Università degli Studi di Padova

Head Office: Università degli Studi di Padova

Department of Comparative Biomedicine and Food Science

Ph.D. COURSE IN VETERINARY SCIENCES

XXXV SERIES

SAFE USE OF BENTONITE IN DAIRY COWS. ASSESSMENT OF PHYSIOLOGICAL RESPONSES BY STUDYING METABOLOME AND MINERAL CONTENT IN BIOFLUIDS AND MILK CHARACTERISTICS.

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Index

Summary

Additives based on bentonite and other clay minerals are widely used in dairy farms to counteract mycotoxin contamination of feed, which negatively affects animal health and production and poses potential risks to consumers' health. However, some studies reported negative effects of clay minerals in different animal species and cell cultures. Furthermore, these additives appear capable of interacting with gut and ruminal microbiota and, based on anecdotical observations reported by cheesemakers, there is concern that bentonite administration to dairy cows may negatively affect milk characteristics and cheesemaking. Therefore, the aim of this project was to investigate possible undesirable effects of bentonite addition to the diet of dairy cows. For this purpose, three different experiments, two *in vitro* and one *in vivo*, were conducted using a commercial bentonite product. The *in vitro* experiments aimed to assess potential alterations in rumen fermentation, metabolome, and mineral content, while the aim of the *in vivo* experiment was to investigate any possible side effects of the additive on dairy cows' health, physiology, and production, as well as on milk characteristics and cheesemaking. In particular, in the first *in vitro* study, five doses of bentonite (0, 2.5, 5, 10, and 50 mg) were incubated at 39 °C for 24 h in fermentation bottles, each containing 1 g of total mixed ration (TMR), 100 mL of medium solution, and 50 mL of rumen fluid collected from a dairy cow. Gas production was monitored in continuous and, at the end of the incubation, ^{1}H NMR spectroscopy and ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy) were used for the study of the metabolome and mineral content, respectively. The second *in vitro* experiment was performed using three doses of bentonite (0, 5, and 50 mg) and three biological replicates (rumen fluid collected from three different dairy cows). The incubation protocol and gas production monitoring system used were the same as in the first experiment. The metabolomic study was in this case performed combining LC/DFI-MS/MS (Liquid Chromatography/Direct Flow Injection-Tandem Mass Spectrometry) and ^{1}H NMR spectroscopy, while the trace element analysis was conducted by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry). Lastly, in the *in vivo* experiment, six multiparous lactating Holstein cows were treated with three bentonite doses (0, 50, or 100 g/cow/d) in a 3 × 3 Latin square crossover design with three 19-d treatment periods interspersed by 9-d washout periods. At the end of each experimental period, BCS (Body Condition Score), daily feed intake, daily rumination time, and daily milk yield were recorded and rumen fluid, blood, and milk samples were collected. Various analytical methods were employed in order to evaluate: *i)* the metabolome and mineral content of rumen fluid, blood serum, and milk whey, *ii)* oxidative stress biomarkers in plasma and milk, and *iii)* quality parameters, leukocyte subpopulations, proteome and peptidome, coagulation properties, and aptitude for cheese-making of milk. The results obtained both *in vitro* and *in vivo* suggest that bentonite can induce slight modifications of the metabolome and mineral content (mainly microelements) of the rumen fluid, even at the doses set by the European Union. Moreover, the highest dose of bentonite

used in the second *in vitro* experiment significantly reduced gas production. However, cows' homeostatic mechanisms seem able to counteract slight changes induced by bentonite in the rumen. Indeed, negligible or null effects of the treatments were found *in vivo* on the metabolome and mineral content of blood serum and milk whey, as well as on the other parameters considered. In conclusion, the use of bentonite in dairy cows' farms, within the limits set by the European Union and for short-term treatments, can be considered a safe practice for animal health and production, human consumption of milk, and cheesemaking. Further studies would be needed to evaluate potential side effects of long-term administration of bentonite in dairy cows.

Keywords: Animal Health, Bentonite, Dairy cows, Metabolomics, Milk characteristics, Ruminal fluid

Riassunto

Gli additivi a base di bentonite e altri minerali argillosi sono ampiamente utilizzati negli allevamenti da latte per contrastare la contaminazione da micotossine dei mangimi, che influisce negativamente sulla salute e sulla produzione degli animali e comporta potenziali rischi per la salute dei consumatori. Tuttavia, alcuni studi hanno riportato effetti negativi dei minerali argillosi in diverse specie animali e colture cellulari. Tali additivi, inoltre, sembrano in grado di interagire con il microbiota intestinale e ruminale e, sulla base di osservazioni riferite dai casari non suffragate da studi scientifici, si teme che la somministrazione di bentonite nelle bovine lattifere possa influire negativamente sulle caratteristiche del latte e sulla caseificazione. Pertanto, lo scopo di questo progetto è stato quello di indagare i possibili effetti indesiderati dell'integrazione di bentonite nella dieta delle bovine da latte. A tal fine, sono stati condotti tre diversi esperimenti, due *in vitro* e uno *in vivo*, utilizzando un prodotto commerciale a base di bentonite. Gli esperimenti *in vitro* miravano a valutare possibili alterazioni a carico delle fermentazioni, del metaboloma e del contenuto di minerali a livello ruminale, mentre scopo dell'esperimento *in vivo* era indagare I possibili effetti collaterali dell'additivo sulla salute, la fisiologia e la produzione delle bovine lattifere, nonché sulle caratteristiche del latte e sulla caseificazione. In particolare, nel primo studio *in vitro*, cinque dosi di bentonite (0, 2.5, 5, 10 e 50 mg) sono state incubate a 39 °C per 24 ore all'interno di bottiglie di fermentazione, ciascuna contenente 1 g di *unified*, 100 mL di *medium* e 50 mL di liquido ruminale prelevato da una bovina da latte. La produzione di gas è stata monitorata in continuo e, al termine dell'incubazione, la spettroscopia ^{1}H NMR e l'ICP-OES (*Inductively Coupled Plasma-Optical Emission Spectroscopy*) sono state utilizzate rispettivamente per lo studio del metaboloma e del contenuto di minerali. Il secondo esperimento *in vitro* è stato condotto utilizzando tre dosi di bentonite (0, 5 e 50 mg) e tre repliche biologiche (liquido ruminale prelevato da tre diverse bovine da latte). Il protocollo di incubazione e il sistema di monitoraggio della produzione di gas sono stati gli stessi impiegati nel primo esperimento. Lo studio metabolomico è stato eseguito in questo caso combinando LC/DFI-MS/MS (*Liquid Chromatography/Direct Flow Injection-Tandem Mass Spectrometry*) e spettroscopia ^{1}H NMR, mentre l'analisi degli elementi in traccia è stata condotta mediante ICP-MS (*Inductively Coupled Plasma-Mass Spectrometry*). Infine, nell'esperimento *in vivo*, sei bovine Holstein pluripare in lattazione sono state trattate con tre dosaggi di bentonite (0, 50 o 100 g/capo/giorno) utilizzando un disegno *crossover* a quadrato latino 3 × 3 con tre periodi di trattamento di 19 giorni intervallati da periodi di *washout* di 9 giorni. Al termine di ciascun periodo sperimentale, sono stati registrati il BCS (*Body Condition Score*), l'ingestione giornaliera di alimento, il tempo di ruminazione giornaliero e la produzione giornaliera di latte e sono stati prelevati campioni di liquido ruminale, sangue e latte. Vari metodi analitici sono stati impiegati per valutare: *i)* il metabolome e il contenuto di minerali di liquido ruminale, siero di sangue e siero di latte, *ii) biomarker* dello stress ossidativo in plasma and latte, and *iii)* i parametri qualitativi, le sottopopolazioni leucocitarie, il proteome e il

peptidome, le proprietà di coagulazione e l'attitudine casearia del latte. I risultati ottenuti sia *in vitro* che *in vivo* suggeriscono che la bentonite può indurre lievi modifiche del metaboloma e del contenuto di minerali (soprattutto microelementi) del liquido ruminale, anche ai dosaggi stabiliti dall'Unione Europea. Inoltre, la dose più alta di bentonite utilizzata nel secondo esperimento *in vitro* ha ridotto in maniera significativa la produzione di gas. Tuttavia, i meccanismi omeostatici delle bovine sembrano in grado di contrastare lievi cambiamenti indotti dalla bentonite a livello ruminale. Infatti, effetti trascurabili o nulli del trattamento sono stati riscontrati *in vivo* sul metaboloma e sul contenuto di minerali del siero di sangue e latte, come pure sugli altri parametri considerati. In conclusione, l'uso di bentonite negli allevamenti delle bovine da latte, entro i limiti fissati dall'Unione Europea e per brevi periodi di trattamento, può essere considerata una pratica sicura per la salute e la produzione animale, il consumo umano di latte e la caseificazione. Sarebbero necessari ulteriori studi per valutare possibili effetti collaterali della somministrazione di bentonite a lungo termine nelle bovine da latte.

