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Effect of mycotoxin binder Bentonite on the active microbial communities of bovine milk and cheese

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ABSTRACT

Six Italian Simmental cows were randomly assigned to 1 of 3 feed additive treatment sequences with Bentonite mycotoxin binders (B) arranged in a balanced 3 × 3 Latin square design with three 19-d experimental periods (T1, T2, T3), to investigate modifications in their metabolically-active bacterial communities using the RNA-based amplicon sequencing method.

The bacterial community of milk was most dominated by four phyla: *Firmicutes, Poteobacteria, Bacteroidetes* and *Actinobacteria,* corresponding to the results reported from previous studies associated active milk flora using DNA-based methods.

The bacterial community of milk was most dominated by three phyla: *Firmicutes*, *Actinobacteria,* and *Proteobacteria,* corresponding to the results reported from previous studies associated active cheese flora using DNA-based methods.

In conclusion, the anti-mycotoxin Bentonite dietary additives resulted in neutral effect in metabolically-active microbial communities in milk and cheese.

Keywords: Mycotoxin; Bentonite; active microbial community; Cow milk; Cheese; 16S rRNA

1. INTRODUCTION

1.1. The value of Milk and Cheese products as nutritious and healthy foods

1.1.1. Overview of Milk products

Milk is a composite physiological fluid secreted by female mammals' mammary glands to assist neonatal adaptation by delivering bioactive components and nutrients at the same time. [7][41] Human milk feeding promotes the development of a digestive system and a healthy microbiome. Milk components contribute to the baby's immune homeostasis and defense against pathogenic bacteria. [6] Animal milk, along with breast milk, is one of the most important sources of nutrition for infants and babies. [41] The term "milk" regularly refers to cow's milk. When other animals' milk is sold commercially, it is spelled out, for instance, sheep milk or goat milk. [7] Milk and its myriad of products are not only the main constituents of the daily diet for people belonging to vulnerable groups like children and the elderly, but they also play an important role in nourishment and development throughout adult human life. Milk is, without a doubt, nature's most complete food with a rich supply of energy, protein, fats, lactose, vitamins, minerals, and essential amino acids. [41][42]

The comprehensive analysis demonstrated that the benefits of drinking milk outweigh the harms when it comes to health-related outcomes. Positive associations were found for cardiovascular disease, stroke, high blood pressure, colorectal cancer, metabolic syndrome, obesity, type 2 diabetes mellitus, osteoporosis, and Alzheimer's disease. (Figure 1.1) [99][118] High-quality dairy proteins play a vital contribution in weight loss and later weight maintenance, as well as the prevention of obesity-related metabolic disorders, by regulating satiety and avoiding excessive energy consumption. Proteins simultaneously improve body composition, i.e., reduce body fat mass and maintain lean body mass. [99] Furthermore, recent cohort studies have reported that fermented dairy products, particularly cheese and yoghurt, have a neutral or inverse correlation with the incidence of type 2 diabetes may be due to their favorable effect on the gut microbiota. [86][98] Other research has found that whey protein and its constituent amino acids can stimulate insulin secretion as a mechanism to improve postprandial glycemic control and lower type 2 diabetes risk. [86]

The overall evidence suggests that high consumption of dairy products, especially the low-fat, calcium-rich types, does not increase the risk of cardiovascular disease.

Specifically, with a milk intake of 200-300 ml/day, there is a risk reduction of hypertension and stroke. [99] Some constituents of milk such as casein and whey protein, unsaturated fatty acids, polar lipids have shown beneficial relationships with intermediary cardiometabolic endpoints. [86] Most of the bioactive peptides of milk proteins are hidden or partially visible in the native protein, but they can be activated when food is digested or fermented with lactic acid bacteria to liberate bioactive peptides. [83] Once bioactive peptides are released, they inhibit the angiotensin-converting enzyme (ACE), lowering blood pressure. [75] In addition, calcium and probiotic bacteria can help to prevent excessive cholesterol absorption into the bloodstream, which lowers the risk of cardiovascular disease. [41][108] The role of cow's milk and its products in cardiometabolic health is an area of active and ongoing research. [86]

Milk provides a variety of nutrients that are necessary for building children's bone strength and density and their maintenance in adulthood, with the goal of preventing bone loss and osteoporosis in old age. [42] Calcium, protein, phosphorus, magnesium, manganese, zinc, vitamin D, and vitamin K are all required for normal bone health, according to the European Commission. With the exception of vitamin D, all these nutrients are naturally present in milk and dairy products. [99] Since fluid milk does not naturally contain vitamin D, it can be fortified with vitamin D by law in some countries to aid your body in absorbing calcium. [22][51] Even though calcium and/or vitamin D supplements are beneficial for those who are unable to meet their calcium requirements through food or who limit their sun exposure, current evidence does not support routine calcium or vitamin D supplementation for healthy adults without a specific bone pathology because it is not helpful and potentially harmful. [91]

Dairy has been linked to a variety of cancers, both positively and negatively, but most of them are based on limited evidence and further studies are needed. The positive effects on carcinogenesis may be connected to calcium, lactoferrin, and fermentation products, while the negative effects may be related to insulin-like growth factor I (IGF-1). According to current meta-analyses, the association between milk intake and a reduced risk of colorectal cancer is primarily attributed to calcium from dairy products, with a 900 mg/day dairy-calcium intake resulting in a 24% risk reduction. On the other hand, high consumption of dairy products, low-fat milk, cheese, and calcium may raise 3–9% of the risk of prostate cancer; however, the evidence is inconsistent. For female consumers, dairy products offer significant health benefits by reducing the risk of common and severe

colorectal cancer and potentially breast cancer as well. From the male consumer side, the protective effects of dairy products in colorectal cancer are believed to outweigh the potential increased risk of prostate cancer. [42][99][118]

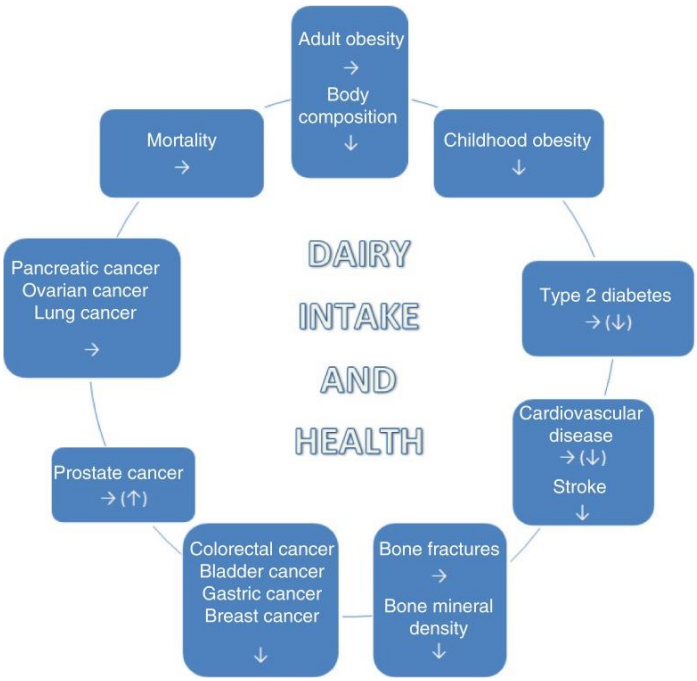

Figure 1.1. Overall association between dairy product consumption and health outcomes
↓ positive effect; ↑negative effect; → no effect

Additionally, a high-level intake of milk might slightly increase the risk of Parkinson's disease, acne, and iron deficiency anemia. Caution should be exercised in the presence of a potential allergy or intolerance to lactose. [118] A majority of the ingredients in milk and dairy products are easily absorbed by humans. However, lactose is intolerant by some individuals who are encouraged to choose fermented milk products with little or no lactose, are easy to digest, and include probiotic bacteria, a health-promoting agent. [42] Many factors can influence the milk yield and composition dynamics such as nutrition, genetics, environments, level of milk production, stage of lactation, disease (related to breast health), season, as well as age of the cow. [69]

1.1.2. Overview of Cheese products

Cheese has long been a staple of the human diet, especially in the Americas and European countries. It was originally produced as a method of preserving milk. [110] Basically, cheesemaking is the process in which liquid milk (an unstable, bulky but nutrient-dense raw material) is converted into cheese (a more stable, flavorful, and concentrated product), in order to extend its shelf life, improve food's palatability and

increase consumer satisfaction. [68] Cheese is also popular for its nutritional value and health benefits. Cheese contains some of the same essential nutrients as milk because it is made primarily of ruminant milk. These nutrients include proteins, bioactive peptides, amino acids, fat, fatty acids, vitamins, and minerals. [73][110]

One of the significant advantages of cheese is that it has less lactose than raw milk, making it more acceptable for lactose-intolerant people. The main step of cheese production is the coagulation of the casein micelles to form the structure of the cheese, which can be accomplished in one of three techniques: a) by adding enzymes (rennets), b) by adding acids or a starter culture, or c) by acidification mixed with heating. [47] Along this process, the solid components (curds), which contain casein, calcium, and fat, are separated from the liquid parts (whey), which contain whey proteins (WP), lactose, and some minerals. Whey is drained off before cheese is formed resulting in the removal of a significant amount of lactose. Since the curds used to make hard cheeses contain less moisture than those used to make soft cheeses, hard cheeses have less lactose than soft cheeses. Moreover, lactic acid bacteria are able to digest the residual lactose in cheese curds during ripening. The longer a cheese is aged, the less lactose is left in the final product. Parmigiano Reggiano PDO is an example of a lactose-free hard-matured cheese. Lactose-free cheese can also be produced by incubating the cheese milk with lactase prior to renneting. [32]

Controversy remains about the presence of saturated fatty acids (SFAs), trans-FAs, and cholesterol in dairy products, which has long raised concerns about the increased risk of cardiometabolic disorders. [86][92] However, evidence from randomized controlled trials over the last 5 years has indicated that high-fat dairy products, cheese in particular do not seem to raise total and harmful LDL cholesterol levels to the extent expected. Cheese consumption has also shown an inverse association with incidence of diabetes, stroke, adiposity, and inflammation in most published trials, implying that dairy fat affects cardiovascular health in an optimistic way. [42] Cheese is an outstanding example of a hypothesis that mitigates the predicted negative effects of SFAs on metabolic health when these fats are ingested within complex dairy food matrices. [86] Moreover, several studies have highlighted the bioactivity of specific milk fat components that may be beneficial to human health. For instance, butyric acid and conjugated linoleic acid, as well as different phospholipids and sphingolipids located on the milk fat globule membrane are molecules with promising anticancer action. [92]

1.1.3. Global and EU markets for bovine milk and dairy products

Current American Heart Association (AHA) and European Society of Cardiology (ESC) diet and lifestyle recommendations continue to emphasize the consumption of fat-free and low-fat (1%) dairy products in a healthy eating pattern, while liquid milk and yogurt remain part of joint American Diabetes Association (ADA)/European Association for Study of Diabetes (EASD) nutrition guidelines, and as part of a "no-one-size-fits-all" answer to diet and T2D by the ADA's 2019 Consensus Report. [86][104]

The milk delivered to dairies is processed into a wide range of fresh and processed products. During the decade of 2020, the world's per capita consumption of fresh dairy products is expected to rise by 1.0% per year. The highest demand comes from India, Pakistan and Africa, while demand in Europe and North America remains stable and shows a tendency downward. Meanwhile, consumption rates of processed dairy products (particularly cheese) vary across regions, depending on preferences and urbanization levels. The majority of total cheese consumption takes place in Europe and North America, where per capita consumption is projected to increase further. [30][76] To meet the large demand, global milk production in 2020 was recorded to increase by about 2% compared to 2019, reaching nearly 906 million tonnes. [35] The European Union is the second-largest milk producer and the largest milk exporter with almost 30% of global exports. [18] Furthermore, with stringent guidelines for bacteria and somatic cell counts in raw milk, the EU is committed to ensuring safe dairy products derived from healthy animals. [16]

On a global scale, bovine milk still remains the most commonly produced and consumed milk thanks to some cows' advantages over other dairy animals in regard to udder size, milk storage capacity, ease of milking, as well as milk yield. [30][34] The Holstein-Friesian breed accounts for 90% of the total cattle's milk production and is the breed with the highest milk production. [10] Despite the fact that developed countries have fewer milking cows than developing countries, their livestock gives high milk yield owing to breeding selection and improved diets. [34] Table 1.1 reveals the milk yield of cows in Europe from 2017 to 2020, as well as the rate of milk self-sufficiency calculated using the formula:

$$\% \text{ Self-sufficiency Milk} = C/(C+I-E)$$

where C is Milk Deliveries, I is Import in Milk Equivalent, and E is Export in Milk Equivalent. [19]

Table 1.1. Annual average cow milk yield in EU-28 (kg/year) and the milk self-sufficiency rate (2017-2020)

Year	2017	2018	2019	2020
Dairy Cows ('000 head)	23.312	22.906	22.627	20.565
Milk deliveries (Tonnes)	156.086	157.415	157.874	160.404
Cow milk yield (Kg)	6.696	6.872	6.977	7.800
% Self-sufficiency rate	113,7%	112,9%	115,6%	114,8%

Obviously, in recent years, the volume of milk production has been steadily increasing, reflecting the growing impact of this agri-food industry in the EU market. [16] EU dairy industry mainly focuses on exports due to the fact that the overall milk consumption has been declining and the self-sufficiency rate is greater than 100%. [18]

1.2. Mycotoxins

1.2.1. Mycotoxins contamination in forages and silages, the main component of dairy cow diets

Mycotoxins are typically described as small molecules of secondary metabolites that are generated by moulds (e.g., filamentous micro-fungi), of which *Aspergillus*, *Fusarium* and *Penicillium* genera are considered to be the main manufacturers whose spores are widespread and, in some conditions, can develop on vegetal foods and feeds. (Figure 1.2) [9][84][85]

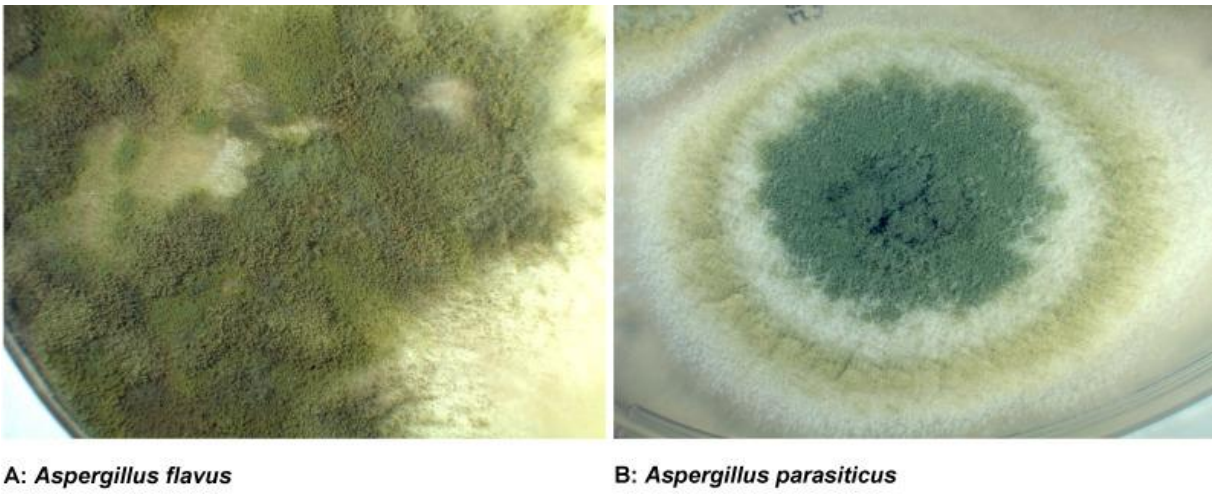

Figure 1.2. Cultures of Aspergillus flavus and Aspergillus parasiticus, respectively, both producers of aflatoxins, on Sabouraud Agar

Mycotoxin-contaminated foods and feeds have been shown to pose potential risks to the safety and quality of products in the food chains, human health, animal welfare, economic growth, domestic and international trade. [27][29] According to the Food and Agriculture Organization of the United Nations (FAO), at least 25% of the food crops over the world are facing mycotoxin problems, including cereals, nuts, oilseeds, fruits, beans, spices, and a variety of forages. [29][90] In addition, the result of a ten-year (2008–2017) survey conducted by BIOMIN Research Center on the global incidence of mycotoxins reported that nearly 88% of animal feed and feed raw materials samples that were collected from 100 countries tested positive for at least one mycotoxin, and 64% of these samples were co-contaminated with more than two mycotoxins. The analysis also revealed regional differences in occurrence trends, with climate and weather playing a crucial role in governing these trends. [43] Approximately 400 fungal metabolites are now recognized as mycotoxins, of which aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEN), fumonisins (FBs), trichothecenes such as deoxynivalenol (DON) and T-2 toxin (T-2) are the most concerning in terms of food safety and government regulation. (Figure 1.3, Table 1.2) [84]

