 and 7.8% in mixed diets at T1, and in *Bacteroides*, with a mean of frequency value from 12.9%, 14.2% and 9.7% in

formula milk, water buffalo milk and mixed milk groups at T1, respectively. The comparison of relative frequencies in the three groups showed increased values of *Lactobacillus and Akkermansia* in formula and in water buffalo milk groups and *Bifidobacterium* particularly in formula and mixed diet, where values exhibited a sixfold increase.

	mean relative frequency %			
Genus	**All To**	**Formula milk T1**	**Water buffalo milk T1**	**Mixed diet T1**
g__Escherichia-Shigella	38,3	9,5	8	7,8
g__Bacteroides	21,9	12,9	14,2	9,7
g__Butyricicoccus	3,9	1,2	1,3	1
g__Lactobacillus	3,2	7	9,5	8,6
g__Clostridium_sensu_stricto_1	2,4	1,1	0,3	0,4
g__[Ruminococcus]_gnavus_group	2,4	0,7	0,2	0,2
f__Lachnospiraceae;__	2,3	2,1	2,3	2
g__Parabacteroides	1,5	2,6	2,2	1,4
g__Akkermansia	1,5	5	4,3	1,8
g__Subdoligranulum	1,3	2,2	3,9	4,3
g__Bifidobacterium	0,8	6,9	1,1	6,1
g__Collinsella	0,8	2,9	1,6	2,1
g__Pseudomonas	0,8	2,4	0,1	0,1
c__Bacilli;__;__;__	0,6	2,5	3,4	3,8
g__Faecalibacterium	0,5	4	8,6	7,5
g__Clostridia_UCG-014	0,3	6,6	4,5	7,6
g__Phascolarctobacterium	0,2	1	1,7	2,9
g__Muribaculaceae	0,1	2,4	1,8	3
g__Gastranaerophilales	0,1	0,7	1,7	2,6
g_[Eubacterium]_coprostanoligenes_group	0,1	1,1	4,9	3,8
g__Christensenellaceae_R-7_group	0	2,2	2,5	1,4
Others	16,7	22,9	21,9	21,9

Table 9. Relative abundance of genus in all sample a T0 time and in each group at T1 time.

The identification of taxa differentially expressed between groups were carried out using R software using a generalized linear model with negative binomial distribution. In particular, due to the presence of low abundant microbes in some sample the Zero-inflated Negative Binomial modeling (ZINB) was applied (Zhang et al., 2016).

The analysis highlighted the presence of different bacterial genera among groups. Indeed six genera were found as differentially expressed among group (table 10), represented by *Faecalibacterium* (p-value 0.048), *Clostridia_UCG-014* (p-value 0.035), *Bifidobacterium* (p-value 0.017), *Parabacteroides* (p-value 0.035), *Eubacterium_coprostanoligenes_group* (p-value 0.000) and *Collinsella* (p-value 0.033). The results are shown in the table:

Genus	mean	ds	ChiSq	ProbChiSq	Highest abundance Group
g__Faecalibacterium	0.036	0,086	6,094	0,048	WB
g__Clostridia_UCG-014	0.033	0,052	6,732	0,035	FM
g__Bifidobacterium	0.028	0,059	8,142	0,017	FM
g__Parabacteroides	0.018	0,034	6,717	0,035	FM
g__Eubacterium_coprostanoligenes_group	0.017	0,035	33,224	0,000	WB
g__Collinsella	0.015	0,024	6,837	0,033	FM

Table 10. Table shows genera differentially expressed in the three groups fed with different diets.

DISCUSSION

The present study describes the influence of different diets on body weight gain and gut microbiota development in water buffalo calves. For this purpose, three groups of calves were included, fed with natural water buffalo milk, formula milk and mixed milk (one feed with natural milk and one feed with formula milk daily), respectively, for a period of 3 weeks after 3 days of colostrum administration. Our results showed that calves fed with formula milk displayed the highest body weight gain. Moreover, the three different diets did not induce significant differences in the species richness in gut microbiota, even if significant differences were found in the structure of gut microbiota, related to differential abundance of specific genera among groups.

Immediately after birth calves are fed with maternal colostrum because it is high in nutrients and antibodies. Time is important because a newborn calf's digestive tract allows antibodies to pass directly into the blood. After 24-36 hours, the calf's intestine cannot absorb antibodies, therefore, farmed calves are exclusively fed with formula milk. Indeed, water buffalo calves and dairy calves are two of the few animal species subjected to restricted maternal milk intake in early life. The pre-weaning period of water buffalo calves is essential for an adequate adaptation to solid diet. It is therefore of utmost importance the selection of an appropriate substitute of natural milk for proper calves' development and growth.

In our study, over the experimental period we observed the highest body weight gain in calves fed with mixed diet, where the increase in body weight was 21.84 kg compared with 20.8 kg and 18.61 kg of water buffalo and formula milk fed groups, respectively. This finding is consistent with the previous data published by Bhatti et al. (2012), where dairy calves fed with milk replacer during the pre-weaning period had lower weights at weaning and thus ate less than heavier calves at weaning. Khan et al. (2007) reported that calves weaned at a not appropriate weight can show a depression in solid feed intake post weaning. Depressed feed intake can be avoided if the calves are weaned gradually .