Parole chiave: Bentonite, Bovine da latte, Caratteristiche del latte, Liquido ruminale, Metabolomica, Salute animale

PREAMBLE

The present dissertation is the result of my research activities conducted during the Doctoral Course in Veterinary Sciences (XXXV cycle) in the Research Area "Large Animals Physiology, Reproduction and Medicine" with the Research Topic "Physiological mechanisms of animal adaptation to farming conditions and confinement" at the University of Padua as of October 1, 2019.

The two Sections of this dissertation are entirely devoted to the main object of my research activity: the investigation of the effects on dairy cow's physiology of the administration of clay mineral-based products commonly used as feed additives for mycotoxin decontamination of feedstuffs. This investigation was largely part of the project "BENFELAT – Interventions in the supply chain aiming at improve the technological quality of milk: bentonite-based additives and lactic fermentations" (DG - DISR 2018), funded by the Italian Ministry of Agriculture, Food, Forestry and Tourism (MIPAAFT – Ministero delle Politiche Agricole, Alimentari, Forestali e del Turismo) and coordinated by Professor Enrico Novelli. It was an interdisciplinary project, which involved a large and diverse research team for the use of several techniques and methods. Among them, flow cytometry, ^{1}H NMR spectroscopy for metabolomic/metabonomic studies and ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy) for mineral content analysis in biofluids have definitely had a central place in my training and experimental activities.

More in detail, Section 1 of my dissertation is introductory in nature, aimed at justifying the experimental activities carried out for the project BENFELAT. It reports (with minor modifications editorial of the text) the information published as a review paper in the journal Frontiers in Veterinary Science (Damato A, Vianello F, Novelli E, Balzan S, Gianesella M, Giaretta E and Gabai G, 2022. Comprehensive Review on the Interactions of Clay Minerals With Animal Physiology and Production. *Frontiers in Veterinary Science,* 9, 889612. https://doi: 10.3389/fvets.2022.889612). On the other hand, Section 2 of this dissertation constitutes the experimental part of this dissertation. It reports three different experiments (two *in vitro* and one *in vivo*) conducted in order to fill gaps in knowledge of possible side effects of dietary administration of bentonite to dairy cows. The two *in vitro* experiments focused on possible interactions of bentonite with rumen fermentations, metabolome, and mineral content. The first of these experiments is described in Chapter 1 of Section 2 by reporting the same data published as a short communication in the journal Research in Veterinary Science (Damato A, Vanzani P, Giannuzzi D, Giaretta E, Novelli E, Vianello F, Tagliapietra F, Zennaro L, 2022. Bentonite does not affect *in vitro* ruminal gross fermentations but could modify ruminal metabolome and mineral content. A proof of concept. *Research in Veterinary Science*, 144, 78-81. https://doi.org/10.1016/j.rvsc.2022.01.012). This experiment represented a pilot study and was followed by the second *in vitro* experiment, aimed at verifying the previous

results and obtaining a better picture of the effects of bentonite on rumen metabolome. The second *in vitro* experiment, described in Chapter 2 of Section 2, was partially carried out in collaboration with the research group led by Dr. David Wishart at The Metabolomics Innovation Centre (TMIC), located on the Campus of the University of Alberta (Edmonton, Alberta, Canada), where I spent four months as a visiting PhD student. There, I had the opportunity to learn more in depth metabolomics techniques (especially NMR spectroscopy) and the related methods for data analysis. The third chapter of Section 2 is dedicated to the *in vivo* experiment, consisting in the administration of different dosages of bentonite to six mid-late lactating Holstein cows in a 3 x 3 Latin square crossover design in order to investigate possible side effects on the physiology and production of cows and on milk characteristics and cheesemaking properties. In particular, my main contribution has been to investigate potential physiological alterations and metabolic responses to dietary bentonite in ruminal fluid, blood, and milk samples. All the above research activities were conducted after the approval by the OPBA (Organismo Preposto al Benessere degli Animali) of the University of Padua (Prot. n. 0197903, 16 May 2019).

There are two appendixes to this dissertation. Appendix 1 provides additional details on the materials and methods used for the experiments related to my main research topic, while Appendix 2 collects other research projects in which I have been involved. In particular, I was co-author of the review article: "Mateo-Otero Y, Yeste M, Damato A and Giaretta E (2021) Cryopreservation and oxidative stress in porcine oocytes. *Research in Veterinary Science*, Volume 135, Pages 20-26, ISSN 0034-5288, https://doi.org/10.1016/j.rvsc.2020.12.024". The abstract of the above review article is reported in the first paragraph of Appendix 2. The second paragraph coincides with the abstract "Giaretta E, Gabai G, Mislei B, Bucci D, Damato A, Vigolo V, Falomo M E and Zennaro L (2022) Evaluation of metabolomic profiling and oxidative stress markers of bull seminal plasma and their relationship with sperm motility before and after thawing. *Animal Reproduction Science*. Volume 247, 107107, ISSN 0378-4320, https://doi.org/10.1016/j.anireprosci.2022.107107", published in a special issue of the journal Animal Reproduction Science, which represents the proceeding of the 13th Association for Applied Animal Andrology Biennial Meeting (24-26 June 2022, Bologna, Italy, and Virtual Conference). Indeed, during my PhD Course I also collaborated to the project "Evaluation of metabolomic profiling and oxidative stress markers of bull seminal plasma and their relationship with sperm quality and oxidative stress status before and after thawing", supported by the Department of Comparative Biomedicine and Food Science (2020 Prot. BIRD203729) and coordinated by Professor Elisa Giaretta. My main contribution was the development and the application of a protocol for the metabolomic study of bull seminal plasma samples by ^{1}H NMR spectroscopy.

For editorial needs, in this dissertation the References are given at the end of each chapter/appendix.

SECTION 1 – General introduction

The information reported in the present section are taken from a review paper published in the journal Frontiers in Veterinary Science (with minor modification of the text for editorial reasons): Damato A, Vianello F, Novelli E, Balzan S, Gianesella M, Giaretta E and Gabai G (2022) Comprehensive Review on the Interactions of Clay Minerals With Animal Physiology and Production. Front. Vet. Sci. 9:889612. https://doi: 10.3389/fvets.2022.889612.