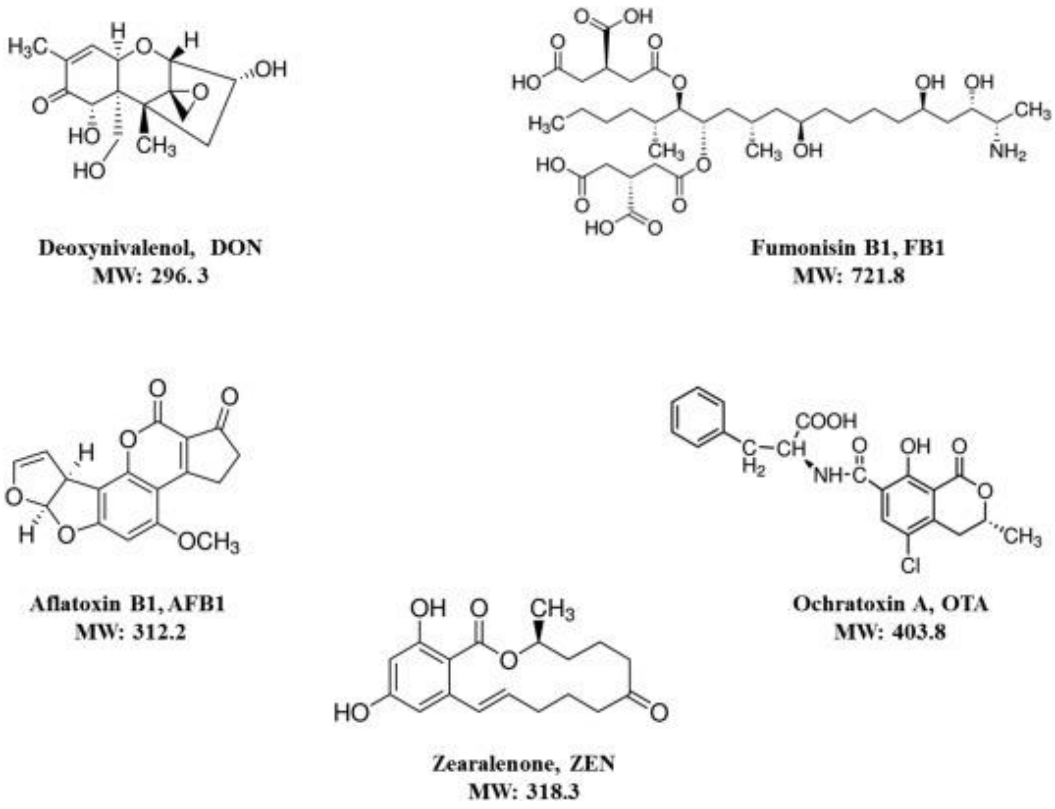

Figure 1.3. Chemical structures of major mycotoxins found in food and animal feed

Table 1.2. The most relevant mycotoxins in animal farms and associated mycotoxigenic fungi

Major classes of mycotoxins	Representatives in animal feed	Mycotoxigenic fungi	Main effects in animals
Aflatoxins	AFB1, AFB2, AFG1, AFG2	*Aspergillus flavus*, *Aspergillus parasiticus*	Hepatotoxicity, carcinogenicity and teratogenicity
Ochratoxins	OTA	*Penicillium verrucosum*, *Penicillium viridicatum*, *Aspergillus ochraceus*	Nephrotoxicity, mild liver damage, immune suppression.
Zearalenone	ZEN	*Fusarium graminearum*,	Estrogenic effects (edema of vulva, enlargement of uterus), atrophy of ovaries and testicles, abortion.
Fumonisins	FB1, FB2	*Fusarium verticillioide*, *Fusarium proliferatum*	Pulmonary edema (pig), Leukoencephalomalacia (horse), nephrotoxicity, hepatotoxicity.
Trichothecenes	DON, 3- or 15-Ac-DON, NIV (type B) T-2, HT-2 (type A)	*Fusarium graminearum*, *Fusarium sporotrichioides*, *Fusarium poae*, *Fusarium equiseti*	Immune effects, haematological changes, dermatitis, oral lesions, gastrointestinal disorders (diarrhoea, reduced FI), gastrointestinal haemorrhage, edema.

Mycotoxins enter the ration of farm animals through fungal infection of plants and subsequent utilization of mouldy crops as ingredients of feed. [107] A wide range of mycotoxin-producing moulds can naturally contaminate forages and cereals both before and after harvest, during transport and storage, or after ensiling during feed-out. [77] Mycotoxin mixtures are made up of pre-harvest toxins that are mostly produced by *Fusarium* species in crops, or post-harvest toxins that are frequently produced by *Aspergillus* and *Penicillium* species in silage or hay under storage conditions. [59] There are some physicochemical factors that influence the incidence of fungal infections and development, including the temperature, humidity, water activity (aw), the presence of oxygen, the nature of the substrate, and pH conditions. [23] Rodents, birds, and insects may contribute to infestation by producing physical lesions on plants, allowing fungal spores to penetrate. [115] Besides that, poor harvesting methods coupled with inappropriate drying, handling, packaging and transportation conditions also enhance the risk of mycotoxin formation. [107]

The ensiling process of forages can eliminate some mycotoxigenic fungi due to low oxygen and low pH conditions. Normally, *Fusarium* spp. thrive in the field but cannot survive in preserved forages that have been compacted and sealed hermetically; in contrast, *Penicillium* spp. and *Aspergillus* spp. as well as a few *Fusarium* species (e.g., *F. oxysporum*, *F. solani*, or *F. verticillioides*) can endure the silage conditions and may produce additional mycotoxins. Furthermore, mycotoxins produced prior to ensiling may remain stable and unchanged during the silage process; for example, ZEN formed from the field can be detected in well-preserved silages devoid of *F. culmorum* activity. [103] In particular, the availability of oxygen in ensiled forages that has not been fully pressed and sealed, or the entry of oxygen through the cutting edge during feed-out, could allow the growth of *Fusarium* spores, or reactivate of inactive fungal spores in silage. [40]

1.2.2. The negative impact of mycotoxins on human and dairy cow health

Effects on cow health and performance

The exposure of mycotoxins in animals is almost often unintentional, through ingestion, inhalation, skin contact, or other routes, resulting in the diseases collectively called mycotoxicoses. The majority of incidents in both animals and humans are caused by the consumption of contaminated food. [74] Ruminants are better protected from mycotoxin effects than monogastric thanks to symbiotic bacteria in their rumen can break down or deactivate poisonous compounds and hence reduce contamination contents. [77] However, ruminant diets are more diverse than monogastric such as swine and poultry, including cereals, protein feeds, grazed forage, sorghum silage, and other compositions; as a consequence, increases the risk of mycotoxin exposure. [40] The rumen fluid, which contains a varied microbiome, is regarded to be the first line of defense against mycotoxins such as ZEN, OTA, and T-2 toxin, but it is useless against AFB1, fumonisin, and patulin. This defense barrier can be damaged in certain scenarios, such as animal disease, dietary changes, and high levels of mycotoxin contamination. [5]

On both humans and farm animals, mycotoxin has a diverse mode of action includes cytotoxicity, hepatotoxicity, nephrotoxicity, teratogenicity, mutagenicity, carcinogenicity, immunosuppression, and estrogenic effects. After ingesting one or more mycotoxins even at low concentrations, infected cows may experience anorexia (lack or loss of appetite for food), reproductive and metabolic disorders, reduced milk output or poor weight gain, get sick, or even death. (Table 1.3) [63][84] Such effects result in serious economic losses, especially significant domestic animal mortality, increased veterinary care expenses and

decreased production efficiency. [23] Commonly, clinical signs and pathological lesions of mycotoxicoses in dairy cows can vary greatly depending on several factors such as the type, dose, duration of intoxication; species, breed, sex, age of cows; as well as synergistic effect along with other toxins and/or pharmacologically active substances. [23][84][105] In addition, because feed storage conditions can result in the presence of mycotoxigenic moulds, cows fed fresh pasture are assumed to be less susceptible to mycotoxins than cows fed dry or concentrated feed, primarily corn silage and hay. [5]

Mycotoxicoses syndromes can lead to acute toxicity with a sudden onset and apparent signs of disease in liver, kidney, epithelial tissue, immune system, and central nervous system, or even mortality. [9][85] In livestock farm environments, mycotoxin effects are rarely acute but predominantly chronic, defined by low-dose exposure over a long period and concealed disorders with impaired digestion, productivity, and fertility. [9][40] The primary chronic toxicity is the induction of cancer, particularly of the liver (e.g. FB1 or AFB1). Some mycotoxins (AFB1 is one example) can interfere with DNA replication, which can result in mutagenic or teratogenic effects. [85] Some toxic residues will transfer into certain compartments of ruminants and will present in edible animal products, such as meat, offal, milk, and eggs, causing food safety problems and endangering human health. [59]

Table 1.3. Summary of effects of common silage mycotoxins in ruminants

Mycotoxin	Effects in ruminants
Aflatoxin B1, B2, G1, G2	Decreased feed efficiency and weight gain. Decreased milk production. Decreased milk quality and safety. Compromised immune. Liver malfunctions.
Ochratoxin A	No significant toxicity to cows when fed alone in naturally occurring doses. Carry-over of the toxin into milk is minimal.
Zearalenone	Infertility, decreased milk production, and hyperestrogenism. Carry-over of the toxin into milk is negligible.
Fumonisin B1, B2, B3	Decreased performance probably due to feed refusal. Mild liver disease. Extent of transfer into milk of ruminants is negligible.
Deoxynivalenol	Gastrointestinal problems and decreased performance probably due to feed refusal. No evidence of carry-over into milk of cows.
T-2 toxin	Immunosuppression in cattle because of decreased antibody production, neutrophil function, and lymphocyte blastogenesis. Infertility and abortion in late gestation. Extent of transfer into milk of ruminants is negligible.

Effects on human health, especially carcinogenic effect

Human exposure to mycotoxins can occur directly through eating cereals or indirectly through digesting animal products, resulting in a wide range of negative consequences. (Table 1.4) [65] Due to the obvious toxicity and carcinogenicity of mycotoxins in food, at least 99 nations (representing around 87 percent of the world's population) proposed mycotoxin regulations for food or animal feed by the end of 2003. [39][102]

Table 1.4. Mycotoxins and their effects on human health

Mycotoxin	Effects in ruminants
Aflatoxin B1/M1	Liver cancer
Ochratoxin A	Possible carcinogen, kidney damage
Zearalenone	Natural oestrogen (effects undefined)
Fumonisin B1	Possible carcinogen, kidney/liver damage
Deoxynivalenol	Nausea, diarrhoea, vomiting and headache
T-2 toxin	Nausea, diarrhoea, vomiting and headache

Many studies have relied on the description of aflatoxin B1 metabolism to illustrate the metabolic fate and toxicokinetics of mycotoxins in human and animals. (Figure 1.4) [72] The completeness of absorption of aflatoxin B1 after oral exposure via food products have been shown using radiolabelled aflatoxin B1 in some experimental animals such as rats and monkeys. Since AFB1 is lipophilic xenobiotic, it can also be absorbed rapidly, by passive diffusion, from the small intestines (especially the duodenum) into the plasma, and then distribute to liver, which is regarded as the main site of aflatoxin transformation. [101] Metabolism of AFB1 divided into two phases: Phase I involves oxidative reactions by cytochrome P450 enzymes, to bioactivation of AFB1 and gives rise to several reactive metabolites/intermediates (epoxides), phase II generally serve as a detoxifying step that involves conjugation of the mycotoxins or their metabolites to another molecule, such as glucuronide or amino acids. Currently, the hazardous or carcinogenic effects of AFB1 are thought to be connected to both the rate of activation and the rate of detoxification at phase I and phase II of metabolism. [25]

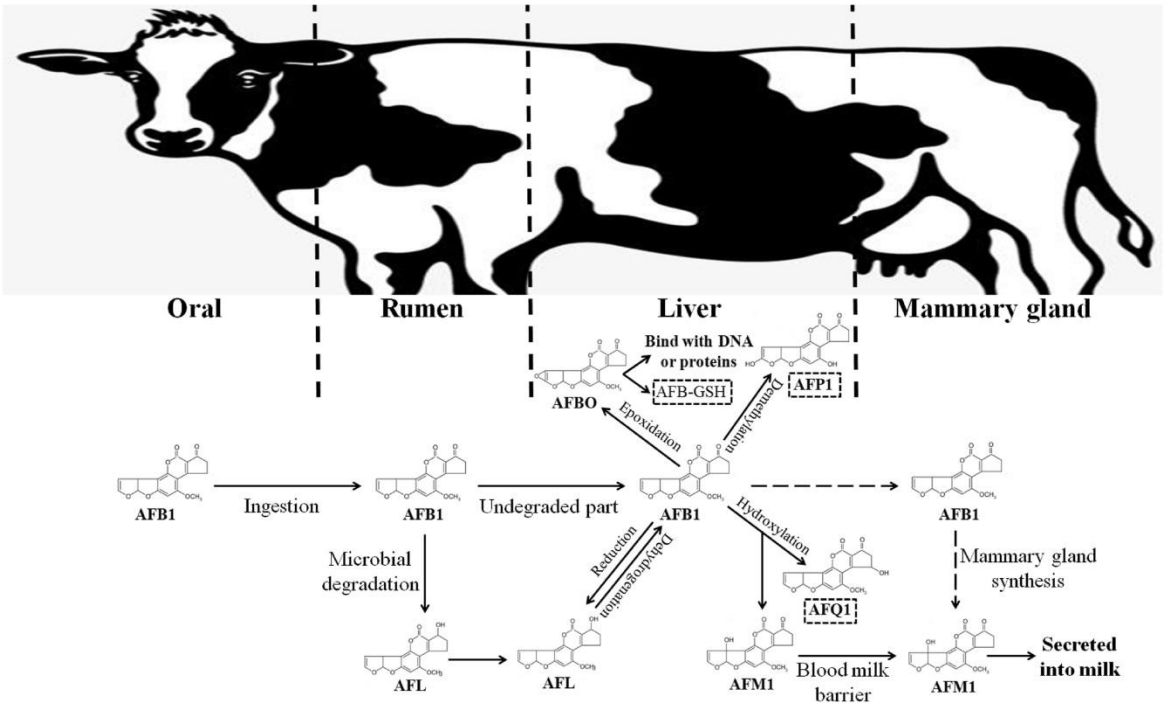

Figure 1.4. The metabolism and biotransformation pathways of AFB1 in lactating dairy cows

Aflatoxins are very toxic compounds that the International Agency for Research on Cancer (IARC) has designated as Group 1 carcinogens in humans. Aflatoxin exposure in the diet is one of the major risk factors for hepatocellular carcinoma. Among AFs, AFB1 is the most potent carcinogen. Moreover, AFB1 can act as "pro-carcinogen" because it will be enzymatically bioactivated by hepatic CYP450 to form reactive AFB1-8,9-epoxide (AFBO), the ultimate carcinogenic form. This resulting reactive epoxide can intercalate the DNA and binds covalently, upon alkylation reaction, to the N^7 position of guanine, causing DNA damage. The three AFBO-induced DNA lesions (AP, AFB1-N7-gua, and AFB1-FAPy) have been known as the main precursors of AFB1 genotoxic and carcinogenic effects. [8][56] AFs exposure is thought to be responsible for roughly 26% of the 550,000-600,000 new cases of liver cancer diagnosed each year around the world. [3] All nations that enacted regulations related to mycotoxins in foods and feeds in 2003 have regulatory limitations for aflatoxin B1 alone or the sum of the aflatoxin B1, B2, G1 and G2. [39]

Fumonisin B1 (FB1) belongs to Group 2B possible carcinogen to humans, though it does not appear to interact with DNA. FB1 exposure has been shown in animal studies to cause neural tube abnormalities, raising worries that this mycotoxin could have comparable consequences in humans. OTA has the potential to be nephrotoxic, hepatotoxic, neurotoxic, carcinogenic, and immunotoxic. ZEN and its derivatives are not

considered carcinogenic; however, they have estrogenic and anabolic activity, which causes precocious puberty in children. DON (vomitoxin) can inhibit protein synthesis, resulting in acute vomiting, diarrhoea, abdominal pain, headache, and fever. [5][85]

1.2.3. Milk and cheese products contaminated with aflatoxin M1 pose a health risk to consumers

Normally, mycotoxins found in dairy products can originate from one of two sources: (1) indirect contamination occurs when lactating animals consume feed containing mycotoxins, which are metabolized and pass into the milk, such as aflatoxin M1 (AFM1); or (2) direct contamination occurs when molds are intentionally or unintentionally grown on dairy products. [95] In raw milk, aflatoxin M1 (AFM1) is the most frequently detected mycotoxin and raised concern about the likelihood of exceeding the EU limit of 0.05 μg/ kg in milk, indicating a significant threat to dairy consumers. (Figure 1.5) [3][20]