The data presented in this study indicate that all animals had an adequate daily weight increase, suggesting that all the diets were consistent with animals' nutritional requirements. However, the highest increase in body weight observed in calves fed with mixed diet compared with formula ones (p-value < 0.005) suggest a synergistic action between natural and formula milk.

There are several possible reasons for this result.

Increased growth and feed efficiency of calves fed with formula milk may be due to coagulation of milk proteins in the abomasum in the presence of gastric acids, determining a delay of digestion, thus allowing for absorption of amino acids (Bartlett et al., 2006). In addition, milk fat fraction contains a greater proportion of medium-chain fatty acids (MCFA) as compared with long-chain fatty acids (LCFA) typically used in milk replacers. MCFA are hydrolyzed

more rapidly and completely than LCFA found in most milk replacers. They are also oxidized more rapidly than LCFA probably leading to differences in energetic efficiency of the utilization of dietary fat (Bascom et al., 2007). Therefore, the greater increase in weight reported in mixed diet was likely due to the higher proportion of MCFA in this group.

The second aspect examined during the present study was the gut microbiota characterization. This study showed that the composition of the microbiota dramatically changes after birth during the first weeks of life, and this result was observed in all the groups under study. Moreover, our analysis highlighted differences in microbial composition related to the presence of differentially expressed taxa among the three groups at the end of the experimental period.

A source of bacteria and nutrients that can help to shape the initial composition of the gut microbiota is colostrum. Potentially, colostrum has a beneficial effect on the calf gastrointestinal microbiota for the whole life of the animal, as suggested in the study of Yeoman et al. (2018) or may influence the intestinal epithelial microbiota, which has not been examined in the present study.

Another recent study showed an association between the colostrum microbiota and fecal microbiota. Indeed, in the former, *Streptococcaceae*, *Enterococcaceae*, and *Enterobacteriaceae* constituted up to 90% of the relative abundance, whereas these groups were present in about 30% relative abundance in fecal samples at 7-days-old dairy calves (Liu et al., 2019).

The initial colonization of the gut microbiota was explored in order to identify factors contributing to its composition and links between microbiota and host health. The development of the gastrointestinal tract (GIT) in neonatal humans and animals is a highly dynamic process that is influenced by genetic and environmental factors, nutrition, and the concomitant development of the intestinal microbial communities (Amin et al., 2021).

Our results displayed a lower Firmictes/Bacteroidetes (F/B) ratio in animals fed with formula milk, while those fed with mixed milk diet displayed the highest F/B ratio.. It has been proposed that the Firmicutes were more effective in extracting energy from food than Bacteroidetes, thus promoting a more efficient absorption of calories and the subsequent weight gain (Krajmalnik-Brown et al., 2012). This evidence might support the observed highest increase in body weight gain reported for animals included in the mixed milk diet group. F/B ratio is considered as an important parameter to define the health status both in humans and animals (Tseng et al., 2019) and has been shown to be affected by the presence of different nutrients, such as antioxidants (Sinisgalli et al., 2020) fat content and carbohydrates in the diet (Hills et al., 2019).

In particular, in the present study ,the impact of different diets was assessed and six genera were identified as differentially expressed in feces samples: *Faecalibacterium, Clostridia_UCG-014, Bifidobacterium, Collinsella, Parabacteroides, Eubacterium_coprostanoligenes_group.*

The genus *Faecalibacterium* belongs to the phylum *Firmicutes* and is an obligate anaerobic, Gram-positive, rod-shaped, butyrate producing microorganism that is abundant in the feces of several animal species (Lopez-Siles et al., 2019 and Oikonomou et al., 2013). This genus has been reported as a promoter of beneficial effects on energy metabolism and on the prevention of colonization of pathogens in the intestine. The ability to produce butyric acid and its gut colonization display anti-inflammatory effects, therefore this genus has been proposed as a potential probiotic for treatment of gut inflammation (Zou et al., 2020). For this reason, this bacterial species represents a taxon normally present in the gut microbiota of healthy animals. Recent study, in addition, found that a high prevalence of *Faecalibacterium* spp. in early life is associated with a lower incidence of diarrhea in calves (Hang et al., 2020) and an increase in weight gain in pre-weaned dairy Heifers (Foditsch et al., 2015). Moreover, in some studies conducted on the infant gut microbiota, low levels of *Faecalibacterium* have been found in patients with asthma (Fujimura et al., 2016), and Crohn disease (Sokol et al., 2008).In the present study we found the presence of this taxon in all the analyzed samples, with an higher abundance in samples collected a T1 time (p-value = 0,048). In particular the most abundant frequency of *Faecalibacterium* was found in calves fed with water buffalo milk suggesting, therefore, that the composition of natural milk likely creates a more favorable environment to its colonization.