1. Introduction

Clays are naturally occurring rock or soil materials primarily composed of fine-grained minerals, characterized by high plasticity when hydrated and hardness in the dry form. The most widespread criterion for clay classification is based on their particle size, even though a general agreement has not been achieved (Moreno-Maroto and Alonso-Azcárate, 2018), and sedimentologists, geologists, and colloidal chemists classify as *clays* materials with particle size smaller than 4, 2, and 1 μm, respectively (Guggenheim and Martin, 1995). Clays are readily available in nature, but their properties vary considerably depending on the geological origin and on post-extraction treatments (EFSA FEEDAP, 2012; Garcia-Romero et al., 2021; Phillips et al., 2019). Depending on their structure and physicochemical properties (particle size, surface charge and adsorption capability), clay minerals can be used for a wide range of applications. Montmorillonite, bentonite, kaolinite, and illite belong to the phyllosilicate family and are authorized as feed additives by the European Commission, and assigned to one or more functional additive groups (**Table 1**) (European Commission, 2021). As an example, bentonite is an essentially impure smectite clay, mostly consisting of montmorillonite, with the ability to absorb large quantities of water and possessing a high cation exchange capacity (CEC) (Adamis et al., 2005). Three types of bentonite are recognized: calcium, sodium, and potassium bentonite. Sodium and calcium bentonite are the two types employed in the feed industry. Also, tectosilicates belonging to the zeolite subfamily [e.g., clinoptilolite (CPL) of sedimentary origin] are authorized by the European Commission as feed additive (European Commission, 2021) (**Table 1**).

It was reported that clay minerals administered as additives in animal feed exert beneficial effects on animal physiology (Ghadiri et al., 2015; Nadziakiewicza et al., 2019; Slamova et al., 2011), although some adverse effects have been documented (Elliott et al., 2020). Interestingly, in humans, geophagy is still in use in many parts of the world and specifically selected soils are consumed for medical reasons or as part of a regular diet (Reilly and Henry, 2000; Wiley and Katz, 1998). Geophagy is commonly observed in animals, which are hypothesized to consume clays as a source of dietary minerals and to remove toxins possibly present in food or to treat gastrointestinal

Table 1. Characteristics of the clay minerals authorized by the European Commission as feed additives (European Commission, 2021).

Additive	Additive category	Additive characteristics	Maximum Content (%) in complete feedstuff with 12% moisture	EU ID
Montmorillonite-illite	Binders Anticaking agents	≥ 75% phyllosilicates ≥ 35% montmorillonite-illite (swellable) ≥ 30% illite/muscovite ≤ 15% kaolinite (not swellable) ≤ 20% quartz Average 3.6% iron (structural) Free of asbestos	2	1g557
Clinoptilolite of sedimentary origin	Binders Anticaking agents	≥ 80% clinoptilolite (hydrate sodium calcium aluminosilicate) ≤ 20% clay minerals (free of fibers and quartz)	1	1g568
Illite-montmorillonite-kaolinite (natural mixture)	Binders Anticaking agents	≥ 40% illite ≥ 10% montmorillonite ≥ 8% kaolinite Average 10% iron (structural) Asbestos free	5 (fattening poultry, ruminants, pigs; weaned piglets) 2 (other animal species)	1g599
Bentonite	Reduction of mycotoxin contamination	≥ 70% smectite (dioctahedral montmorillonite) < 10% opal and feldspar < 4% quartz and calcite BC_{AfB1} > 90%	2	1m558
Bentonite	Binders Anticaking agents	≥ 50% smectite (dioctahedral montmorillonite)	2	1m558i
Bentonite	Control of radionuclide contamination	≥ 50% smectite (dioctahedral montmorillonite)	–	1m558i

ailments (Ghadiri et al., 2015; Slamova et al., 2011). Similarly, clay minerals have long been used by traditional medicine both as topical applications and to alleviate intestinal ailments, such as diarrhea (Dupont and Vernisse, 2009; Ghadiri et al., 2015; González et al., 2004; Nadziakiewicza et al., 2019; Pavelić et al., 2018).

In animal production, clay minerals are primarily used as binders for the production of pelleted feed and as adsorbents for mycotoxins and heavy metals (Ghadiri et al., 2015; Nadziakiewicza et al., 2019; Slamova et al., 2011). Indeed, diet supplementation with clay minerals, such as bentonite, is a recognized effective method to counteract the toxic effects of mycotoxins in both ruminant and monogastric species (Adegbeye et al., 2020). Clays can also sequester phytotoxins, enterotoxins, bacteria, and viruses in the gastrointestinal tract of animals, favouring their expulsion from the body. However, each type of clay has its own specific binding capacity, and even clays from the same family may have different efficiencies against the same substance (Nadziakiewicza et al., 2019).

An important aspect that deserves attention, still poorly understood, is represented by the multiple ways of interaction between clays and gut microorganisms (Cuadros J., 2017). This area of investigation is of the utmost importance considering the relevance of the intestinal and/or ruminal microbiota-host interactions for animal physiology (Gerstner and Liesegang, 2018; Neubauer et al., 2019; Prasai et al., 2016). Indeed, several studies suggested that the addition of different types of

clays as feed additives can positively affect the performances and health of different production animals, likely by modulating intestinal and/or ruminal microbiota (chicken: Chalvatzi et al., 2016; swine: Wang et al., 2012; cattle: Humer et al., 2019; Neubauer et al., 2019). Clays can also be applied as inorganic carriers for bioactive compounds with anti-bacterial activity (Malachová et al., 2009) and these products may be widely employed in the future to manipulate the intestinal/ruminal microbiota.

Despite the considerable number of studies reported on the advantages of administering clay minerals for human and animal health, it should be considered that others suggested that these agents might also cause undesirable effects. A series of *in vitro* and *in vivo* studies documented that clay administration led to mineral and vitamin unbalances, interactions with veterinary drugs, intestinal toxicity, hepatic damage and decreased growth performances (Elliott et al., 2020). In addition, anecdotal observations made by cheesemakers suggested that clay administration to cows may affect milk characteristics and cheese making properties, although, to the best of our knowledge, these observations were not supported by scientific evidences.

To further complicate the landscape, recently nanotechnologies have made available a wide range of applications in several fields and, nowadays, a wide choice of nanomaterials-based strategies is suggested to circumvent the limitations of treatments with conventional materials (Magro and Vianello, 2019). Indeed, various types of clay nanoparticles can be produced and combined with organic polymers to produce hybrid materials, which can be used for biomedical applications and for food industry (Bellmann et al., 2015; Ghadiri et al., 2015; Kantesaria and Sharma, 2020; Mousa et al., 2018; Uddin F., 2008; Uddin F., 2018). Manufactured clay particles are in the ultrafine-size range with diameters ranging from few to hundreds nanometers, which, depending on their properties, can be absorbed by cells leading to modifications of viability, proliferation, and functions (Hu et al., 2009). Conversely, larger clay particles (above micron size) or incorporated in polymeric products are traditionally considered as bio-inert or even biocompatible, and raise considerably less toxicity and health risk concerns (Jayrajsinh et al., 2017; Uddin F., 2008; Uddin F., 2018). Moreover, ingested clays are exposed to different environments (ruminal, gastric, intestinal), which may modify their characteristics and structure (Bellmann et al., 2015; Elliott et al., 2020; Tateo and Summa, 2007), thus potentially expose the intestinal cells to different clay structures.

In the near future, climate change will likely influence mycotoxin contamination of cereals and, consequently, animal feed (Medina et al., 2017). In addition, the development of strategies to limit greenhouse gas and urea emission will also be required (Gerber et al., 2013; Llonch et al., 2017; Shen et al., 2020). As clay minerals possess mycotoxin adsorptive properties and can modulate the intestinal and ruminal microbiota composition and metabolism (Chalvatzi et al., 2016; Humer et al., 2019; Neubauer et al., 2019; Prasai et al., 2016), an increased use of clay minerals in different forms

is conceivable. In this context, a better understanding of the animals' physiological responses to clay mineral administration will be of the utmost importance to guarantee animal health and the safety of products of animal origin.