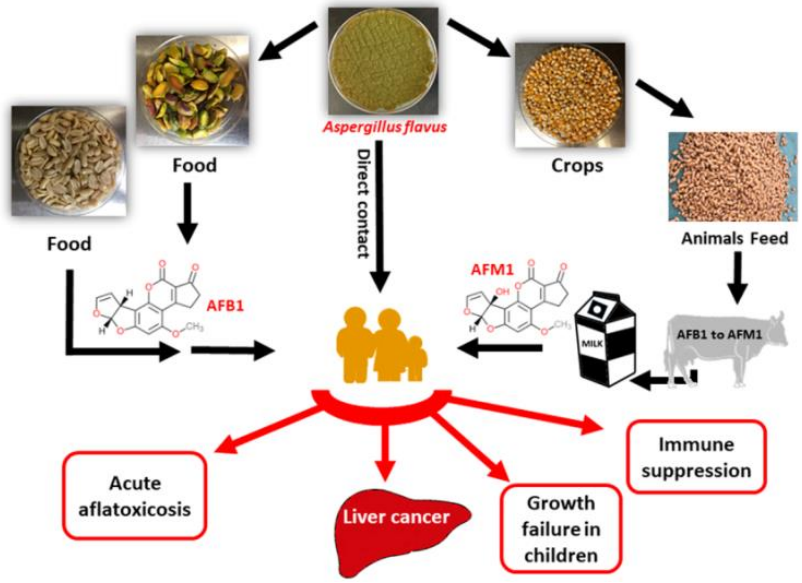

Figure 1.5. Major AFB1 and AFM1 contamination routes and health effects on humans

AFM1 is a hydroxylated metabolite of AFB1, which is biotransformed by cytochrome P450 enzymes in the liver of dairy cows fed with AFB1 contaminated feed. (Figure 1.6) [3][48] AFM1 was identified in milk 12-24 hours after the first AFB1 ingestion, and after a few days, it reached a high level. [36] Some investigations demonstrated that when cows eat AFB1-contaminated feeds, a portion of the AFB1 is digested in the rumen, forming aflatoxicol. The remaining fraction is passively diffused in the gastrointestinal tract and hydroxylated to AFM1 in the liver. [37] Due to the hydroxyl group, these compounds are highly hydrophilic, allowing their elimination through urine,

bile, feces, and milk. [5] (Figure 1.6). Risk assessments revealed that AFM1 can leads to carcinogenicity, mutagenicity, genotoxicity, teratogenicity, and immunosuppression. [72] AFM1 is heat resistant and chemically stable within the range of conventional food processing conditions (80–121°C); therefore, it still persists throughout boiling and frying, or even not completely inactivated by pasteurization, sterilization, or other milk treatment processes. [5][82]

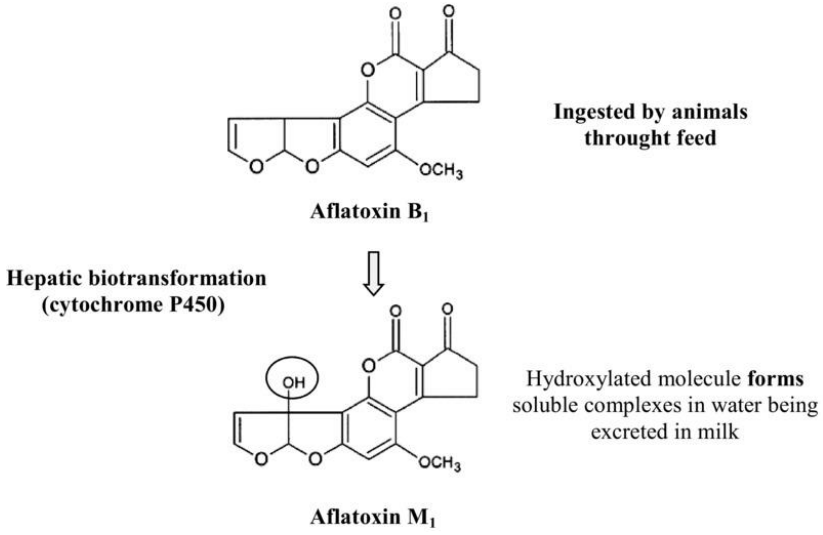

Figure 1.6. Hepatic biotransformation of aflatoxin B1 into aflatoxin M1

Furthermore, milk is not only consumed as liquid milk but is also used as an ingredient in the production of related products, such as cheese. AFM1 is relatively stable in raw and processed milk, so cheese prepared from such milk will be contaminated as well. AFM1 contents in cheeses can remain constant even during processing and ripening. [95] Mycotoxins in cheese vary depending on cheese type or processing parameters such as temperature, pH, pressing time, and so on. [65] Some other mycotoxins detected in cheese are the consequence of the accidental proliferation of wild strains such as *Penicillium* species. Sometimes, moulds are purposefully introduced into some cheeses, such as Camembert and Roquefort. The risk of mycotoxins in cheese increases dramatically if toxigenic molds are allowed to develop during manufacture and storage. [65][95]

Given the adverse effects mycotoxin may cause in milk and cheese consumers (especially children and old age), many countries, particularly European countries, have established regulations relating to mycotoxins, including the maximum residue levels (MRLs) of AFM1 in milk and dairy products, in order to protect the public health. (Firgure 1.7) [67][102] According to the European Commission, the MRL for AFM1 is

0.05 µg/L in milk and 0.025 µg/L in milk-based infant meals. MRL levels in other nations such as Syria, the United States, China, and Brazil, are higher (for example, in the US, the limit is 0.5 g/L). From a global viewpoint on the prevention of toxicity, the management of mycotoxins levels, in particular AFM1, in milk is a mandatory requirement for health and regulatory objectives. [52] Infants' exposure to AFM1 is more concerning since their ability to metabolize carcinogens is generally lower than that of adults, making them more susceptible. [48]

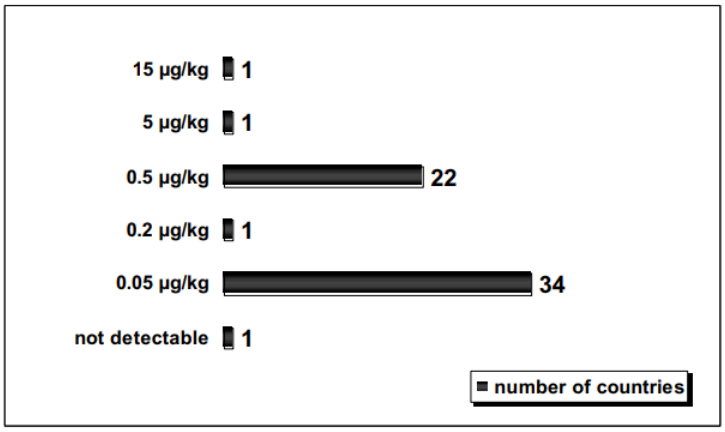

Figure 1.7. Worldwide limitation of aflatoxin M1 in milk

1.3. The addition of mycotoxin binder to cattle feeds to reduce mycotoxin exposure

Although the agricultural and feed industries' primary goal is to prevent mycotoxin infection in the field and during storage by employing good agricultural and storage practices (GMP), the complete absence of mycotoxins in dairy cow feed can be difficult to achieve, necessitating the use of detoxification methods. [107] A variety of methods have been applied to decrease the presence of mycotoxins in feed; however, dietary addition of adsorbent clays is the most prominent approach and is extensively utilized by farmers and fodder manufacturers. Clay minerals are finely granulated earthy substances (diameter less than 2 mm) that exhibit plasticity when moistened or non-plastic and hard when dried. [23] These substances are usually added to animal feed as a non-nutritive additive to prevent feedstuff from lumping, which is a risk of microbial contamination. [27] Moreover, mineral adsorbents are abundant in nature, are affordable, can be chemically manipulated to enhance mycotoxin-adsorption capacity. Several studies have examined the potential of mineral adsorbents to bind mycotoxins in vitro, and their partly or fully protective efficacy against mycotoxicosis in vivo. [27] The use of high-dose mycotoxin binder can also diminish the concentration of AFM1 that carry-over into the

milk and further increase the clearance rate. [88] Since 2009, the European Commission has officially authorized the use of such anti-mycotoxin agents through the amendment and issuance of regulations related to the new technological feed additives. (EC No 386/2009). [27]

Mycotoxin binders are added to the cattle feed to prevent mycotoxins from being absorbed in the gastrointestinal tract, as well as their transport to the blood circulation and target organs, decreasing their bioavailability after ingestion, and thereby limiting the deleterious effects of mycotoxicosis in individuals. [61] The main mechanism of these binders is forming mycotoxin-adsorbent complexes by different types of interactions such as hydrophobic binding, hydrogen bonds, or ion-dipole interactions. These complexes is able to pass through the animal and is then excreted via the faeces. [107] Table 1.5 presents a summary of the major clay adsorbents currently used for sequestering mycotoxins [27]. According to EFSA in 2009, mycotoxin-binding agents can be categorized into silica-based inorganic compounds or carbon-based organic polymers (Figure 1.8). [15]

Table 1.5. Summary of physicochemical properties of mineral adsorbents commonly used for binding mycotoxins

Adsorbent	Structure	CEC (cmol/kg)	Surface area (m2 /g)	Mode of formation
Bentonite	2:1 Lattice	53–83	370–490	Decomposition of volcanic ash in marine environment or silica bearing rocks such as granite and basalt.
Kaolinite	1:1 Lattice	3–15	5–20	Rock weathering or the hydrothermal alteration process at high temperature or the alteration of primary minerals at low temperature (such as feldspar).
Montmorillonite	2:1 Lattice	80–100	70–800	Weathering products in soils at moderately high temperature (200°C)
Palygorskite	2:1 Lattice	4–40	300–600	Alteration of precursor minerals or precipitation from rock solution.
Activated carbon	Pore	–	300–4000	Pyrolysis of organic materials such as lignin, coconut shell, peat, hard and soft wood, lignite coal and carbonaceous materials.
Zeolite	1:2 Lattice	180–600	500–700	Rock interaction with aqueous solution

(Clinoptilolite)	or fluid in a wide variety of geochemical environments.

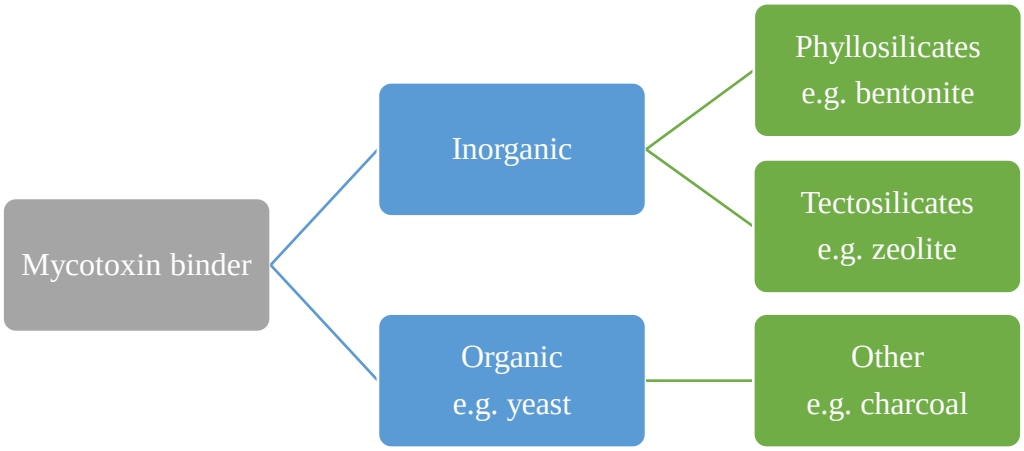

Figure 1.8. Classification of mycotoxin binders (adapted from EFSA, 2009).

Bentonite is a type of phyllosilicate clay with a layered crystalline architecture that permits other molecules to be absorbed and a strong tendency to inflate when wet. [23] A basic structure of Bentonite (primarily made up of montmorillonite) is composed of several tens of stacked nanolayers (tactoids). Each platelet is made up of three layers, an octahedral layer comprising of Mg, Fe, Al and OH-groups, sandwiched between two tetrahedral layers with Si-O tetrahedrons. (Figure 1.9) [26][27][46] The performance of bentonite adsorption is highly influenced by its montmorillonite content and interchangeable ions. [107] Around 80 percent of the exchangeable cations are found in the interlayer spaces, which help to balance out the negative charges in the reticulated region. The enormous surface area and strong cation exchange capacity of the smectite group, along with this structure, make these compounds suitable adsorbents for low polar mycotoxins, enabling both cations and polar molecules to pass through. [23] The adsorption ability is often influenced by adsorbent parameters such as total charge, charge distribution, pore size, and accessible surface area, in addition to the physicochemical properties of target mycotoxins. [87] In numerous in vitro and in vivo trials, Bentonite clays have shown high efficacy in forming and firmly binding mycotoxin-adsorbent complexes to limit toxin bioavailability. [107] These clays have the capacity to bind minor mycotoxins like aflatoxin and ochratoxin A, but not bigger compounds like those found in Fusarium toxins. [87] AFs can adsorb at multiple sites, particularly the interlayer region, but also at edges and external basal surfaces. [27][107]

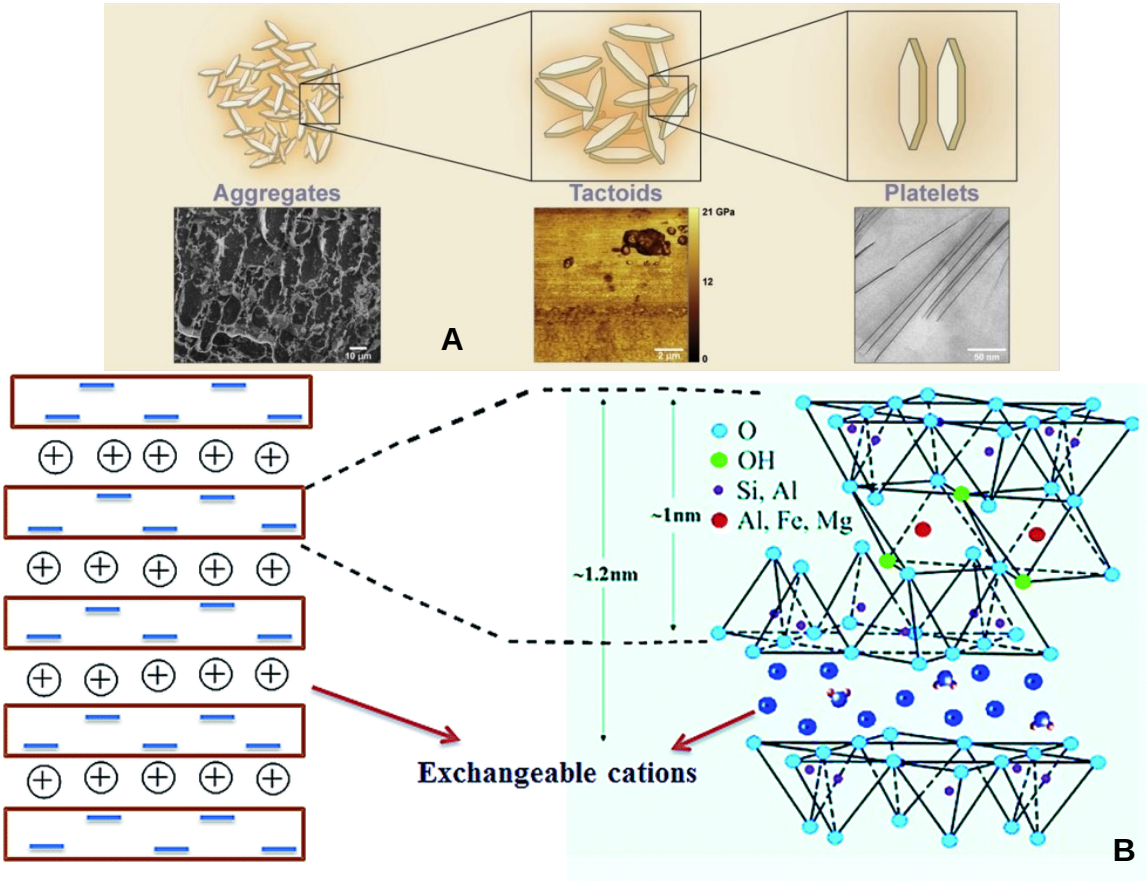

Figure 1.9. Graphical abstract (A) and basic structure (B) of Bentonite clays

Concerning the safety and efficacy of bentonite detoxifying agents, The European Food Safety Authority (EFSA) assessed and suggested that after being employed as a technological feed supplement, bentonite was observed to be non-genotoxic and non-absorbable, offering no direct toxicological risk to the animal (EFSA, 2011). [107] On the other hand, there is still considerable debate about whether commercial mycotoxin binders, such as Bentonites, can have negative repercussions for the health and production of farm animals. In addition, there is a scarcity of research data regarding the impact of mycotoxins on animal products, such as cow milk. As a result, farmers and the feed industry must be cautious when supplementing these anti-mycotoxin mineral adsorbents, and further studies are needed in this area. [27]