Clostridia_UCG-014 is an anaerobic bacterium commonly present in the intestinal mucosa. In our study, this genus resulted significantly more abundant in the animals fed with mixed milk diet. The relative abundance of this genus as indicated by Song et al. (2018) is lower in newborn calves and increases over the first few weeks of life. A recent study has reported that bacteria belonging to the *Clostridia* family can modulate the expression of mucins-related genes and increase mucin production in the intestine (Graziani et al., 2016), suggesting that these species may play a role in increasing host resistance to pathogenic bacterial invasion through reinforced barrier functions at 21 days.

The microbes in the large intestine are mostly anaerobic bacteria and can use diet components to produce short chain fatty acids (SCFAs), thus providing energy. It was reported that about 90% of SCFAs, in the form of acetate, propionate and butyrate, could be absorbed through intestine and may play different roles. For instance, butyrate provides energy to intestinal epithelial cells whereas acetate and propionate enter the fatty acid synthesis and gluconeogenesis pathways, respectively. *Clostridium* species are chemoorganotrophic bacteria and can ferment a variety of nutrients, like carbohydrates, proteins, organic acids and other organics, to produce acetic acid, propionic acid, butyric acid, and some solvents, such as acetone and butane. In animal and human intestine, *Clostridium* species utilize indigestible polysaccharide and, most of the metabolites they produce bring out many

benefits to host gut health (Guo et al., 2020). In our study we found that the diet associated with the highest body weight gain was the mixed milk one, even though it is interesting to note that the diet with formula milk only induced the highest abundance of *Clostridia_UCG-014.*

Bifidobacterium is a genus of gram-positive, non motile, anaerobic bacteria. They are ubiquitous inhabitants of the gastrointestinal tract of mammals, including humans, and they are regarded as being beneficial to host health (Duranti et al., 2019). In our study we have found a higher abundance of this taxon in animals fed with formula milk. *Bifidobacterium* is able to ferment milk oligosaccharides (OS) as primary substrate and this ability confers it a significant competitive advantage during the pre-weaning period when milk is the primary nutrients source (Badman et al., 2019). The presence of this specialized bacteria suggests that milk OS present in formula milk may influence the differences in gut microbiota reported in this study. In water buffalo milk the presence of free OS is very low and this can explain why the greater proliferation of *Bifidobacterium* occurs in calves fed with formula milk.

Our results also showed a significant difference in the relative abundance of *Parabacteroides* (p-value =0.035) and *Collinsella* (p-value =0.033).

These two taxa are often referred to as markers of gut microbiota in healthy calves (Alipour et al., 2018) because they are normal commensal. Both taxa in our study were found at a higher abundance in formula milk fed animals. The reason is likely linked to formula milk which reduces the microbial richness (as

suggested by the bray curtis analysis: water buffalo milk vs mixed p-value = 0.002) thus causing a greater proliferation of certain bacterial species thanks to the increased availability of nutrients.

Finally, *Eubacterium_coprostanoligenes_group* is a Gram-positive, anaerobic, non-fermenting bacteria, that we found more abundant in fecal samples collected from animals fed with natural water buffalo milk only.

Several members of this genus produce butyrate, which plays a critical role in energy homeostasis, colonic motility, immune-modulation and suppression of inflammation in the gut. The bacteria of the genus *Eubacterium* also carry out bile acid and cholesterol transformations in the gut, thereby contributing to their homeostasis.

Several studies focused their attention on variation of this microorganism as a function of the diet. Duncan et al. showed that the *Eubacterium_coprostanoligenes_group* decreases with increasing fat percentage in diet (Duncan et al., 2007); Ghosh et al. assert that the Mediterranean diet, which is well established as a diet that can contribute to health, has been shown to increase *Eubacterium* spp. populations in the gut (Ghosh et al., 2020). This consideration allows us to hypothesize that probably in our study the high percentage of *Eubacterium_coprostanoligenes_group* (4.9%) observed in animals fed with water buffalo milk might be related to the high fat content of this food. This deduction is supported by the lower mean

percentages observed in mixed diet group (3.8%) and in formula milk fed animals (1.1%).

This study provides further knowledge concerning the composition and development of gut microbiota in water buffalo calves.

We report that the development of gut microbiota in water buffalo calves, as already described for human gut microbiota in early life, begins in the first weeks of life and is strongly influenced by the type of feeding (natural milk or formula milk).

The study points out that there is a close interaction between the development of the gut microbiota and the metabolism of fats, which especially in the early life, establish the development of bacterial species.

Moreover we found that formula milk can negatively impact the richness and variability of the gut microbiota in buffalo calves , and this could lead to an inadequate development or late development of gut microbiota during the weaning phase.

Therefore, further studies will be necessary to set up strategies to balance this negative aspect linked to the use of formula milk in newborn calves, such as the administration of probiotics to promote the development of a more balanced bacterial community in intestine, and at the same time inhibit the growth of bacterial pathogens responsible for infections and inflammation in young calves.

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