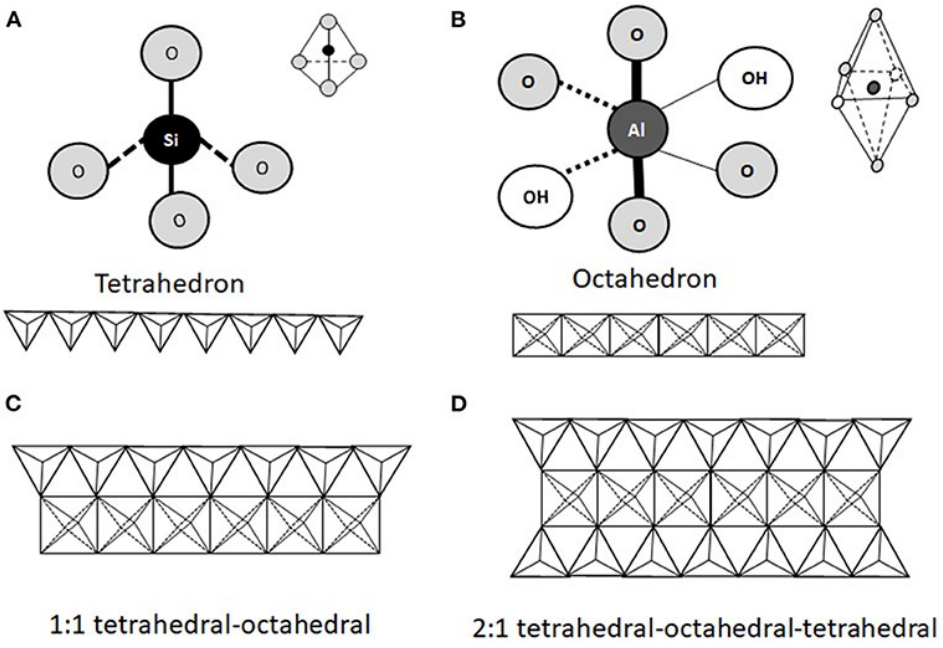
The present review represents a preliminary reflection for a conscious use of clay minerals in farm animals, with a special focus to ruminants. Indeed, the use of clays as feed additives in animal production deserves proper attention, considering that animal nutrition and feeding have significant implications not only on the livestock sector, but also on public health, trade, economy, and environment (Gerber et al., 2013; Makkar H.P.S., 2016; Thumbi et al., 2015). Authors' aim focuses on the possible actions of different types of clay minerals not only as mycotoxin binders, but also in affecting animals' physiology. Gaps in knowledge about these topics are also highlighted to help designing the best and safest use of these feed additives.

2. Structure, classification and physical-chemical characteristics of clays

The structure and composition of different types of clay minerals have been already reviewed (Nadziakiewicza et al., 2019; Uddin F., 2008; Uddin F., 2018), and a thorough discussion about the classification and the physical-chemical characteristics of clays is beyond the scope of the present review. In this context, it is worthwhile to provide a brief overview of the structure of clay minerals, and underline the gross differences among the different classes of clays. Authors believe that this helps the readers in understanding the different effects of clays reported in scientific literature and in suggesting to increase the awareness that an accurate description of the clay product is an important prerequisite to explain its biological effects. Differences of structure, compositions, and industrial treatment clearly provide different physicochemical properties to clays and confer different characteristics to these feed additives, which can affect the expected results (Elliott et al., 2020; Ghadiri et al., 2015; Nadziakiewicza et al., 2019; Uddin F., 2018). As an example, among industrial treatments, commercial clays are commonly subjected to various procedures, such as contaminant removal, grinding to a finer powder and sieving to increase particle size uniformity (Phillips et al., 2019).

From a mineralogical point of view, clay minerals belong to the phyllosilicate family, which are characterized by parallel layers of hydrated aluminosilicates. In natural soils, clay minerals are rarely present as pure or homogeneous mixtures of single groups of minerals (Barton and Karathanasis, 2002). The basic structural unit of aluminosilicate clays is a combination of tetrahedral silica and octahedral aluminum sheets. The tetrahedral sheets are composed of SiO_4 units, which share three out of four oxygens (**Figure 1A**). The octahedral sheets are composed of aluminum (or manganese) bound to oxygen and hydroxyl groups (**Figure 1B**). Tetrahedral and octahedral sheets form two

Figure 1. Schematic representation of the structural units of phyllosilicates. The structure consists of a combination of tetrahedral silica **(A)** and octahedral aluminum **(B)** oxide sheets exposing hydroxyl groups. Two types of phyllosilicates can be distinguished. The 1:1 tetrahedral-octahedral (T-O) type consists of one sheet of tetrahedral SiO_4 joined to one octahedral aluminum (or manganese) sheet **(C)**. The 2:1 tetrahedral-octahedral-tetrahedral (T-O-T) type consists in one octahedral aluminum (or manganese) sheet between two tetrahedral SiO_4 sheets **(D)**.



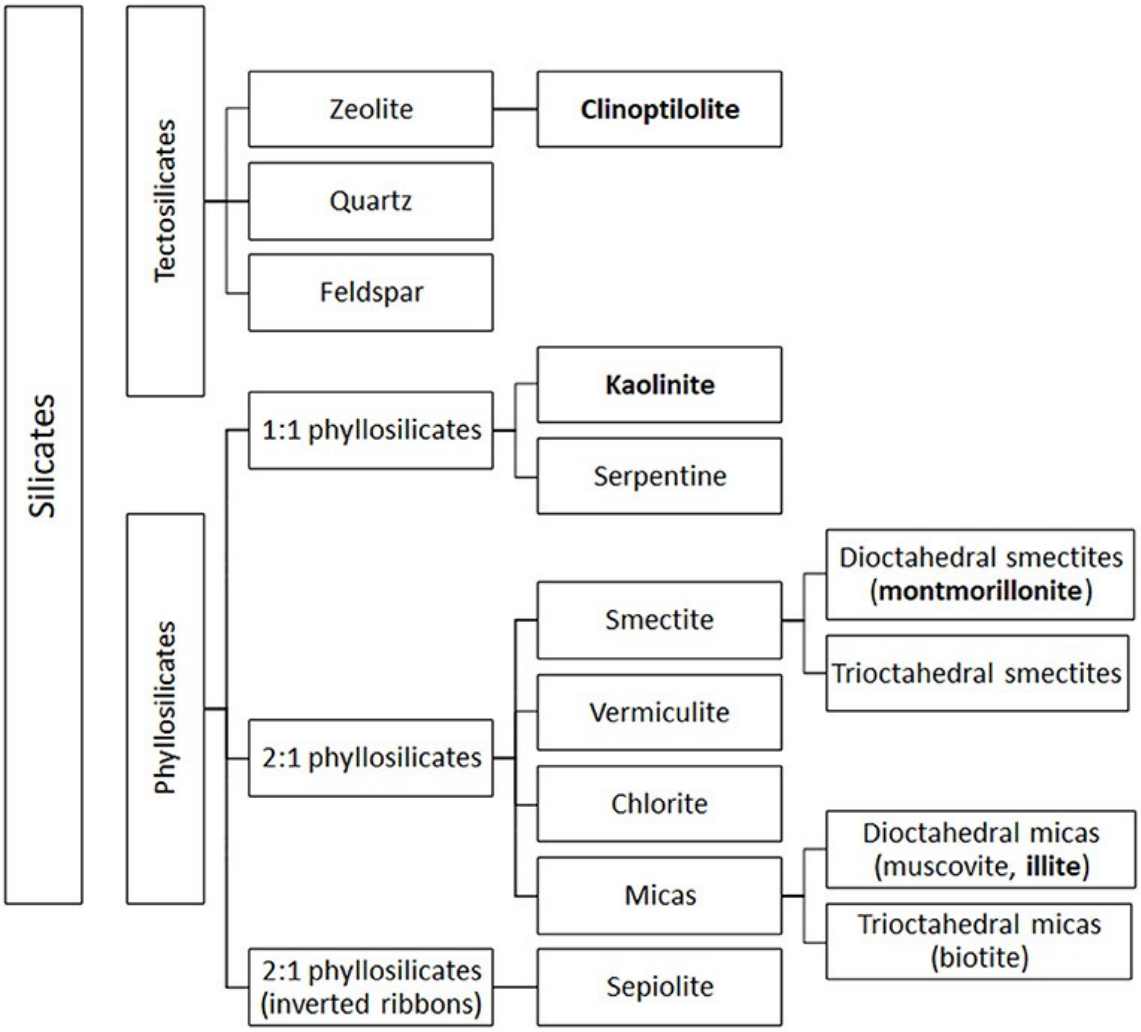
main types of phyllosilicate layers: the 1:1 tetrahedral-octahedral type (T-O; **Figure 1C**) consisting of one sheet of tetrahedral SiO_4 joined to an octahedral aluminum (or manganese) sheet; the 2:1 tetrahedral-octahedral-tetrahedral type (T-O-T; **Figure 1D**) consisting of one octahedral aluminum (or manganese) sheet between two tetrahedral SiO_4 sheets (Elliott et al., 2020; Ghadiri et al., 2015; Nadziakiewicza et al., 2019). The unit layers (T-O or T-O-T) of phyllosilicates are stacked repeatedly and the distance between two adjacent layers varies depending on the type of clay. The space between the two layers, called basal spacing or interlayer, can be occupied by water and/or different ions, which confer different properties to the specific clay (Nanzyo and Kanno, 2018; Uddin F., 2018). Phyllosilicate classification is based on the arrangement of the tetrahedral and octahedral layers (**Figure 2**). Trioctahedral layers are characterized by the occupation of divalent cations in all the octahedral sites. Conversely, in dioctahedral layers two third of the octahedral sites are occupied by trivalent cations. Under natural conditions in soils, some cations of both tetrahedral and octahedral sites are replaced by other metals of the same size even with different charge, and this phenomenon is called isomorphic substitution. In the tetrahedral sites, Si^{4+} can be partially replaced by Al^{3+} while, in the octahedral sites, Al^{3+} can be partially replaced by Mg^{2+} or Fe^{2+}. Isomorphic substitution does not significantly affect the crystal structure of clays, but leads to a permanent surplus of negative charges in the aluminosilicate layers, which is balanced by the absorption of exchangeable cations, such as Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+} (Barton and Karathanasis, 2002;