1.4. Microbial community of bovine milk and cheese

1.4.1. Milk microbiota investigation in the omics era

As stated above, milk is a nutrient-rich material with a neutral pH and high water activity, providing a perfect environment for the growth of a wide range of microorganisms. In the past, it was considered that a healthy udder and the milk it contained were sterile and the microorganisms detected in milk were assumed to have

been introduced from numerous external contamination sources. [89] On the other hand, with the great developments in science and technology and the introduction of next-generation sequencing (NGS) techniques, the existence of a diverse and complex microbial population in cow's milk was revealed, even in healthy mammary glands. [80] The methods used to investigate milk-associated microbiota evolved from traditional cultivation-based analysis to the most recent culture-independent, DNA-based techniques, which typically rely, at least in part, on the use of polymerase chain reaction (PCR) technology. One of the main benefits of this alternative is the ability to correct inaccurate outcomes from traditional research since it can identify even non-culturable species due to unknown nutritional needs. [89] DNA sequencing allows us to exactly specify the order of the nucleotides that make up the nucleic acid's primary structure, from that we can acquire the sequences of previously unknown species or to accurately identify particular samples by comparing them to databases. [62] The large-scale sequencing (NGS) can create more rapid, high-throughput tests by generating millions of sequenced reads in only a single run, allowing for far more detailed and precise estimation of microbial diversity. One limitation of NGS is that it requires advanced platforms and IT tools for the re-processing of the enormous quantity of data that is generated. [89] Futhermore, the availability of sequence databases and bioinformatics tools offers a great chance for taxonomic assignment of the microorganisms present at a high level of precision. [89]

The majority of published milk microbiome data has resulted from DNA-based methods with amplification and sequencing of 16S rRNA genes. [1][21][89] The prokaryotic 16S rDNA, which codes for the small subunit (SSU) of ribosomal RNA, is made up of nine hypervariable regions (V1-V9) flanked by regions of highly conservative sequence. Because it is highly conserved across members of the domains *Bacteria* and *Archaea*, allowing the design of "universal" PCR primers, and its hypervariable regions show significant sequence diversity among different species, providing species-specific signature sequences, this gene is commonly used in microbial community fingerprinting and taxonomic classification. [49] Figure 1.11 shows the variable regions of 16S rDNA and the PCR universal primers. [1][55] To simplify the large datasets, the amplified pool of 16S rDNA fragments was grouped/clustered based on similarity to form operational taxonomic units (OTUs). Representative OTU sequences were compared to reference databases and used to compare biodiversity within and between samples. [111]

Several fingerprinting approach have been widely used such as denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP), or microarrays. [49] In recent years, next-generation sequencing (NGS) technologies have empowered deeper sequencing and data analysis of various types of environmental samples than was previously possible using standard molecular biological techniques. [97] The commercially available Illumina MiSeq platform poses the great potential for 16S rDNA sequence studies, as it allows for relatively long reads of up to 600 bp. Additionally, the newly designed universal PCR primers Pro341F and Pro805R targeting the V3-V4 region of bacterial rDNA were modified based on previously published universal PCR primer sets, to improve the coverage of existing sequences in the database and so obtain better result of prokaryote detection. [49]

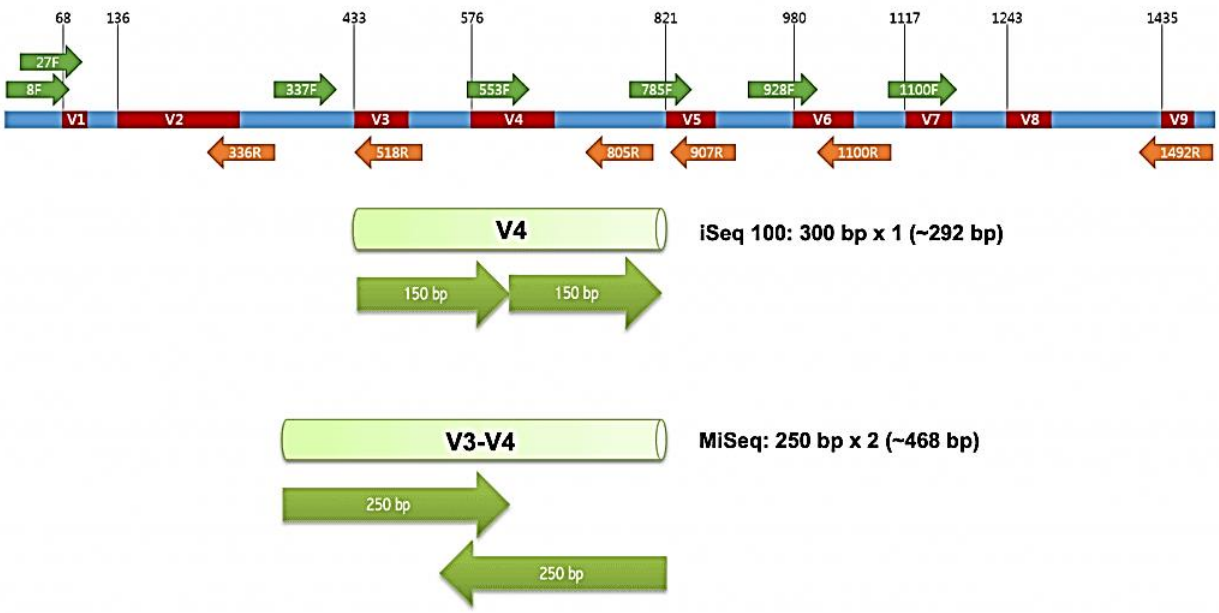

Figure 1.10. Hypervariable regions of 16S and PCR primers to amplify them

In recent years, a wide range of meta-omics analyses have been widely applied and have substantially expanded our understanding of the milk microbial communities, including metagenomics, metatranscriptomics, metaproteomics, and metabolomics, which target DNA, RNA, proteins, and metabolites produced by the entire microbial community, respectively. (Table 1.6) [1]

Table 1.6. Features of the -omics investigations available for studying microbial communities

Approach	Target molecule(s)	Information provided	Drawbacks
16S metagenomics	16S rRNA gene (or its hypervariable regions)	Taxonomic distribution	Only bacteria are characterized
Metagenomics	Community DNA	Taxonomic distribution and gene potential	Issues with sequence annotation and costs
Metatranscriptomics	Community RNA (or mRNA)	Taxonomic distribution and gene expression	Issues with RNA stability and data analysis
Metaproteomics	Community proteins	Taxonomic distribution and protein expression	Issues with protein dynamic range and data analysis
Metametabolomics	Community metabolites/organic compounds	Metabolic fluxes	No direct link between metabolite and microbial taxonomy

Although DNA-based methods can provides important information about community taxonomy and its functional potential, there is a lack of information related to microbiota activities under specific conditions or during specific periods. Due to the inability to differentiate among the genes originating from active, inactive, lysed, or dead cells, this method cannot determine the viability of the detected microbial communities, as well as their biological and metabolic activities. Therefore, when it comes to the scenario of the functional activity of milk microbial communities, this approach is deemed not feasible. The alternative RNA-based approach offers more promise thanks to the existence of a positive association between the content of ribosomal RNA (rRNA) and the metabolic activity of microorganisms, allowing the prediction of metabolically-active microbiome structures. [1][4] Following extraction, total RNA is reverse-transcribed to cDNA, and cDNAs are analyzed using high-throughput sequencing technologies (RNA-seq). However, many challenges remain in the research using this method, particularly sample preparation and handling caution due to RNA's low stability and the ubiquitous presence of RNases, as well as bioinformatics issues relating to sequence reconstruction, annotation, and statistical analysis. [60]

Some recent reports have noticed the discrepancies in the community profile between DNA and RNA-based studies. This finding demonstrated that DNA profiling data alone can lead to an underestimating of active members in the community, emphasizing the significance of using a complementary method to gain a broad general overview of not only of total and active members but also in the expected functionality. [4][93] Nevertheless, only a few studies have conducted RNA-based profiling of the microbial community found on milk and cheese to date. This could be due to the methodological and technical challenges the researcher encounters, such as developing a uniform process that evenly harvests all RNA transcripts from all species while simultaneously preventing degradation.

1.4.2. Functions of microbial community in milk and cheese

The active microbial community of breast milk is thought to contribute significantly to the development of the neonates' GI tracts as well as the health of nursing mothers' breasts. The abundance of milk bacteria is estimated to be around 10^3–10^4 CFU/ml in healthy human breast milk. [53] With respect to the cows' milk microbiota, most research has focused on how the microbial flora of milk changes as it is transformed into a food product, either for immediate intake or for transformation into specific dairy products (cheese for example). [89] Moreover, the analysis may be also interested in knowing how microbial species differ between cheese kinds and different cheesemaking procedures. [100] Some bacteria harbored within milk is able to transform the milk components such as lactose, to produce metabolic secondary products that could further be used as a substrate for the growth and the metabolism of other microorganisms. The diverse milk-associated microbiota also plays an active role in determining cheese composition as well as specific cheese features, for instance, flavors and aromas. At the same time, bacteria capable of consuming milk sugars also became more abundant in composition. [89][100] In addition to its endogenous microbiota, once milked, raw milk is easily colonized by a range of other bacteria from the breast skin, milking machines, storage tanks, or the surrounding environment. Also, milk and cheese can be contaminated with potentially pathogenic bacteria (or their toxins) and hence can have serious effects on human health. [1] Moreover, sometimes microorganisms are introduced into dairy products on purpose, such as in mould-ripened cheese, or the strictly controlled starter cultures. [11][12] Indeed, by understanding the dynamics of the dairy microbiota, people can better control the qualitative, textural, sensorial, and biosafety characteristics of dairy products. [100]

Therefore, it is necessary to cultivate more knowledge about the change of microbial composition in raw milk throughout milking, transportation, storage and processing. [1]

Over the years, the importance of the microbiome in milk and milk products has been increasingly appreciated, so this microbiome has become the subject of many different articles. Despite the fact that the use of various investigation methods and the influence of various factors (both endogenous and exogenous) make a direct comparison and the integration of the obtained results more complicated, the typical composition cow milk microbiota registered a heterogeneous composition characterized by the high abundance of LAB such as *Lactococcus spp.* (10^1 –10^4 CFU/mL), *Streptococcus spp.* (10^1 –10^4 CFU/mL), *Lactobacillus spp.* (10^2 –10^4 CFU/mL), *Leuconostoc* (10^1 –10^3 CFU/mL) and *Enterococcus spp.* (10^1 –10^3 CFU/mL). In addition, bacteria with a fluctuating load, such as psychotropic *Pseudomonas, Acinetobacter*, and *Aeromonas*, have been found in the microbiota of cow milk. Furthermore, recent research using more sensitive methods than traditional ones revealed the presence of anaerobic bacteria such as *Bacteroides*, *Faecalibacterium*, *Prevotella*, and *Catenibacterium*.[89][100]

To date, despite the recognition of the importance of microbial activity in dairy products, there is still relatively little research in this area. Most concerns are linked to the microbial ecology of raw milk, rather than their behavior in the context of animal health and physiology, or when cow diets change. [78][79] Associated with diet change, Zhang et al. used pyrosequencing of the 16S rDNA to evaluate the impact of two dairy cattle diets (high concentration versus low concentrate diet) on milk microbial populations. Generally, *Actinobacteria, Firmicutes, Proteobacteria*, and *Bacteroidetes* dominated the milk microbiota regardless of diet. The authors also suggested that differences in feeding strategy can associate differences in milk microbial communities. [117] Table 1.7 summarizes some current findings on the composition of the microbial community in healthy cow milk.

As far as cheeses are concerned, regardless of whether they are fermented in a natural manner or with the help of starter cultures, most cheeses contain a diverse combination of microbial populations that develop and alter during cheese manufacturing and ripening. Generally, the microbiome in cheese is diverse and varies from one type to another, although the predominant microorganisms for each type of cheese are often similar. Prokaryotic *Archaea* and *Bacteria*, eukaryotic yeasts and fungi, as well as viruses (mainly bacteriophages) are all make up the cheese microbiota. Cheese bacteria are

mainly dominated by the *Firmicutes* (LAB, enterococci, staphylococci), *Actinobacteria* (corynebacteria, propionibacteria, bifidobacteria), and *Proteobacteria* (enterobacteria) phyla as showed in the research of Mayo et al. with the use of DNA-based profiling. [71]

Table 1.7. Composition of the healthy cow milk microbiota

Study	Most revelant genera
Kuehn et al. [64]	*Ralstonia, Pseudomonas, Sphingomonas, Stenotrophomonas, Psychrobacter, Bradyrhizobium, Corynebacterium, Pelomonas, Staphylococcus*
Oikonomou et al. [79]	*Faecalibacterium, unclassified Lachnospiraceae, Propionibacterium, Aeribacillus, Bacteroides, unclassified Clostridiales, Staphylococcus, Streptococcus, Anaerococcus, unclassified Xanthomonadaceae, unclassified Bacteroidales, unclassified Bacteria, Lactobacillus, Porphyromonas, Comamonas, Fusobacterium, Enterococcus, unclassified Carnobacteriaceae, Asticcacaulis*
Zhang et al. [117]	*Chryseobacterium, Streptococcus, Enterococcus, Stenotrophomonas, Brevundimonas, Lactococcus, Sphingomonas, Prevotella, Sphingobacterium, Helcococcus, Leucobacter, Butyrivibrio, Atopostipes, Bosea, Alcaligenes, Ruminococcus, Facklamia, Actinomyces, Sphingobium, Trueperella, Pseudomonas, Enterobacter, Comamonas, Megasphaera, Salinicoccus, Ochrobactrum, Lactobacillus, Mogibacterium, Peptococcus, Succiniclasticum, Myroides*

Diet modifications are expected to have an impact on the active microbiota of milk and cheese. Moreover, there are few comparative studies on the effects of the mycotoxin binder Bentonite on the community profile of the milk and cheese microbiota, and this area needs to be explored to determine better management strategies regarding the use of this substance as a dairy feed additive. In this study, we hypothesized that Bentonites feed additives may not cause negative effects on the phylogenetic diversity of dairy flora. If this hypothesis is confirmed, it will provide a theoretical basis for the safety monitoring of using this mycotoxin binder in bovine feed.

2. AIM OF THIS WORK

The role of mycotoxin binders in recent years has been highly appreciated as an effective method to prevent the negative effects of mycotoxin in human and animal healths, especially to prevent AFM1 toxicity in cow's milk fed with fungal feed. The addition of Bentonites toxin binders to dairy feeds is widely used worldwide; however, there is still a lack of studies evaluating the impact of this substance. The metabolically active microbiota in the milk and cheese matrix plays an important role in determining the quality of the product, including composition and flavor of dairy products; at the same time, they may contribute to the human gut microbiota diversity. The question is whether after dairy cows consume food supplemented with Bentonites, will this substance enter the milk and affect the microflora of milk?

The aim of this thesis is to investigate the effect of Bentonite in cow's dietary supplement on the structure of metabolically active milk and cheese microbiota, by using a high throughput, RNA-based approach. Specifically, this method includes total RNA extraction, cDNA synthesis, and subsequent two-step PCR amplification of RNA-derived transcripts–amplicons using primers targeting bacterial 16S ribosomal RNA genes, followed by Illumina MiSeq platform sequencing. The 16S rRNA gene is profiled using the V3-V4 region as a marker for the phylogenetic diversity of the expressed bacterial community. This approach aims to allow a comparison among the relative amounts of active microbial taxa within different amounts of Bentonites added to the bovine diet. To the best of our knowledge, there are currently no studies reporting changes in the structure of the active microbial communities in cow's milk and cheese associated with the mycotoxin binder Bentonites.

3. MATERIALS AND METHODS

3.1. Animals, treatments and experimental design to investigate the effect of Bentonite clay

The study was conducted to investigate the effect of Bentonite clay added to the cows' diet on the active microbial communities of milk and cheese from November 2020 to June 2021. A total of six lactating multiparous (parity 2 to 3) Italian Friesian cows in plateau lactation phases (219 ± 37 DIM) were randomly assigned to 1 of 3 feed additive treatment sequences (B) arranged in a balanced 3 × 3 Latin square design with three 19-d experimental periods (T1, T2, T3). A Latin square design means the arrangement of t group of trials, each of which is repeated t times, in the design so that each group of trials appears accurately once in each row and column. This experimental design is useful because it can aid the case we have several factors and we want to keep them separate, it allows experiments with a small number of runs and decrease systematic error due to rows and columns. [24][112] The first 24-d acclimation was used to ensure animal adaption to a new diet. A 9-d washout interval between periods was imposed to minimize carryover of treatment effects between periods. Figure 3.1 depicts a schematic diagram of the experimental phases.

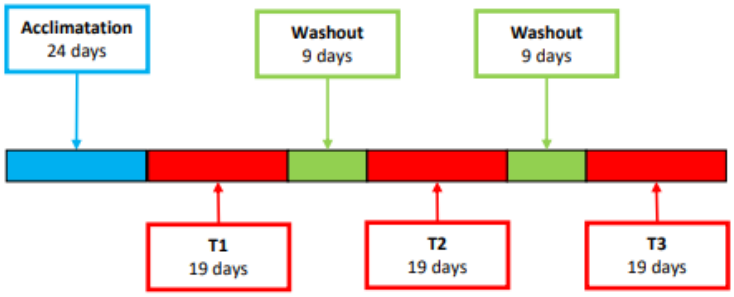

Figure 3.1. Schematic design of each experimental period and its sampling time points

All cows used in this study were housed in identical rearing conditions at the Experimental Farm of University of Padova, Italy. Cows were provided the same TMR basal diet to meet their nutritional requirements [4] and were fed ad libitum. From d 1 to 19 of each additive treatment period, Bentonite clay was top-dressed on the respective basal diet. Each cow was assigned an ID number, 88, 313, 437, 578, 632, and 988, respectively. The feed additive treatments were formulated to either: (1) the control B0 group (basal diet without Bentonite additive); or (2) the B50 group (with 50g/day Bentonite additive); or (3) the B100 group (with 100g/day Bentonite additive). The exact protocols of feed additive treatments can be found in Table 3.1.

Table 3.1. The Bentonite additive treatments during three 19-day periods (T1, T2, T3)

Period / Treatment	T1		T2		T3	
B0	88	578	313	437	988	632
B50	632	437	88	988	313	578
B100	313	988	632	578	88	437

3.2. Sampling procedures

3.2.1. Milk sampling and pretreatment

Each period of the Latin square lasted 19 d, with milk samples collected on d 17 in the morning between 6:00 and 7:00 (before morning feeding) for analysis of the active milk microbial community. An aliquot of 50 ml was obtained from each cow under sterile conditions by washing and drying cow's teat with pre-dipping (Figure 3.2) and alcohol solution to prevent teat apex bacterial contamination of the milk, followed by manual removal of the initial 3 streams of milk. Milk sample collections were transported and preserved in frozen conditions for further analysis.

Figure 3.2. A rapid-acting foam pre-dipping

In the lab, milk samples were centrifuged at 2683g for 20 min (at 4°C), and then discarded the cream (fat) and the supernatant. The pellets were re-suspended with 1 ml of PBS (phosphate-buffered saline) which is an isotonic and non-toxic buffer solution. Re-suspended mixture was centrifuged at 12000g for 1 min (at 4°C), removed supernatant and washed with 500 µl of PBS. The obtained re-suspensions were separated into two sterile tubes (250 µl each) and following by the centrifugation at 12000g for 1 min (at 4°C). Supernatant was eliminated and pellet was mixed with 1 ml of TRIZOL Reagent that is useful for isolating total RNA by preserving the integrity of RNA during tissue

homogenization and cell disruption. Cells were disrupted with Zirconia Silica beads (0.25mm) on a TissueLyser (Qiagen, Hilden, Germany) in frequency of 30 Hz (1/s) x 1 min. Final pre-treated milk samples were stored at -80 °C to be used in further RNA extraction.

3.1.1. Caciotta cheese making, sampling and pretreatment

Table 3.2 summarizes some information related to the cheese-making process using raw milk that was carried out in this study.

Table 3.2. Summary of Caciotta cheese making

Boiler ID	Cow	Treatment	Milk used (kg)	pH at 22^{0}C	^{0}SH /50 ml	Average weight (g)	Standard deviation weight (g)	Boiler yield
CALDAIA 5	88+578	B0	36	6.6	3.2	681.763	24.810	11.4%
CALDAIA 6	313+437	B50	37	6.12	5.3	752.543	51.099	12.2%
CALDAIA 7	988+632	B100	36.9	6.5	3.8	780.960	14.517	12.7%
CALDAIA 8	437+632	B0	36	6.74	3.5	704.293	38.740	13.7%
CALDAIA 9	88+988	B50	34	6.8	2.8	612.680	12.171	10.8%
CALDAIA 10	313+578	B100	36	6.69	3.3	542.957	17.673	10.6%
CALDAIA 11	313+988	B0	36	6.67	3.6	634.513	25.282	10.6%
CALDAIA 12	578+632	B50	30	6.62	3.5	621.488	14.983	12.4%
CALDAIA 13	88+437	B100	24	6.68	3.0	856.410	21.420	10.7%

Individual cow's milk collected from the milking of the evening d 16 and the morning d 17 was stored at 4°C until the cheesemaking of the 17th day. Milk from cows in the same treatment group was pooled in equal amounts to reach the volume required for cheesemaking (34±4 L). Milk acidity traits averaged 6.6 (pH at 22°C) and 3.6 Soxhlet-Henkel degrees (^{0}SH)/50 mL (TA). Milk was heated to 39°C and a starter culture (TB1/A-D, Bioagro srl, Thiene, Italy) was inoculated at concentration 0.03 g/L. To be noted, this starter culture contains a mixture of strains of streptococci and thermophilic lactobacilli; thus, they were predicted to be presented in the results.

It needs to wait 40 min as an adaptation period. Then, calf rennet (MRI 8/2, Bellin Pietro, Vicenza, Italy) was added and sit quiet for 45 min. After the end of coagulation, curd was cut into walnut size and cooked at 42°C for 15 min. The curd was extracted and

collected in Caciotta cheese molds until the pH reached to around 5.2. After the addition of salt, cheese was stored at 13.5°C, 80% relative humidity for 21 days.

In the sampling process, cheese samples were obtained from the core of the cheese in three points as described by Figure 3.3. The samples were then crumbled and mixed well. 150mg cheese was weighed and transferred to each sterile tube, and mixed with 1 ml of TRIZOL Reagent. Cells were disrupted with Zirconia Silica beads (0.25mm) on a TissueLyser (Qiagen, Hilden, Germany) in frequency of 30 Hz (1/s) x 1 min. Final pre-treated cheese samples were stored at -80 °C to be used in further RNA extraction for analysis of the active cheese microbial community.

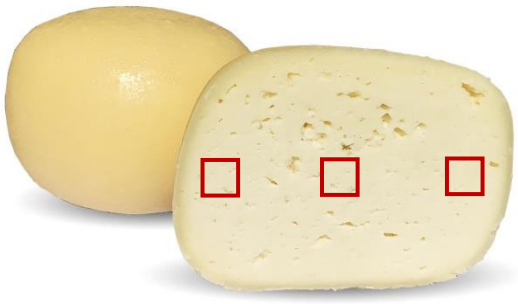

Figure 3.3. Cheese sampling regions

3.3. RNA extraction and cDNA synthesis

Each pre-treated milk sample was added 200 µL chloroform and vortexed for 15 sec, left on ice for 10 min and centrifuged at 12000g for 15 min (at 4°C). The extracted RNA was purified using the RNeasy Minikit (Qiagen, Hilden, Germany) with an on-column DNase digest step, following the instruction of manufacturer. Following centrifugation, the solution was separated into three phases, RNA remains only in the upper aqueous phase. The 450 µL of the upper aqueous phase was transferred to a fresh tube and mixed with 350 µL of 100% ethanol. This mixture (800 µL) was added to the column provided in the RNeasy Minikit (Qiagen) and centrifugated at 14000rpm for 30 sec.

For RNA extraction of cheese samples, the first step were required is homogenization with Zirconia Silica beads (0.25mm) on a TissueLyser (Qiagen, Hilden, Germany) (30 frequency 1/s, 1 min each), to facilitate stable emulsion consists of a uniform mixture. This step is only required for cheeses; for milk, we proceed directly to the RNA extraction. The further steps were the same as those described in the previous section on milk samples.

One of the most important factors for good RNA extraction and handling is maintaining an RNase-free working environment such as work in a separate and aseptic area, use dedicated RNase-free equipment and reagents, wear gloves whenever handling RNA and reagents, as well as keep all tubes tightly closed when not in use. Also, RNA should be extracted as quickly as possible after obtaining samples, and always keep the sample tube on ice during experiments.

The extracted RNA was evaluated using a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The Nanodrop is an instrument that accurately measures the RNA concentration (ng/µL) contained in the eluate while using only 1.3 µL of material. In addition, the output of absorbance ratios 260/280 and 260/230, which refer to the presence of proteins and solvent residues, respectively, is used to assess RNA purity. The closer the absorbance ratios are to the value 2, the purer the RNA.

The SuperScript IV Reverse Transcriptase cDNA Synthesis protocol (ThermoFisher Scientific) was used to conversing purified RNA into single-stranded cDNA by reverse transcription.

3.4. Library preparation for 16S microbial communities

For the construction of the libraries, a two-step PCR amplification are provided, each followed by an electrophoretic run in 1.5% agarose gel necessary to check the amplification and a purification propedeutic for the following step. An illustrative example of this method is shown in Figure 3.4. [33]

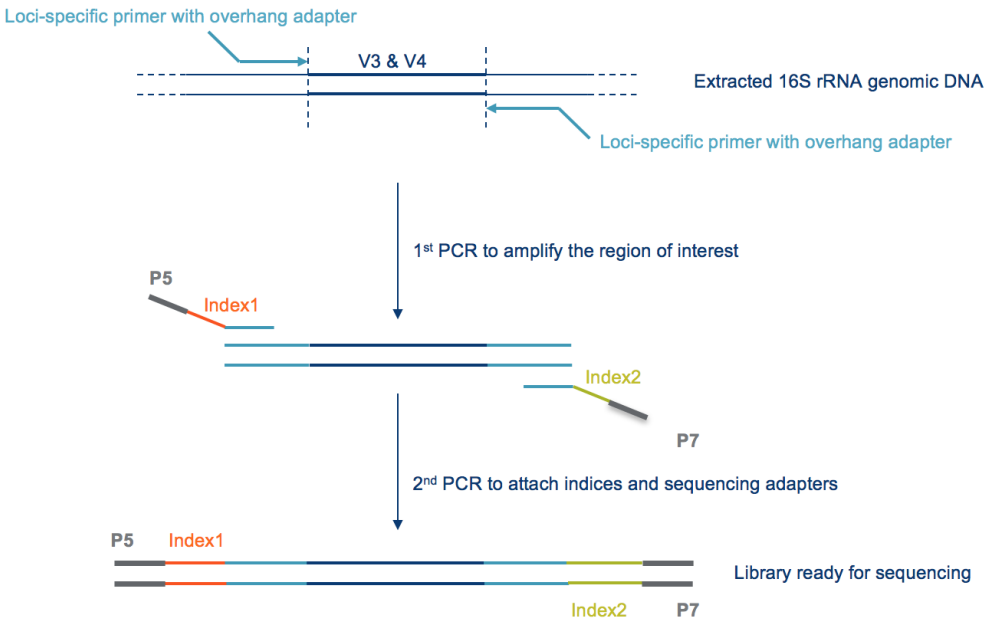

Figure 3.4. Library preparation using a two-step PCR amplification protocol

3.4.1. First PCR step

The first PCR amplification involves the pair of tailed primers Pro341F/ Pro805R which contains a part of the sequence capable of specific binding for V3–V4 hypervariable region of *Prokaryote* 16S rRNA gene, and a part that acts as an overhang adapter for the binding of primers that will be used in the second PCR step. Table 3.3 shows the sequences of the primers were used. [97] The primers were selected to amplify simultaneously *Bacteria* and *Archaea* genomes, followed by sequencing on an Illumina MiSeq platform.

Table 3.3. Primers used for the first PCR step

16S_PCR_Forward (Pro341F)	5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG **CCT ACG GGN BGC ASC AG**-3′
16S_PCR_Reverse (Pro805R)	5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G**GA CTA CNV GGG TAT CTA ATC C**-3′

Table 3.4 below shows the quantity of the individual reagents used in the first PCR reaction mixture. Sample amplification occurred in triplicate to increase the likelihood that even less represented sequences will be amplified. On the other hand, to reduce errors associated with taking incorrect amounts of Taq polymerase, a single mix is prepared for all samples. 20 µL of cDNA templates were added to the mix, at the most suitable dilution. The cDNA templates were diluted with Rnase-free water to obtain a final concentration of 50 ng cDNA/µL.

Table 3.4. Reaction mixture for the first PCR step

Reagents	Volume 1X (µL)
HF Buffer 5X	12
dNTPs 25 mM	0.6
Primer 16S_PCR_Forward 10 µM	1.5
Primer 16S_PCR_Reverse 10 µM	1.5
Tag Phusion	1.5
H_20 BDH (Rnase-free water)	22.9
cDNA template	20
Total	60

Specifications of PCR thermocycler comprised an initial denaturation at 94°C for 3 min, followed by 35 cycles of annealing at 94°C for 45 sec, 57°C for 60 sec, and 72°C for 90 sec and a final extension at 72°C for 10 min.

To verify the accurate amplification of the 16S rRNA, after PCR step, the triplicates were pooled in a single pool, from which a volume of 5 µL was taken for the 1.5 % agarose gel electrophoresis. The gel obtained with 150 ml of agarose solution 1.5% (2.25 g) and 15µl of cyber safe to verify the amplification. The 100 bp marker has been loaded in 1.3 µl. Samples were loaded using 5 µl of amplicon DNA (18-01-2020) and 5 µl of Loading dye 2X. The run was performed at 120V for 32 min. In our study, we wanted to find a band that was somewhat smaller than 500 bp, therefore the 100 bp molecular weight marker was chosen.

Once the presence of the bands and the correct size have been verified, it is necessary to proceed with the purification step to select the amplicons of about 500 bp and to eliminate excess dNTPs, primer dimers, and the Taq polymerase. Purification was performed with the SPRIselect reagent Kit (Beckman Coulter Genomics), which is magnetic beads-based purification. The protocol provides for the withdrawal of all the amplified available, and therefore in our case of 55 µL, to which the magnetic beads at a concentration of 0.8X are added. Using the pipette to mix up and down in order to facilitate the binding of the amplicons with the beads and, to further increase the binding efficiency, then vortexing and incubating the suspension for 3 minutes at room temperature. The mixture was then placed on a magnetic station, at which the magnet will attract and settle to the SPRI beads. The amplicons were linked to the beads, while the transparent supernatant containing dNTP's, primer dimers and Taq polymerase was subsequently taken and eliminated. The beads were then washed with 180 µL of 85% ethanol at room temperature for 30 seconds. The elution of the amplicon from the beads occurs with 52.5 µL of BDH water, which has a greater affinity with DNA than the beads. After placing the water in the well, it is necessary to mix well, holding the plate out of the magnet, and making sure that the suspension is well resuspended; at this point the plate can be transferred again into the magnet which will attract the beads allowing us to transfer 48 µL of supernatant, composed only of BDH water and amplicons, into a new plate.

3.4.2. Second PCR step

In the second PCR step, two pairs of primers were used, one of which is characterized by seven nucleotides that differ for each sample and is called the Barcoding primer (Primer BC). Thanks to the different sequence, the BC primer allows us to uniquely identify the sample even after running the Pool, or having gathered libraries of multiple samples in a single tube. The two primer pairs shown in table 3.5 are bound to the amplicon obtained from the first amplification step.

Table 3.5. Primers used for the second PCR step

2bRAD_ampl_F	5′-AAT GAT ACG GCG ACC ACC GA-3′
2bRAD_ampl_R	5′-CAA GCA GAA GAC GGC ATA CGA-3′
2bRAD_BC	CAAGCAGAAGACGGCATACGAGATcatcttGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
2bRAD_for	AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC T

Table 3.6 below shows the reaction mix for the second PCR step. The 50 µL obtained from the mix were separated into three different wells and placed in the thermal cycler where the amplification took place following the thermal protocol comprised an initial denaturation at 98°C for 3 min, followed by 10 cycles of annealing at 98°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec and a final extension at 72°C for 7 min.

Table 3.6. Reaction mixture for the second PCR step

Reagents	**Volume 1X (µL)**
HF Buffer 5X	10
dNTPs 25 mM	0.625
Primer 2bRAD_ampl_F 10 µM	1
Primer 2bRAD_ampl_R 10 µM	1
Primer 2bRAD_BC 10 µM	2.5
Primer 2bRAD_for 10 µM	2.5
Tag Phusion	1
H_20 BDH (Rnase-free water)	26.375
cDNA template	5
Total	50

After amplification, the triplicates of each sample were combined in a single pool from which 5 µL were taken to perform the amplification control in 1.5% agarose gel electrophoresis. With the second amplification the expected size of the amplicons increased to approximately 620 bp as the added primers have a very long sequence.