Garcia-Romero et al., 2021; Ghadiri et al., 2015; Nanzyo and Kanno, 2018; Uddin F., 2008). Phyllosilicate clays have also a pH-dependent charge. Indeed, the Al and Si-bound hydroxyls at the edges of clay crystals present acid-base properties and develop a negative charge at high pH and positive charge at low pH (Shainberg and Levy, 2005).

In 1:1 phyllosilicate, such as kaolinite, the strong interaction (hydrogen bonds) between neighboring octahedral and tetrahedral layers prevents water absorption and the mineral exposes only its external surface to the environment (Trckova et al., 2004). Moreover, isomorphic substitution is absent, leading to low cationic exchange capacity (CEC) (1–10 $cmol_c/kg$, expressed in centimole of charge per Kg), low surface area (20–50 m^2/g) and no swelling in water (Ghadiri et al., 2015; Mousa et al., 2018; Shainberg and Levy, 2005). Conversely, in 2:1 phyllosilicates, isomorphic substitution accounts for most of the total charge, and the different subfamilies (**Figure 2**) display different degrees of isomorphic substitution, resulting in a wide range of surface charges, interlayer spaces and swelling property (Barton and Karathanasis, 2002; Garcia-Romero et al., 2021; Mousa et al., 2018). For instance, in illite, belonging to mica group, the replacement of Si with 20% of Al atoms creates an important negative charge, mainly balanced by K^+ ions, which form bridges between layers hindering mineral expansion (Brigatti et al., 2013; Kumari and Mohan, 2021; Nadziakiewicza et al., 2019; Sparks D.L., 2003). Illite is characterized by an interlayer space of 10 Å, an intermediated surface area, and a CEC of 20–40 $cmol_c/kg$ (higher than kaolinite). Its adsorption and swelling capacity are lower than those of a common clay, montmorillonite (Kumari and Mohan, 2021; Shainberg and Levy, 2005). Montmorillonite, belonging to smectite group, is characterized by a low net charge that allows the smectite platelets (the particle units) to undergo complete dissociation by osmotic swelling (Mousa et al., 2018). Exchangeable cations in the basal spacing are responsible for the high expansion of the montmorillonite lattice upon hydration (Barton and Karathanasis, 2002). In fact, attraction between tetrahedral sites of two neighboring layers in montmorillonite is very weak, allowing the entry of water and exchangeable cations in the crystal structure, leading to the expansion of the interlayer space (from 9.6 to 20 Å) (Kumari and Mohan, 2021). Montmorillonite is characterized by high surface area (800 m^2/g), negative charge, high CEC (80–120 $cmol_c/kg$), and high swelling ability (Liu et al., 2021; Shainberg and Levy, 2005).

The 2:1 tetrahedral-octahedral-tetrahedral (T-O-T; **Figure 1D**) layers containing Na^+ or Ca^{2+} ions represent the simple unit of the clay lattice. In naturally occurring smectite clays, several tens of layers stack together to form colloidal particles named “tactoids”, which can be leaf-like, needle-like or plate-like in shape. Tactoids display a typical lateral dimension of 100–200 nm, a layer thickness of 10 nm, and an interlayer spacing of about 1 nm and form the basic structure of clays (Faisal et al., 2021; Segad et al., 2012a; Vietti and Dunn, 2006). Tactoids can gather into different orientations and form clusters (or flocculi) displaying diameters in the sub-micrometer (nanoparticles) to micrometer range, depending on the physical–chemical conditions of the

Figure 2. Simplified classification of silicates. Clay subfamilies and species used and present in feed additives are indicated in bold. Most clay minerals used as animal feed additives belong to the phyllosilicate family. The figure includes also the classification of tectosilicates of sedimentary origin, such as CPL (clinoptilolite), a natural zeolite that may be used as a technological additive in animal feed.



environment in which they are dispersed (OECD, 2015; Tan et al., 2017). As examples, the size of illite and kaolinite particles displays a bimodal distribution showing higher frequencies at the sub-micrometer and at 1–2 µm size. The size of calcium and sodium montmorillonite particles displays a unimodal size distribution around 1–2 µm (Tan et al., 2017). A set of bentonite samples examined by the European Food Safety Authority (EFSA), aimed at expressing an opinion about the safety and efficacy of bentonite as feed additive (EFSA FEEDAP, 2017), was characterized by a particle size lower than 10 µm in 3–25% of the material depending on the analyzed batch. These minerals are characterized by small particle size, high porosity and high CEC, which provide the ability to react with inorganic and organic polar reagents (Garcia-Romero et al., 2021). Indeed, clay nanoparticles can be produced by the modification of their layered structure, and a flourishing industry has developed various types of nanoclays (predominantly montmorillonite-based products). Moreover, nanoclays can be combined with organic polymers to produce hybrid materials, called clay

nanocomposites (Amari et al., 2021), which can be used as vehicles for drugs and other bioactive substances, and for the development of biomedical applications, such as in the fields of regenerative medicine and biosensing (Ghadiri et al., 2015; Kantesaria and Sharma, 2020; Mousa et al., 2018; Uddin F., 2018). In addition, clay nanoparticles may recite a role in food industry providing beneficial properties to food products and improved nutrition (Bellmann et al., 2015).

Finally, although not classified as clays, zeolites are often associated with clay minerals due to their similar properties and applications. Zeolites are silicate minerals belonging to the tectosilicate family, characterized by a 3-dimensional crystalline structure constituted of SiO_4 and AlO_4 tetrahedra joined by oxygen atoms to form large pores. These pores can contain weakly bound water molecules and mobile and exchangeable alkaline cations (e.g., Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+} etc.), which balance the negative charge of the structure (Kaduk and Faber, 1995; Nadziakiewicza et al., 2019; Papaioannou et al., 2005; Trckova et al., 2004). The properties of zeolites are related to their ability to reversibly absorb and release water, maintaining unaltered their crystalline structure, and on their structural pores, which form a kind of molecular sieve able to attract and hold positively charged atoms and molecules (Diaz et al., 2005; Nadziakiewicza et al., 2019; Papaioannou et al., 2005). In some zeolites, pores can form long channels, in which ions and molecules can be easily absorbed and released (Diaz et al., 2005). The best-known natural zeolite is clinoptiolite (CLP), characterized by a biocompatible nanoporous structure (Valpotić et al., 2017).