After verifying the presence of a single band at the correct height, we proceeded with the second purification with the SPRIselect beads method. The protocol is similar to that described for the first purification but in this case the volume of BDH water with which eluting the DNA from the beads is reduced to 27.5 µL and a volume of 25 µL is taken from the well of the plate to transfer it to a new one.

After purification, an electrophoretic run is performed in 1.5% agarose gel to verify the successful elimination of the primer dimers and reagents necessary for the execution of the PCR reaction, and to verify the presence of the band at the expected size.

3.4.3. Library quantification by fluorimetric method and obtaining the pool

We used the Qubit fluorometer (Invitrogen, Life technologies) to quantify the nucleic acids and proteins contained in a sample by exploiting the use of probes capable of binding specific target molecules. When the probes are linked to their respective molecules, they emit fluorescence with an intensity that is a few orders of magnitude higher than the intensity they emit when the bond does not occur. Unlike the Nanodrop, using this method it is possible to quantify only the molecule of our interest, DNA in this case, and not all those molecules capable of absorbing at 260 nm. Another advantage deriving from the use of Qubit is that of being able to quantify only intact DNA and not degraded DNA or single dNTP's since the fluorophore intercalates only at the double helix. It is therefore deduced that the quantitation of Qubit is much more sensitive and specific even at low concentrations of DNA. The reagents used for carrying out the assay are contained in the Qubit Assay Kit BR (Invitrogen, Life technologies) but, having a large number of samples to be analyzed the fluorescence measurement was carried out in the plate using the instrument for real time (Stratagene). Performing the plate measurement using the tool for real time allows us to use a lower sample volume than what would be needed if the measurement were to take place with the Qubit fluorometer.

The kit contains the fluorescent dyes fluorophore, with which to quantify the DNA by measuring the fluorescence, and two standards (STD 100 ng/µL - STD 0 ng/µL) with

which to build the calibration line to obtain the equation, necessary in order to calculate the concentration of the samples starting from the detected fluorescence data.

The quantification mix contained the fluorophore and the buffer in the ratio 1: 200. In the special real-time plate, 19 µL of mix and 1 µL of sample were placed in each well, shaken by vortexing for two minutes and then centrifuged at 3,000 rpm for two minutes, and care must be taken not to expose the fluorophore to light as it is very sensitive to degradation.

The quantification of the DNA contained in each sample is essential for obtaining an equimolar pool, or a pool in which each sample is equally represented. Equimolarity is fundamental in the sequencing phase as it prevents a sample present in greater quantities and therefore more likely to be sequenced than others. Therefore, by quantifying each sample we are able to calculate the volume to be taken from the well necessary to have an equal concentration among all the samples.

3.4.4. Qualitative and quantitative assessment of the Pool

For the qualitative evaluation of the pool, we used Agilent 2100 Bioanalyzer instrument which involves running the samples in a miniaturized Chip45 containing the gel. This method is particularly advantageous since it is quite sensitive and it requires a volume of only 1 µL of DNA. The chip (DNA 1000) has 16 wells, 12 of which are intended for the samples, connected together by glass microchannels containing the Gel-Dye which allows the separation of the sample on the basis of the molecular weight. The principle is similar to that of a classical electrophoretic run but the times of separation are significantly reduced, the sensitivity is much higher and the detection of the DNA fragments takes place through a laser-induced fluorescence that uses a fluorescent dye intercalating the DNA molecule. Figure 3.5 shows the DNA analysis Bioanalyzer chip.

Figure 3.5. DNA analysis Bioanalyzer chip

An amount of 9 µL of Gel-Dye Mix were placed in the well indicated with the letter G circled in black and, by means of the pressure generated by a syringe, pushed for 1 minute, the gel was distributed evenly within the microchannels. Subsequently, another 9 µL of Gel-Dye Mix were placed in the two wells where the letter G is indicated, in the well with the scale symbol, 1 µL of Ladder has also been added. The marker is an internal control that serves to delimit the run while the Ladder is the molecular weight marker that allows identifying the length of the fragments contained in the pool. In each of the 12 wells intended for samples was placed 1 µL of DNA. The Chip was first shaken by vortexing for 1 minute and then inserted into the Bioanalyzer instrument for the run and the relative identification of the fragments present in the sample.

Although the Bioanalyzer also possible to perform a quantification of the sample, by calculating the area under the peak corresponding to the intensity of fluorescence detected at the passage of the amplicon, it was decided to use the Bioanalyzer only to evaluate the quality, and to use the Qubit for quantification. The quantification of the pool is carried out in a single tube using the Qubit Assay Kit BR and not in a 96 plate, using the instrument for real-time, as in the case of the quantification of libraries. The mix consists of 199 µL of buffer and 1 µL of fluorophore, both provided by the Kit. Before quantification, the instrument must be calibrated by inserting standard 1, corresponding to 0 ng/µL, and then standard 2, corresponding to 100 ng/µL, in the appropriate space. After calibration, the pool can therefore be quantified using a solution containing 198 µL of the previously prepared mix and 2 µL of sample.

3.5. Sequencing and Bioinformatics Analysis

3.5.1. Sequencing

The library pool in equimolar ratios (also called balancing) was then shipped to UC Davis Genome Center (California) for sequencing and demultiplexing. The center itself performed the sequencing using the Illumina MiSeq platform with a paired-end 300-cycle run, producing forward and reverse reads of length equal to 300 bp and partly overlapping. To demultiplex sequences we need to know which barcode sequence corresponds to each sample. Using the CASAVA v1.8 (Illumina) instrument, the Illumina MiSeq sequences (2 × 300 bp) were demultiplexed, which means that the reads were separated based on the assigned barcode in order to distinguish the different samples. After demultiplexing, you can figure out how many sequences were obtained per sample and get a summary of the sequence quality distribution at each point in the sequence data.

3.5.2. FASTQC

Output provided by the sequencing center is in FASTQ format, a text format where each nucleotide of the sequence is associated with the quality parameter (Phred Quality Score). The quality parameter expresses, through a numerical value, the accuracy of attribution of each nucleotide, the higher the associated value, the more accuracy (see table 3.7).

Table 3.7. Phred Quality Score

Phred Quality Score	Error	Accuracy (1 - Error)
10	1 in 10 = 10%	90%
20	1 in 100 = 1%	99%
30	1 in 1000 = 0.1%	99.9%
40	1 in 10000 = 0.01%	99.99%
50	1 in 100000 = 0.001%	99.999%
60	1 in 1000000 = 0.0001%	99.9999%

The quality of the raw sequence data received from the UC Davis center was subsequently evaluated using the FastQC (Babraham Bioinformatics) software (version 0.11.7; available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), which provides the Phred Quality Scores Chart for each nucleotide of the reads using a graphic output that divides the plot into three color areas different according to the quality scores.

The processing of the sequences was performed using multiple computer software and the various steps are outlined in Figure 3.6.

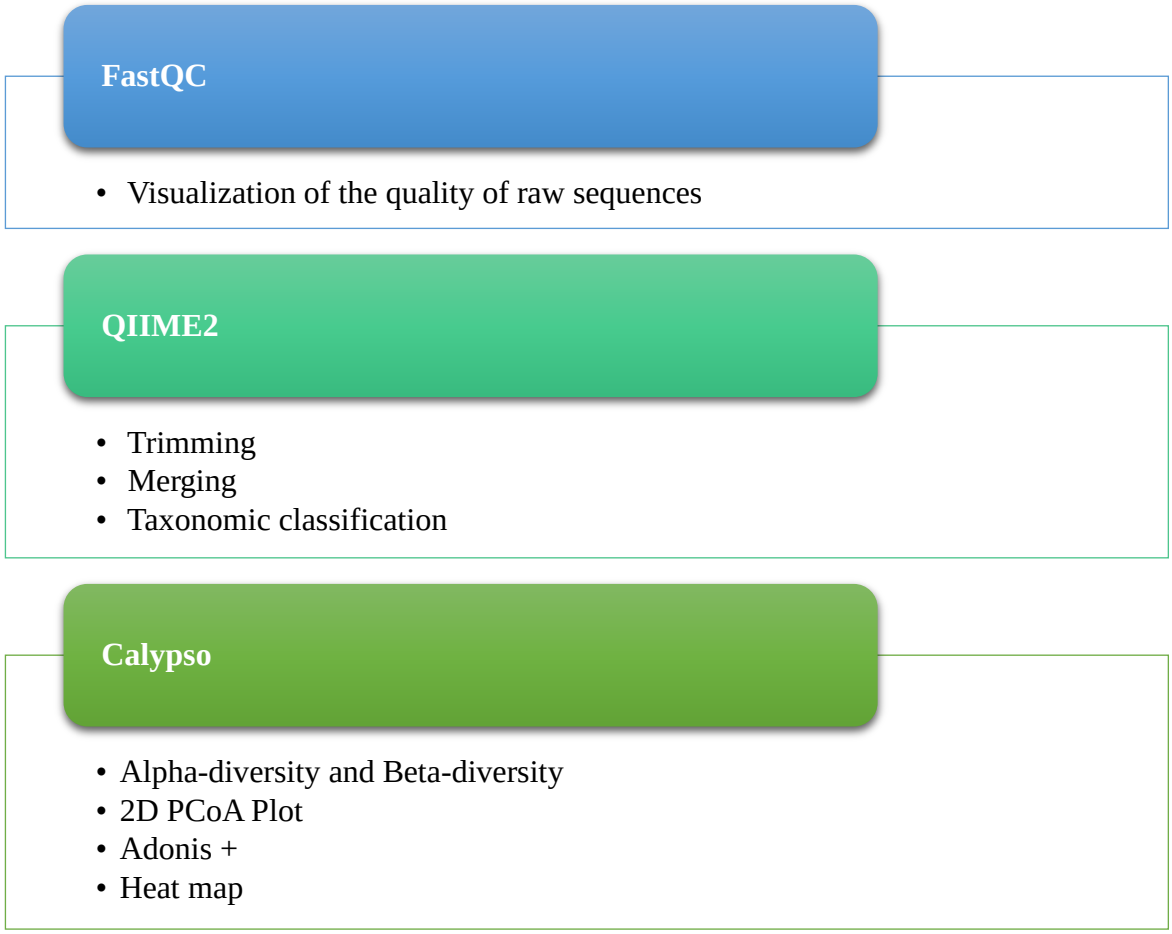

Figure 3.6. Workflow of the bioinformatics analysis performed

3.5.3. QIIME2

The bioinformatic analysis was carried out using Quantitative Insights Into Microbial Ecology version 2 (QIIME2 2021.2) software. This is a bioinformatics pipeline that starts with raw DNA sequences obtained by Illumina or other platforms and works its way to microbiome analysis. [14] A GitHub repository can be found at: https://github.com/beiko-lab/mimb_16S which provides us the scripts for downloading a sample dataset. The software asks for sequence data in FASTQ format that has been named using the Illumina naming convention. [44]

Input/output files in QIIME2 are divided into two categories: QIIME artifacts (.qza) and QIIME visualizations (.qzv). Using the "SampleData [PairedEndSequencesWithQuality]" semantic type, we can import the demultiplexed raw sequence data into the QIIME2 artifact. The residual artificial sequences (barcodes, primers) were then removed from the sequencing data by implementing cutadapt 2021.2.0 in QIIME2 artifact with using q2-cutadapt plugin and trim-paired command with the primer of the first step PCR (forward (17 bp and reverse (21 bp) primes). [70] The

trimmed sequence data files were quality filtered by cutting bases with an average quality score below 20.

Following, denoised paired-end sequences (F280 and R220) were merged by DADA2 pipeline. Non-overlapping regions, chimeric sequences, and singletons were discarded, and FeatureTable[Frequency] and FeatureData[Sequence] QIIME2 artifacts were generated using q2-dada2 plugin which implements DADA2 pipeline in QIIME2 [17].

For taxonomic classification, first, we downloaded the Naive Bayes classifier artifact that was trained on SILVA 16S rRNA reference database (silva_132_99) (available at: https://www.arb-silva.de/) and clustered at 99% sequence similarity. [13] This progress was carried out by using q2-feature-classifier plugin in QIIME2 and the Naive Bayes sklearn-based taxonomy classifier with a default confidence of 0.7.

For more detail, you can see table 3.8 below, which illustrates some useful commands that were used in bioinformatic analysis with QIIME2.

Table 3.8. QIIME2 commands

The commands were used	Functionality
qiime tools import --type 'SampleData[PairedEndSequencesWithQuality]' --input-path sequence_data –input-format CasavaOneEightSingleLanePerSampleDirFmt --output-path reads.qza	Import Data
qiime demux summarize --p-n 10000 --i-data reads.qza --o-visualization qual_viz.qzv	Visualize Sequence Quality
qiime cutadapt trim-paired --i-demultiplexed-sequences reads.qza --p-front-f CCTACGGGNBGCASCAG --p-front-r GACTACNVGGGTATCTAATCC --p-error-rate 0 --o-trimmed-sequences trimmed-seq.qza --verbose	Trimming Primers With Cutadapt
qiime dada2 denoise-paired --i-demultiplexed-seqs trimmed-seq.qza --o-table table.qza --o-representative-sequences representative_sequences.qza --p-trim-left-f 0 --p-trim-left-r 0 --p-trunc-len-f 280 --p-trunc-len-r 220 --o-denoising-stats stats-dada2.qza --verbose	Merging Denoise Sequences With DADA2
qiime feature-classifier classify-sklearn --i-classifier silva-132-99-nb-classifier.qza --i-reads representative_sequences.qza --o-classification taxonomy.qza	Taxonomic Classification

3.6. Statistical Analysis with Calypso software

Two QIIME 2 output files were exported, namely FeatureTable[Frequency] as feature-table.biom and FeatureData[Taxonomy] were imported to Calypso version 8.84 software for statistical analyses purpose. The Calypso software is online, easy for non-expert users to mine, interpret, and compare the taxonomic classification of 16S rRNA genes in metagenomic datasets. [116]

For the 16S rRNA amplicon sequencing dataset, Chloroplast and/or Cyanobacteria were eliminated by using data filtering in Calypso. The OTU abundance data were normalized via the total sum normalization (TSS) method combined with square root data transformation (Hellinger transformation) before doing the calculation of Bray-Curtis dissimilarity.

Adonis is a multivariate method designed to achieve the same goals as multivariate analysis of variance (MANOVA) and based on distance matrices permutations. Adonis can assist us figure out if differences in community composition are due to experimental treatments or control variables. Adonis+ determines if the different explanatory variables can explain the variation in community composition. We determined the p-value using the Adonis+ Multivariate Analysis, which calculates the likelihood of detecting a difference between groups, and the p-value is supposed to imply statistical significance if it less than 0.05. [96]

Rarefaction analysis can be used to determine how well metagenomic sequence reads cover the microbial communities. Microbial sequences are picked at random from each sample in a rarefaction analysis. The number of observed species is enumerated and shown as a function of the number of sampled sequences for each subsample. If the underlying microbial community is well represented by the sequencing data, the slope of the rarefaction curve reflects that. According to rarefaction curve, the richness was visualized and can expressed the alpha-diversity, which is the index that explains the variability of the microorganisms within the individual samples grouped according to precise categorical variables. [96] A steep slope suggests that a significant portion of the species diversity is still unknown. If the curve flattens out to the right, a sufficient number of sequence reads has been gathered, and additional sampling will likely reveal only a few additional species.

In Calypso software, we can also evaluate alpha-diversity among amplicon sequence datasets based on the construction of HeatMap. The HeatMap allow us to identify the most represented taxa within the dataset and output a graphic representation that uses a color scale to indicate the abundance of individual taxa within the samples. [96]

To evaluate the microbial diversity between the samples, beta-diversity is calculated by producing the 2D PCoA Plot (Principal Coordinates Analysis) which uses orthogonal axes to map the samples; the first axis represents the greatest amount of variability, the second contains most of the remaining variability and so on depending on the number of axes that you want to use. The metric used to obtain the PCoA Plot is the Bray-Curtis which performs a qualitative analysis, considering the phylogenetic information, regardless of the different abundances of OTUs, but only the presence or absence of taxa. [96]

4. RESULTS AND DISCUSSION

4.1. Results

4.1.1. Milk sampling and cheese-making

An aliquot of 50 ml of healthy cow milk was obtained on d 17 in the morning between 6:00 and 7:00 under sterile conditions to make sure that the milk samples had a good quality, limited as much as possible the contamination of exogenous bacteria. The cheese-making was using the raw cow's milk collected from the milking of the evening d 16 and the morning d 17, and then made the cheese on the d 17, with the added starter culture contains strains of streptococci and thermophilic lactobacilli. In general, the quality of the cheese samples was good. The milk and cheese samples were pre-treated as mentioned in section 3.2 of the materials and method chapter, and were stored at -80 °C to be used in further RNA extraction.

4.1.2. RNA extraction and cDNA synthesis

The RNA was extracted from two different matrices, milk and cheese; however, having a fairly similar extraction protocol. Except for the homogenization with Zirconia Silica beads steps prior to RNA extraction, is only required for cheeses. In total, 54 samples were extracted: 36 extracted RNA relating to the testingof active milk microbiota and 18 extracted RNA relating to the testing of active cheese microbiota, of which libraries were built for a single sequencing run within the Miseq Illumina® 2x300 platform. At the end of each RNA extraction session, the quality and quantity of the extracted RNA was evaluated with the Nanodrop ND-1000 according to the protocol was described in the section 3.3 of materials and method chapter.

The two different matrices used do not showed significantly different in term of extraction efficiencies. For the RNA extraction qualitative, both nanodrop output of milk and cheese matrices reports the absorbance ratios 260/280 are quite close to the optimal value 2, while the 260/230 ratios are lower. The absorbance ratios 260/280 and 260/230 are very variable among different samples. It is a normal condition when you extract DNA or RNA from complex matrices. Concerning the quantification of the extracted RNA you usually do not infer from biological explanatory variables. This means that you aspect a range of concentration.

You consider that for the following amplifications performing different tests to understand what dilution of DNA gives you the more suitable outcome. The quality of the extracted RNA and obtained DNA was tested on PCR real-time, using one specific primer for bacteria (Nadkarni) and one specific primer for bovine DNA. The results highlighted a good amplification of the bacterial DNA. Moreover, comparing the output of the primer for bovine DNA and bacteria DNA it was possible to understand that bacterial DNA was advantaged in amplification when the dilution increased.

After reversed-transcription, the cDNA templates were diluted with RNase-free water to obtain a final concentration of 50 ng cDNA/µL.

4.1.3. Library preparation for 16S microbial communities

Despite the low efficiency of RNA extraction from milk and cheese matrices, following the first PCR amplification required for the construction of the sequencing library, all samples show the band at the expected height in 1.5% agarose gel. The bands in milk samples are not clearly visible, while all cheese samples show the clearer bands.

Figure 4.1 shows the photo of a gel obtained following the first amplification step, and it is noted that despite the variability of the intensity of the band, it is present in all the samples and absent in the three sterility controls.

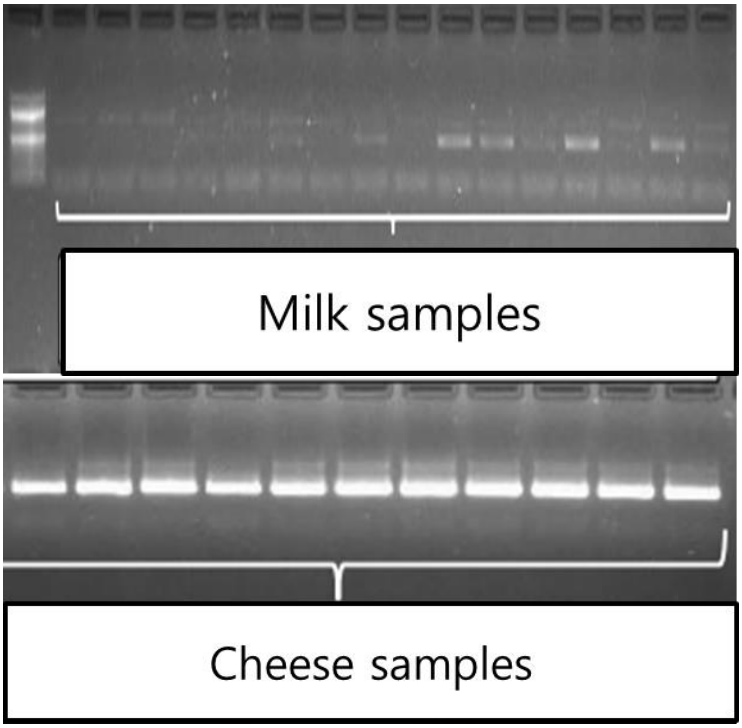

Figure 4.1. First PCR step quality was checked by 1.5 % agarose gel electrophoresis

Continuing with the purification steps and with the second PCR amplification in no case is the presence of nonspecifics observed in the gel, and by comparison with the molecular weight marker, it was possible to check that the fragment increased in size thanks to the bond with the long primers used in the library building process. (Firgure 4.2)

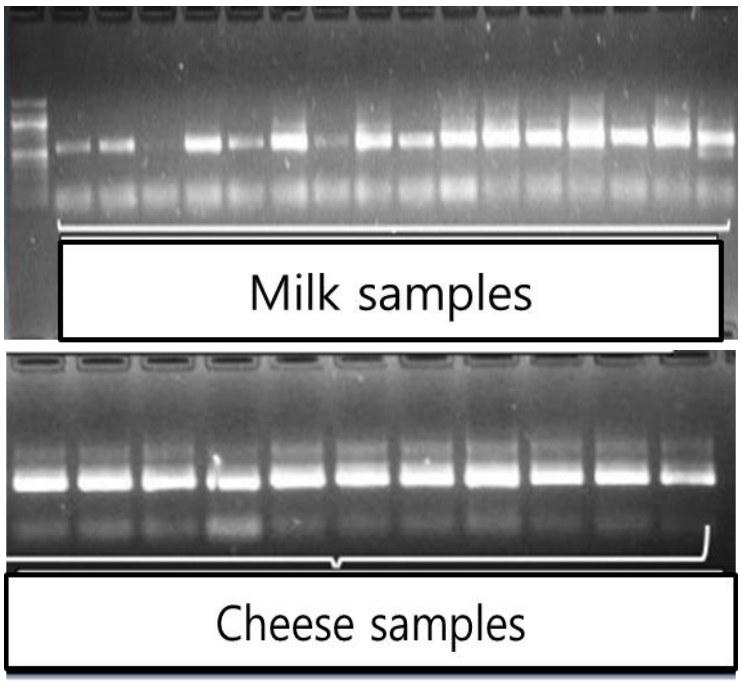

Figure 4.2. Second PCR step quality was checked by 1.5 % agarose gel electrophoresis

After the construction of the libraries, each of them was quantified with a fluorimetric method to obtain an equimolar pool that could be transformed to the sequencing center.

After obtaining the library pool for sequencing, an internal quality control was performed using Bioanalyzer, in order to identify the presence of any non-specific primer dimers and verifying that the fragments present inside were of the expected size.

The output obtained from the Bioanalyzer is shown in Figure 4.3. It identifies the first and last peaks corresponding to the marker, loaded in each well, while the central peak corresponding to the fragments of the libraries contained in the pool. The peaks of the marker represent internal control, which is necessary for the operator to verify that the assay was run properly and there were no microbubbles inside the microchannels of the chip.

The peak corresponding to 596 bp represents the fragments of the amplified V3 and V4 regions of the 16S gene after the two-step PCR amplification. In the Figure 4.3 there is no further peaks are observed that means there is no non-specific DNA molecules, so we can confirm that the primers are highly specific and the purification steps have occurred correctly by selecting only the fragments of interest.

Another characteristic can demonstrate the good quality of the pool is the narrow shape of the peak, which denotes that the fragments present have a very similar length to each other, although not the same since each bacterial species will have a different number of nucleotides.

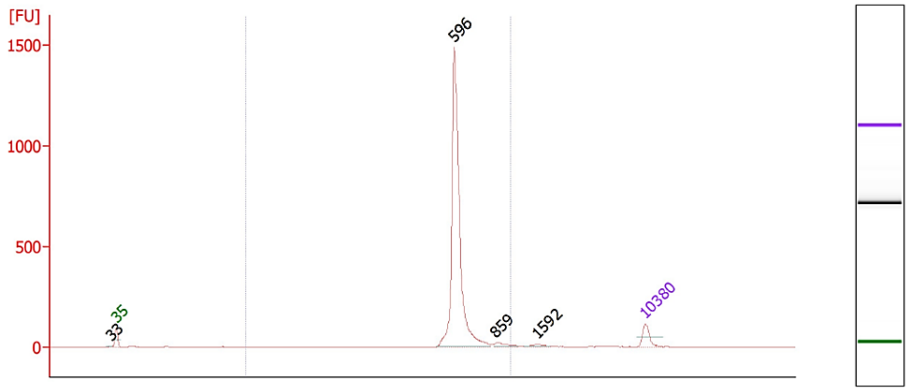

Figure 4.3. Bionalyzer of pool

4.1.4. Sequencing Quality Assessment

The quality of the raw sequences obtained from the sequencing center (sequencing with the Illumina MiSeq 2x300 platform) was evaluated with the FASTQC software which returned the graphic output shown in Figure 4.4. The nucleotide positions of the generated reads are indicated in the horizontal axis; the Phred quality score is shown by the vertical axis. By using this graphic representation, it is possible to define the quality of the sequences for each nucleotide position.

As we can see from Figure 4.4, the terminal nucleotides always have a lower quality than the other positions and the quality of the Reverse sequence (Figure 4.4-B) always tends to decrease some positions earlier than the Forward sequence (Figure 4.4-A).

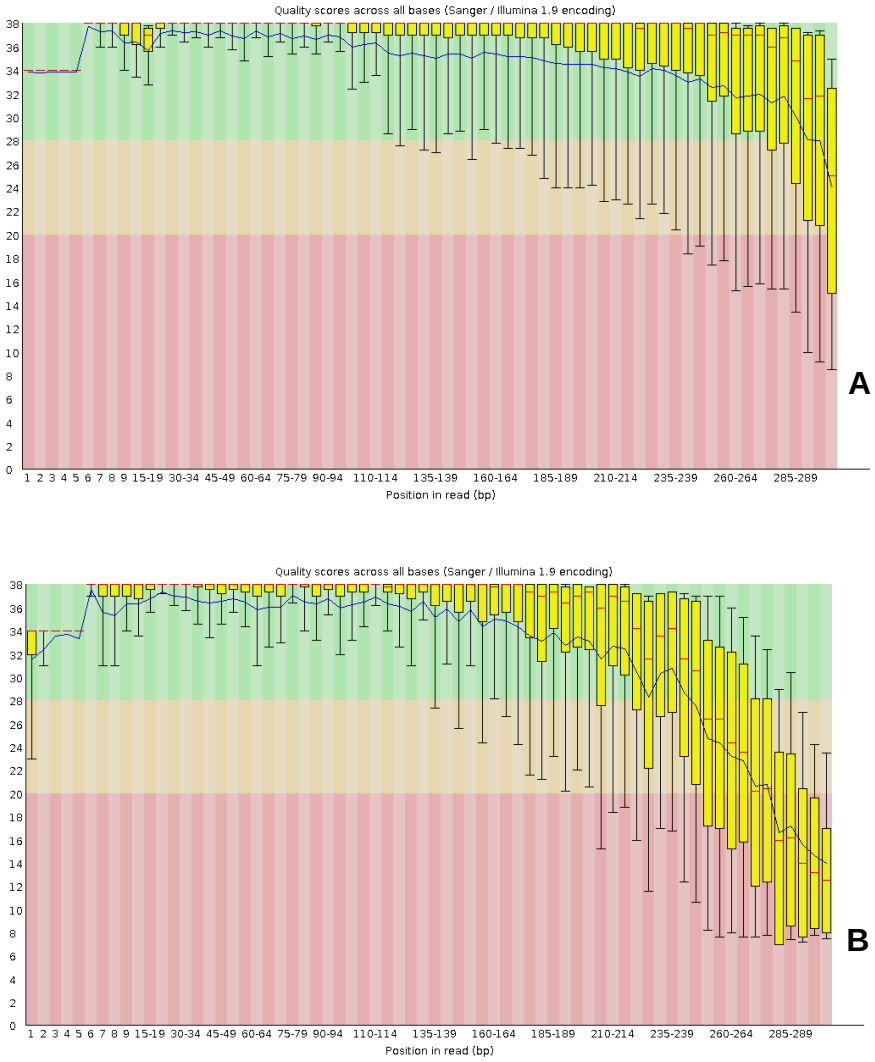

Figure 4.4. Graphical display with FastQC software of the quality of the raw sequences A: Forward, B: Reverse

Clearly, the quality of the raw sequences obtained is good since in almost all the nucleotide positions there was at least one sequence that presented a Phred quality score higher than Q20. The raw sequences were then trimmed and merged with QIIME2, and were normalized and filtered with Calyso.

Sequencing of the 54 samples analyzed in the Illumina MiSeq 2x300 platform generated a total of 4,190,103 raw sequences; after filtering and normalization with Calypso, we obtained a total of 2,252,403 reads representing 12,369 unique features.

4.1.5. Evaluation of the effect of Bentonites additives on bacterial community of milk and cheese

The addition of Bentonites mycotoxin binders into feeds of Italian Friesian cows was found to have no effect on the diversity of active milk and cheese bacterial communities. The p-value of 2.97e-1 was obtained by doing Adonis+ Multivariate Analysis, showing non-significant ($p > 0.05$) and hence suggesting that the diversity of active microbiota in milk and cheese was unaffected by different amounts of Bentonites feed additives.

On the other hand, the variance in microbial composition could be explained by the matrix-based explanatory variables (i.e. milk and cheese) since the p-value of 3.3e-05 shows a statistically significant ($p < 0.05$); thus, the diversity of active microbiota in milk and cheese of cows fed with Bentonites additives was still clear and unaffected. In addition, within three treatment periods, the diversity of active microbiota also indicates significance ($p < 0.05$), indicating that the dose and the time of use of Bentonites did not affect the microbial community.

Table 4.1. p-value obtained by a means of Adonis+ Multivariate Analysis

Explanatory variables	p-value
Bentonites (B0, B50, B100)	0.297
Matrix (milk,cheese)	0.000333
Period (T1, T2, T3)	0.00367

Figure 4.5 shows the rarefaction curve that describes the alpha-diversity index (richness), which is explains the variability of the microorganisms within the individual samples grouped according to categorical variables. The index was calculated according to the matrix-based categorical variables (4.5-A) and the treatment-based categorical variables (4.5-B).

In both cases, the slope of the rarefaction curve obtained tends to flattered to the right, which indicates that a reasonable number of sequence reads has been obtained and more intensive sampling is likely to yield only a few additional species. Therefore, the information contained in the dataset is sufficient to describe the microbial diversity.

Clearly, the bacterial species richness associated with the milk groups is much higher than that of the cheese groups.

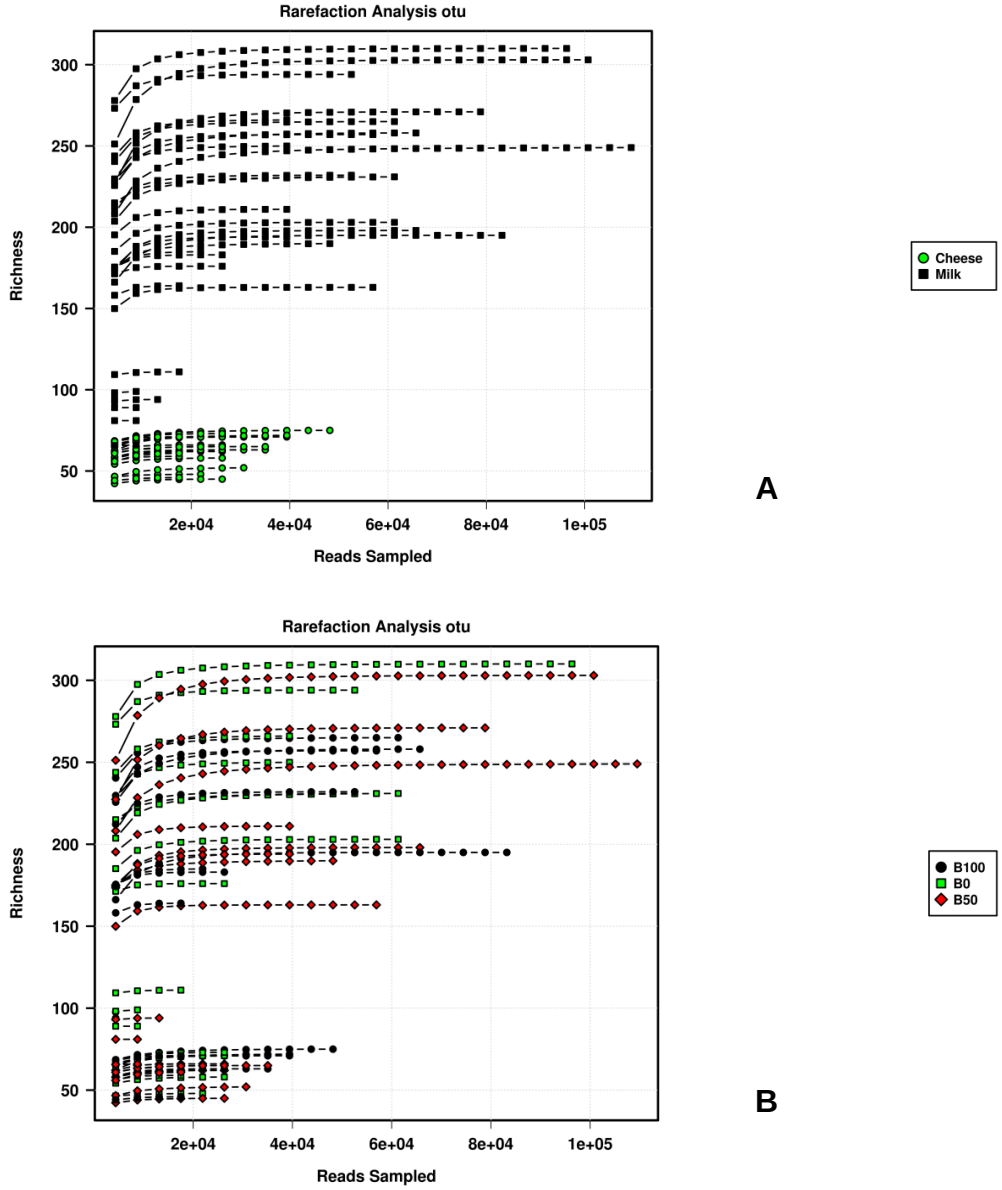

Figure 4.5. Rarefaction curves of alpha diversity index (richness); A: variable matrices, B: variable Bentonite treatments

The groups of samples relating to the two different types of matrices are significantly spaced out (Figure 4.5-A), indicating significant separated clusters between active milk microbiota and active cheese microbiota. From figure 4.5-B it can be seen that the groups relating to Bentonite treated and un-treated do not distance themselves but are superimposed on each other. And so this confirms that the Bentonite feed additives do not affect the diversity of microbial community in milk and cheese.