3. Cell-clay particle interactions

A thorough understanding of the interactions between clay particles and cells is important to explain both the beneficial and adverse effects of clay minerals in human and animal organisms (Elliott et al., 2020; Uddin F. 2008; Uddin F. 2018). Clay particles as bulk materials are traditionally considered as bio-inert or even biocompatible. However, various kinds of colloidal particles with a diameter ranging from several nanometers to few microns can be taken up by cells, which can undergo changes in morphology, viability, proliferation, and functions (Hu et al., 2009; Uddin F., 2018). Most information on the interactions of clay particles with cells and on their potential cytotoxicity derives from studies performed on nanoclays (Connolly et al., 2019; Elliott et al., 2020; Mousa et al., 2018; Sharma et al., 2010; Sharma et al., 2014; Verma et al., 2012). Other information derives from studies on clay-polymer nanocomposites, whose characteristics are very different from those of the natural clay nano or microparticles (Amari et al., 2021).

Importantly, when used as feed additives, clay minerals are ingested mainly as particles of few tens of micrometers. However, their size, structure and surface properties may change during they transit through the gastrointestinal (GI) system and, to the best of our knowledge, little information is available about their fate once they are ingested. Note that swelling and disaggregation

commonly follow hydration of clays. Thus, a clay mineral dispersion within an organism could be a very complex system and may contain both individual clay mineral particles and, most likely, clay mineral aggregates. As an example, particle size of smectite materials show a multimodal distribution with components at <2 μm, primary constituted of clay mineral particles, 10–20 μm flocculi (usually resistant to disaggregation), and less common 50–500 μm micro-aggregates (Tan et al., 2017). This system is further complicated considering that the mechanical behavior of smectite in water is very sensitive to several parameters, such as its concentration, particle size and morphology, nature of exchangeable cations and chemical environment (pH and ionic strength) above all (Paumier et al., 2008). Thus, within the GI system, ingested particles endure the action of physical and chemical players. Moreover, it is conceivable that clays used as feed additives release ions (Tateo and Summa, 2007), and the particle size may be modified during the digestive processes (Bellmann et al, 2015). Likely, mechanical factors, such as mastication and peristalsis, should have a small impact on clay particles size. Conversely, as above mentioned, ion concentrations and pH could have a deeper effect. At acidic pH, clay minerals tend to agglomerate (Bellmann et al, 2015; Tateo and Summa, 2007). Na-montmorillonite platelets dissolve at low pH, and during this process, trivalent Al^{3+} ions are released. As a consequence, silicate platelets aggregate into tactoids (Segad et al., 2015). Conversely, at basic pH values, clay aggregates tend to disassemble to particles in the nanosize range (Bellmann et al, 2015; Tateo and Summa, 2007). In a mixed electrolyte solution, tactoid size depends also on the ratio of divalent to monovalent cations, where divalent cations (Ca^{2+}) favor the formation of tactoids, while monovalent cations (Na^{+}) cause a repulsion between platelets leading to aggregate disassembly (Segad et al., 2012a; Segad et al., 2012b).

In humans and monogastric mammals, the pH of the stomach is definitely acidic, while intestinal pH ranges between 5 and 8 (Bellmann et al, 2015). In ruminants, particles reside for a considerable time interval in the reticulum-rumen, in which clays are exposed to daily pH fluctuations, ranging in dairy cows from 5.5 to 6.5 (Mensching et al., 2020; Palmonari et al.,2010). Thus, feed additives based on clay minerals, such as bentonite, are usually administered for long time intervals to counter mycotoxin contamination, and clay particles possibly require a long period for being washed out from the GI tract (Damato et al., 2021), thus creating a prerequisite for their interaction with GI cells. Moreover, digestive enzymes, microbiota and interactions with biomolecules, such as bile acids, can modify particle surfaces, affecting their colloidal behavior (Bellmann et al, 2015) and their ability to interact with cells. Note that when nanomaterials are dispersed into biological fluids, they are rapidly coated by biomolecules (Vianello et al., 2021). This coating, called corona, influence nanoparticle adhesion to the plasma membrane and, as an example, can alter cellular uptake by human adenocarcinoma alveolar basal epithelial A549 cells (Lesniak et al., 2013). Finally, clay nanoparticles can form a particle layer at the oil/water interface preventing coalescence of emulsion droplets, thus forming pickering emulsions (emulsion stabilized by solid particles) (Lu et

al., 2021). Under simulated gastric and intestinal conditions, different clay mineral microparticles can incorporate lipids depending on their surface chemistry, thus reducing the absorption of the lipid fraction (Dening et al., 2018). Therefore, the interactions between bile acids, ingested lipids and clay minerals may play a role in modulating the effects of ingested clay mineral particles.

Once in the GI tract, and concomitantly with the phenomena mentioned above, the interactions between inorganic particles and cells depend on the cell type and on particle characteristics, and it is worth noting that size, surface properties, and shape of particles play a prominent role (Bellmann et al, 2015; Benne et al., 2016; Hallabad and Jacobs, 2009; He and Park, 2016; Hu et al., 2009). From a mechanistic point of view, clay particles interact with cell membranes by different weak bonds, such as van der Waals forces, electrostatic or hydrophobic interactions, and hydrogen bonding, but the occurrence of specific mechanisms, such as ligand-receptor interactions, could make the clay particles-cell system even more complex. The effects of particle size, shape, and surface characteristics on cell viability was well described for polymeric particles (poly-lactic-co-glycolic acid or polystyrene) (Loos et al., 2014; Mundargi et al., 2008).

Once interacting with cell membranes, particles can be internalized by cells, being endocytosis the preferred internalization mechanism (Bellmann et al, 2015; Chen et al., 2020). Inside cells, nanoparticles could cause cellular toxicity mainly by four mechanisms: reactive oxygen species production, disruption of cell membrane, induction of inflammatory response, and genotoxicity (Zhu et al., 2019), and these effects can be interrelated.

In general, cells *in vitro* are able to internalize particles with a diameter lower than 10 µm and these may produce an inflammatory response. Notably, elongated and metal particles are more pro-inflammatory *in vivo* than spherical and polymeric particles (Hallabad and Jacobs, 2009).

As already described, the basic structural unit of clays is composed of silica and alumina layers held together by electrostatic forces. These materials, in the nano size, have large surface area and high aspect ratio (length ~2–300 nm; thickness ~1 nm), and these characteristics can confer cell toxicity (Connolly et al., 2020; Verma et al., 2012). Indeed, nanomaterials characterized by high aspect ratio can cause concerns, in comparison to isometric nanoparticles, similarly to asbestos fibers (Fubini et al., 2011). Nevertheless, a recent review on the biological effects of clay nanoparticles, including nanocomposites, reported that most studies exploring nanoclay-cell interactions observed only minimal cytotoxicity in all cells tested, and, at the same time, improvements of various cell functions, such as adhesion, proliferation, and differentiation (Mousa et al., 2018). As an example, no genotoxic effects were reported for a natural Na-montmorillonite. Specifically, according to the manufacturer, the particle size distribution of this natural clay is 10% with size <2 µm, 50% with size <6 µm, and 90% with size <13 µm, and the possible content of nanometer-sized particles was not indicated (Maisanaba et al., 2013; Sharma et al., 2010). Differently, the quaternary ammonium salt modified montmorillonite (Cloisite® 30B) was moderately genotoxic on Caco-2 cells (Maisanaba et

al., 2013; Sharma et al., 2010), but no clinical signs of toxicity were observed when Cloisite®30B was administered to Wistar rats (no induction of DNA strand-breaks in colon, liver, and kidney cells was detected), and no increase of inflammatory cytokine markers in blood was found. Moreover, administered clay particles were not absorbed and were found in feces, thus no systemic exposure was reported (Sharma et al., 2014).

Natural montmorillonite at high concentration and after long exposure time was reported to cause cytotoxic effects in human normal intestinal cells (INT-407). The cytotoxicity was probably due to the micron size of administered particles, which coated the cell surface rather than penetrating the plasma membrane. In this case, reactive oxygen species generation was also suggested to be responsible for the adverse effects on cells. Moreover, no remarkable sign of toxicity was found in mice receiving up to 0.1% montmorillonite, and no significant accumulation in any specific organ was detected, nor direct affection of *in vivo* cell viability (Baek et al., 2012). In general, *in vitro* and *in vivo* studies on clay toxicity led to controversial results. Most *in vitro* studies suggested that clays display different degrees of cytotoxicity through different mechanisms (necrosis/apoptosis, oxidative stress or genotoxicity). Conversely, studies performed on laboratory animals did not show clear evidences of systemic toxicity even at very high doses of clays (Maisanaba et al., 2015).

Halloysite is a natural aluminosilicate clay with a hollow tubular structure, constituted of nanotubes of about 0.5–2 μm length and with a 10–20 nm inner luminal diameter that enables the loading and release of different molecules and drugs. Halloysite exhibits a good degree of biocompatibility in Caco-2/HT29-MTX cells under monolayer co-culture. HT29-MTX is a mucous-secreting cell line that provides a model to study the influence of the mucous layer on nanoparticle diffusion. Halloysite nanoparticles did not induce cytotoxicity despite an increased proinflammatory cytokine release (Lai et al., 2013). In the case of clay-polymer nanocomposites, it should be considered that poly-lactic-co-glycolic acid constitutes a potent inflammatory stimulus leading to NF-κB translocation to nucleus and pro-inflammatory cytokines production (Nicolete et al., 2011). As well, polystyrene can interact with dendritic cells depending on the surface charge of the particle (Foged et al., 2005). Furthermore, the uptake of polystyrene was described in human pro-myelocytic cell line HL60 as a function of particle hydrophobicity (Müller et al., 1997).

In the light of the above reported evidences, the possible elicitation of inflammatory response upon clay particles ingestion and interaction with GI and Gut Associated Lymphoid Tissue (GALT) cells is still an open question. Notwithstanding, the uptake of inert particles by the alimentary tract has been documented since the 1960s. It has been well established that nanoparticles can be better absorbed than microparticles (Carr et al., 2012; Chen et al., 2020).

It should be considered that, before interacting with the cell membrane, particles have to cross the mucus layer coating the GI epithelium. The thickness and composition of mucus layer vary considerably among the different GI tract portions. In the stomach and large intestine, the mucus

layer is quite thick and non-adhesive particles could diffuse through it. Conversely, the mucus layer in the small intestine is relatively thin and allows nutrient uptake by the enterocytes, while keeping at bay potentially hazardous large particles, such as bacteria (Bellmann et al., 2015). At the best of authors' knowledge, no specific study is available on clay micro and nanoparticles interaction with GI mucus. Generally, it could be said that pH and density of mucus have a substantial influence on this interaction and nanoparticles have more chances to cross the mucus layer than microparticles. Moreover, non-charged particles present a significant advantage in terms of diffusion in mucus (Chen et al., 2020).

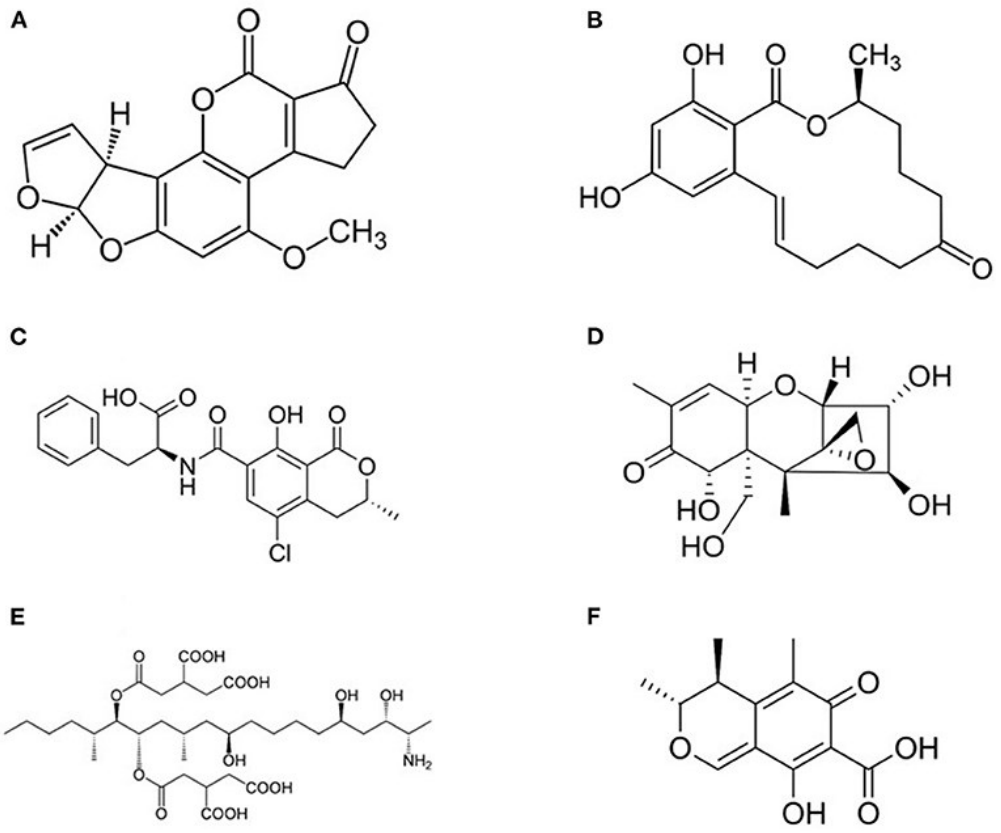
Finally, immune cells, such as macrophages, can modify particle cellular uptake, and an increased intestinal permeability can result from the interaction between enterocytes and GALT. As an example, the exposure of CaCo-2 cells to THP1-derived macrophages increased the uptake of polystyrene micro-particles across the cell monolayer, likely through a macrophage-induced loosing of tight junctions and/or a decrease of epithelial depth (Moyes et al., 2010). Indeed, granular pigments composed of inert inorganic particles (100–700 nm), such as kaolinite and environmental silicates, can be observed into phagolysosomes of macrophages within human GALT (Powell et al., 1996).

4. Clays for animal production

In animal production, clays are primarily used as adsorbents to protect animals, consumers, and the environment against the potentially harmful effects of feed and water contaminants (Abebe et al., 2020; Adegbeye et al., 2020; Alali and Ricke, 2012; Hejna et al., 2018; Howard B., 2021; Liu et al., 2021).

Mycotoxins represent one of the most challenging problems in feed and food chains and every year they appear among the “top ten” hazards reported by the Rapid Alert System for Food and Feed (RASFF) in Europe (Agriopoulou et al., 2020; European Commission, 2020; Iheshiulor et al., 2011; Zain M.E., 2011). They constitute a heterogeneous group of compounds (**Figure 3**) produced as secondary metabolites by filamentous fungi and capable of causing toxic effects (mycotoxicosis) in vertebrates (Bennett J.W., 1987; Bennett and Klich, 2003; Ostry et al., 2017; Zain M.E., 2011). In farm animals, prolonged exposure to low levels of mycotoxins results in increased mortality, predisposition to infections, decreased fertility, decline of growth and weight gain, decreased milk and egg production, and, finally, higher veterinary expenses (Denli M., 2015; Sun et al., 2015; Zain M.E., 2011). The carryover of mycotoxins and their metabolites was reported in milk and dairy products, beef meat, as well as in innards and hen eggs, and in numerous tissues, organs and products from swine (Adegbeye et al., 2020; Becker-Algeri et al., 2016; Völkel et al., 2011).