By the means of 2D principal coordinate analysis (PCoA) plots, beta-diversity can be assessed to establish how microbial diversity varies between samples. Figure 4.6 represents the PCoA carried out with the Bray-Curtis dissimilarity which takes into account the phylogenetic distance between the OTUs present in the samples but not their abundance. From figure 4.6 it can be seen that the groups relating to the three different Bentonite treatments (B0, B50, and B100) do not distance themselves but are superimposed on each other. On the other hand, the groups of samples relating to the two matrices (Milk and cheese) are clearly spaced out.

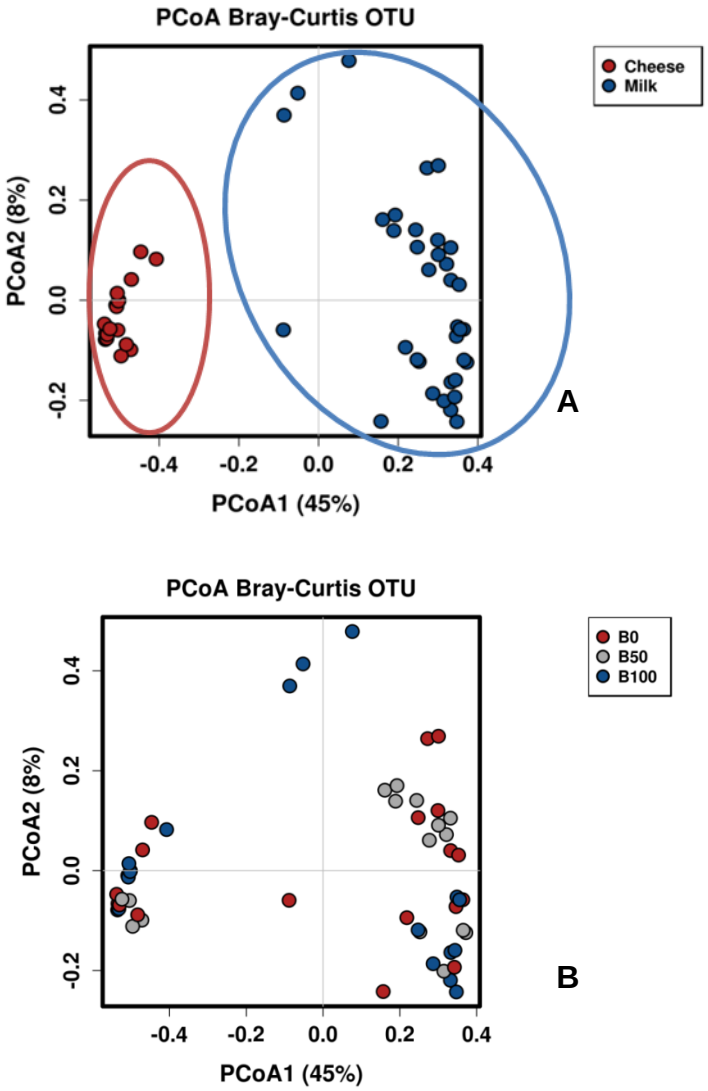

Figure 4.6. Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity A: variable matrices, B: variable Bentonite treatments

Figures 4.7 show the heatmap obtained with the dataset of the experimentation; the 20 most represented bacterial phyla and families are reported by means of graphic output. The heatmap uses a color scale to identify the abundance of each taxa within each sample, thus summarizing in an image the partial microbial diversity within the sample itself, and between the samples. Positioned on the horizontal (x) axis, the grouping is shown based on the compositional similarity between the samples; the grouping carried out according to the similarity of abundance between the taxa is shown on the vertical (y) axis.

At the phylum level, there is no separation shown in the heatmap between milk and cheese matrix; whereas at the family level, there is a transparent distinction between the heatmap of milk and cheese matrix. The bacterial families shown all samples belong mainly to the *Firmicutes, Poteobacteria, Bacteroidetes* and *Actinobacteria* phylum, confirming what is reported in the literature. [71][117]. There is a higher amount of taxa in milk samples in compare to cheese. In cheese, we obtained the *Steptococcus* that was introduced from the starter culture. Moreover, within each taxa, we can see both un-treated (B0) and treated (B50, B100) Bentonites groups includes; thus, it can confirm that Bentonites usage doses did not have an effect in active microbial community.

It can be clearly seen that the active microbiota in milk and cheese after cows were fed a feed supplemented with Bentonites did not change in terms of diversity and gave results corresponding to the active microbial community of healthy cows.

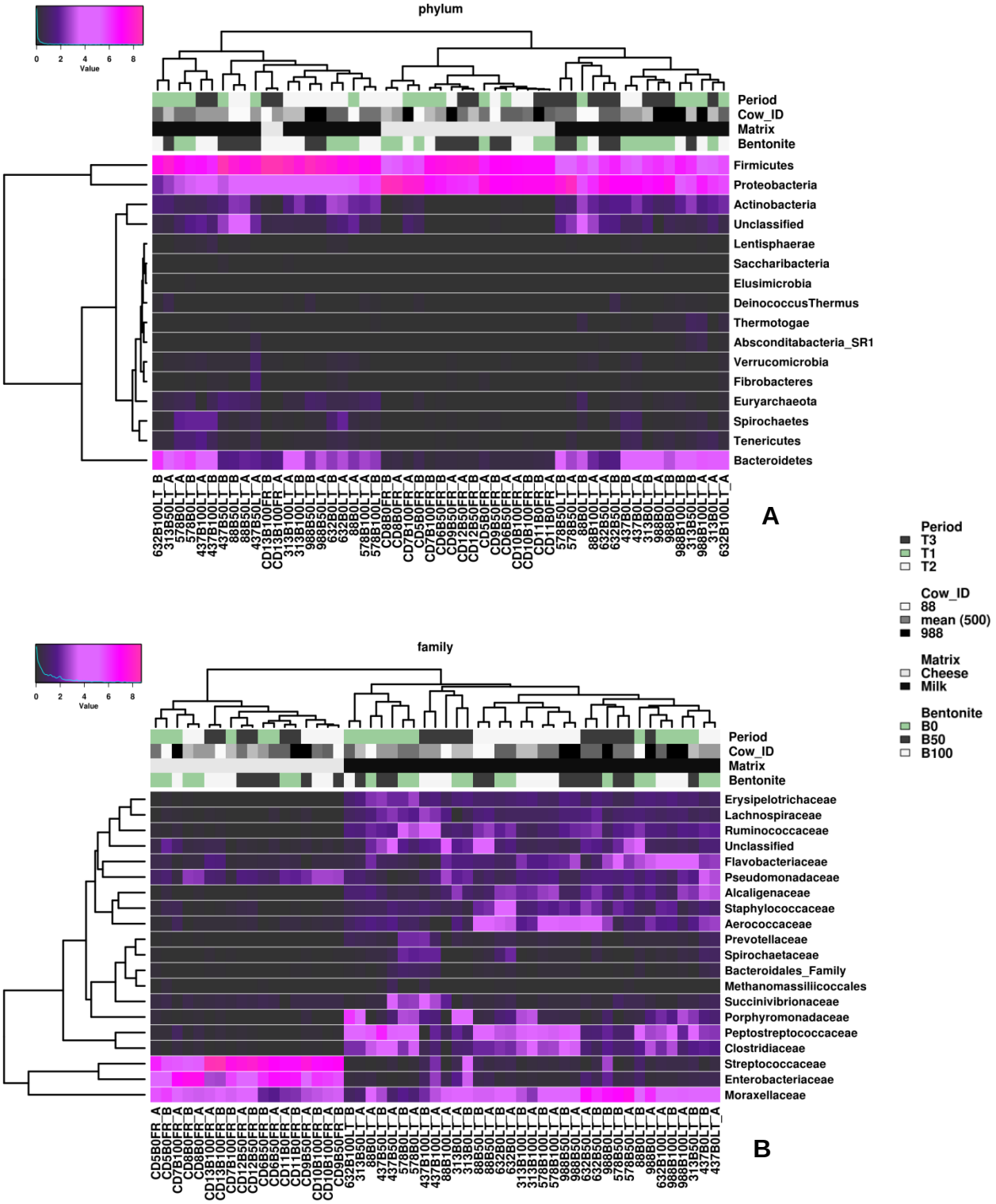

Figure 4.7. Heatmap of active milk and cheese bacterial communities of different Bentonites treatments; A: at phylum-level, B: at family-level

4.2. Discussion

Milk and cheese are nutritious foods that have positive effects on human health, such as preventing cardiovascular disease, maintaining weight; thus, they are widely consumed worldwide. [118] Microbial community in milk is believed to contribute significantly to the establishment of gut microbiota, thereby conferring beneficial effects on consumer health. At the same time, microorganisms also play an important role in the composition and flavor of dairy products, which will determine the choice of consumers. Indeed, effective quality management of dairy products requires knowledge of altered microbiota activity in response to external influences, such as changes in the cow's diet or added food additives. [89][100]

In addition, there is increasing concern about the harmful effects of mycotoxins on consumer health when mycotoxins are contaminated with the milk of cows fed toxin-contaminated feedstuff. In fact, dairy cows are often fed a compound cattle feed that is blended from various raw materials and additives, including grains, forage, silage, agro-industrial by-products, minerals, and vitamins. Each ingredient can be unintentionally contaminated with one or some kinds of mycotoxins. It is an important reason to increasing the risk of mycotoxicoses in cows. [40] Mycotoxins can affect directly to the health and performance of animals, which in turn leads to effects on economic growth and international commerce. Moreover, some mycotoxins can pass into the milk, such as aflatoxin M1 (AFM1); thus, it poses a major public health risk. Dairy products are an integral part of the diets of young children and the elderly, who are less resistant to this toxin, and hence they can be more susceptible to infections.

Since 2009, the European Commission has officially authorized the use of mycotoxin binders in animal feed, as a common and simple method for farmers and fodder manufacturers. [27] Mycotoxin binders are added to the cattle feed to prevent mycotoxins from being absorbed in the GI tract, as well as their move to the blood circulation and target organs, decreasing their bioavailability after ingestion, and thereby diminishing harmful effects of them in individuals. [61] The mechanism of the binders is forming mycotoxin-adsorbent complexes that are able to pass through the animal and is then excreted via the faeces. [107] However, there is a scarcity of research data regarding the impact of mycotoxin binders on animal products, such as cow milk. As a result, farmers and the feed industry must be cautious when supplementing these anti-mycotoxin mineral adsorbents, and further studies are needed in this area. [27] Our research generally

focuses on Bentonite that is a commonly used toxic binder today. Therefore, the obtained results will be a premise for studies on other types of anti-mycotoxin agents.

The introduction of independent culture methods, and in particular of NGS, has made it possible to significantly increase the ability to study complex microbial community. The methods commonly used in the past were not able to make a reliable evaluation of the active bacterial communities because: a culture-dependent approach is not able to identify the bacteria considered non-cultivable and the independent culture methods prior to NGS such as DGGE, T-RFLP, and Sequencing Sanger have low taxonomic resolution and inadequate sampling depth. The use of NGS techniques introduces a lower number of errors than cultivation techniques, thus allowing the definition of the relationships between the sample and environmental factors and a better description of the actual microbial diversity contained [62][80][89] Although NGS techniques are widely used, there are still some aspects that need improvement and fine-tuning.

The majority of published milk and cheese microbiome data commonly based on DNA-based methods with amplification and sequencing of 16S rRNA genes. [71][89][100][117]. However, considering that DNA-based analysis cannot discriminate between active and inactive species, RNA-based amplicon sequencing has become popular in microbial ecology investigations as an alternative method to generate information of active members in the communities of different environments. [93] The RNA-based approach offer more promise thanks to the existence of a positive association between the content of ribosomal RNA (rRNA) and the metabolic activity of microorganisms, allowing the prediction of metabolically active microbial community structures. [1][4] Following extraction, total RNA is reverse-transcribed to cDNA, and cDNAs are analyzed using high-throughput sequencing technologies (RNA-seq). The Illumina Miseq platform was used in this experiment as it is a powerful and rapid sequencing platform for characterizing the microbial composition by sequencing the 16S rRNA gene. [49] The use of an RNA template in a 16S metabarcoding (targeting cDNA obtained through reverse-transcription PCR) analysis provides the relative amounts of the living microbial taxa forming the active part of the microbiota in milk and cheese.

Although 16S rRNA-based metabarcoding shows great potential in microbial taxonomy, there are still many challenges that remain in the research using this method, particularly the difficulty in extracting RNA of good quality, sample preparation and handling caution due to RNA's low stability, and the ubiquitous presence of RNases, as well as bioinformatics issues relating to sequence reconstruction, annotation, and statistical analysis. [60]

For the phylogenetic investigation, the complete gene is not amplified and sequenced but only an amplicon of about 470 bp including the hypervariable regions V3 and V4. In order for samples to be sequenced, it is necessary to build libraries consisting of a set of fragments of nucleic acids representative of the sample itself. The library for the phylogenetic analysis of microbial communities was obtained by means of the convenient two-step PCR amplification reactions that firstly used a pair of 16S universal primers and then secondly used pair of primers capable of being recognized by the Illumina platform and one of the latter contains its own internal a barcode sequence necessary to distinguish the individual samples within the pool. [49][97]

In our current study, at the family level, there is a transparent distinction between the heatmap of milk and cheese matrix. In milk samples of our study, the overall dominance of the phyla: *Firmicutes, Poteobacteria, Bacteroidetes* and *Actinobacteria* are in agreement with the results of previous DNA-based studies. [117] In cheese samples of our study, the overall dominance of the phyla: *Firmicutes*, *Actinobacteria*, and *Proteobacteria* are in agreement with the results of other DNA-based studies. [71] There is a higher amount of taxa in milk samples in compare to cheese. In cheese, we obtained the *Steptococcus* that was introduced from the starter culture. Moreover, within each taxa, we can see both un-treated (B0) and treated (B50, B100) Bentonites groups includes; thus, it can confirm that Bentonites usage doses did not have an effect in active microbial community.

In our study, the diversity and composition of the active microbiota in milk and cheese after cows were fed a feed supplemented with Bentonites was virtually unchanged, demonstrating a neutral effect of this toxin binder. This is also partly supported by the EFSA's previous scientific advice that mycotoxin binder is not absorbed once the animal ingests it; therefore it do not toxic to animals.

The significance of this study suggests that it is possible to safely use mycotoxin binder Bentonites in the feed of dairy cows, as it helps to prevent the risks posed by mycotoxins on cow health and performance, but at the same time, does not reduce the diversity of active microbiota and does not affect the quality of animal products, here in particular, milk and cheese. Based on current result, we can improve our knowledge and confidence in the use of bentonites toxin binders in animal feed. Moreover, it will be the basis for similar studies on other toxic binders, or studies on other livestock such as chickens, pigs or horses.

5. CONCLUSION

The RNA-based amplicon sequencing method enables for precise prediction of metabolically active bacteria and communities in milk and cheese. The capacity to distinguish between active and inactive, lysed or dead microorganisms is very important when studying the influence of various stimuli on active dairy microbial communities. The findings of this investigation revealed that Bentonites feed additives have no effect on diversity and composition of bacterial community.

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