Figure 3. Structure of most common mycotoxins in feed. **(A)** Aflatoxin B1 (2,3,6a,9a-tetrahydro-4-methoxycyclopenta(c)furo(3',2':4,5)furo(2,3-h)(1)benzo-pyran-1, 11-dione); **(B)** Zearalenone [(3S,11E)-14,16-Dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione]; **(C)** Ochratoxin A (N-[(3R)-5-Chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1H-2-benzopyran-7-carbonyl]-L-phenylalanine); **(D)** Nivalenol [(3α,4β,7α)-12,13-epoxy-3,4,7,15-tetrahydroxy-trichothec-9-en-8-one] is a mycotoxin of the trichothecene group; **(E)** Fumonisin B1 [p(2S,2'S)-2,2'-[(5S,6R,7R,9R,11S,16R,18S,19S)-19-Amino-11,16,18-trihydroxy-5,9-dimethylicosane-6,7-diyl]bis[oxy(2-oxoethane-2,1-diyl)]dibutanedioic acid]; **(F)** Citrinin (3R,4S)-8-Hydroxy-3,4,5-trimethyl-6-oxo-4,6-dihydro-3H-2-benzopyran-7-carboxylic acid).



Heavy metal contamination represents another important issue all over the world. Heavy metals are elements with metallic properties belonging to different chemical groups (transition metals, metalloids, lanthanides, and actinides) characterized by high atomic weight and high density (higher than 5 g/cm^3) (Babula et al., 2008; Briffa et al., 2020). In human and animal nutrition, heavy metals have a double significance. Some of them (e.g., Co, Cr, Cu, Fe, Mn, Mo, Se, and Zn) are essential micronutrients and are required in small but critical amounts for normal growth and healthy status. They act as constituents/activators of enzymes, proteins, and hormones and their deficiency can cause several symptoms, such as impaired growth, decreased fertility and immune defense, anemia, neurological disorders, ataxia, and skeletal deformities (Alloway B.J., 2013; Briffa et al., 2020). Other heavy metals are generally considered as non-essential, so-called xenobiotics, as they do not seem to play key roles within organisms or can negatively affect the level of essential elements in the body (Briffa et al., 2020). Therefore, non-essential heavy metals are undesirable substances in animal feed. In addition, some of them (Al, As, Cd, Hg, Ni, Pb) display systemic toxicity

and carcinogenic properties even at very low concentration (Hejna et al., 2018; Tchounwou et al., 2012). Furthermore, heavy metals are subjected to bioaccumulation and biomagnification along the food chain and their carryover is reported in many animal tissues and organs and in products for human consumption (i.e., milk, eggs, meat) (Hejna et al., 2018).

Clay minerals can adsorb mycotoxins, heavy metals, and other toxic compounds (e.g., phytotoxins, diarrheagenic enterotoxins, harmful microorganisms, and radionuclides) by different mechanisms (Aytas et al., 2009; Dominy et al., 2004; Hassen et al., 2003; Liu et al., 2021; Papaioannou et al., 2005; Szajewska et al., 2006; Valpotić et al., 2017). Mycotoxin sequestration involves different types of molecular interactions. Physical interactions are generally reversible and influenced by environmental conditions (e.g., pH and ionic strength) and include hydrophobic bonds, such as Van der Waals forces, and electrostatic forces, such as dipole-dipole interactions. Typically, these interactions are frequently involved in mycotoxin adsorption by clays. Differently, chemical binding is irreversible and occurs when adsorbent and adsorbate form a covalent interaction. The adsorption efficiency depends on the characteristics of both the clay (e.g., electric charge, pore density, and their size and distribution) and the mycotoxin (e.g., polarity, shape, size, and solubility) (Di Gregorio et al., 2014). In general, because of their hydrophilic and negatively charged surfaces, aluminosilicates are more effective in binding polar mycotoxins, such as aflatoxins (AFs), than the less hydrophilic zearalenones (ZEAs), ochratoxin A (OTA), and trichotrecenes (TCTs) (Di Gregorio et al., 2014; Papaioannou et al., 2005). Negatively charged clays are also poorly effective against acidic mycotoxins, such as fumosins (FUMs), which in solution tend to form anionic species by deprotonation of –COOH groups (Matusik and Deng, 2020). Studies on the mechanism of interaction between clays and mycotoxins have focused mainly on smectites and AFs and different adsorption models have been proposed (**Table 2**).

The ability of clays to adsorb heavy metals mainly depends on clay CEC and specific surface area (Papaioannou et al., 2005; Otunola and Ololade, 2020). In general, clay minerals characterized by high porosity (ex. smectites) have a higher surface area than those exposing only the external surface to the solvent (ex. kaolinite, illite) (Dogan et al., 2006; Otunola and Ololade, 2020).

Studies on animals (e.g., mice, pig, and fish) suggested that zeolites, mainly CLP, can be proposed as useful feed additives for the prevention of intoxications by heavy metals (Papaioannou et al., 2005). However, the *in vivo* effectiveness of clays as heavy metal adsorbents should be further in depth assessed. Indeed, the interactions between clays and heavy metals depend on several factors, such as contact time, clay dosage, pH, temperature, the presence of other metal species or organic substances, which could affect the adsorption capacity of clays in the GI tract (Otunola and Ololade, 2020; Chantawong et al., 2003; Abollino et al., 2003). In addition, it is important to bear in mind that clays can also release metal ions (e.g., Al^{3+}) or adsorb essential microelements (e.g., Zn, Cu, and Mg), potentially causing mineral imbalances (Elliott et al., 2020).

Table 2. Adsorption mechanisms of AFB_1 by raw mineral adsorbents determined by *in vitro* trials.

Mineral adsorbent		AFB_1 concentration (mg/Kg)	Experimental conditions	Adsorption efficacy (mg/Kg)	Adsorption mechanism	References
Type	Concentration (%)					
Hydrated sodium calcium aluminosilicate (HSCAS)	Not reported	Not reported	Not reported	131,000[a]	Selective chemisorption by mononuclear bidentate chelation	Phillips et al., 1995
Ca-montmorillonite	0.5	0.0-2.0	Incubation in water (pH 2.0 or 8.0) at 37 °C for 1.5 h under intermittent mixing	613.5[b] at pH 2 628.9[b] at pH 8	Hydrogen bonds on the edges of Ca-montmorillonite	Desheng et al., 2005
Ca-montmorillonite	Not reported	Not reported	Not reported	Not reported	Electron donor acceptor (EDA) on the negatively-charged surface	Phillips et al., 2008
Smectite clays	2.0	0.0-8.0	Incubation in water under shaking for 24 h	18,000-212,000[b]	Coordination bonds, hydrogen bonds with smectite interlayer cations or associated water molecules	Kannewischer et al., 2006; Tenorio Arvide et al., 2008
Ca-smectite	0.03	33.3[c]	Incubation in water under shaking overnight	140,000[b]	Hydrogen bonds with hydration shells of exchangeable cations or coordination with exchangeable cations	Deng et al., 2010
Ca-montmorillonite	0.1	1.0-2.0	Phosphate-buffered solutions at pH 3.5 (gastric conditions), 6.5 (intestinal conditions) and 9, at 37 °C under shaking for 60 min	50[b]	Ion-dipole interactions and coordination with exchangeable cations	Wang et al., 2018
Smectite	1.0	3–10	Incubation in water at 25 °C for 3 days under shaking	3–400	Strong EDA coordination via Ca^{2+}-bridging on the surface	Kang et al., 2016
Illite	1.0	3–20		3–300	A moderate EDA attraction by the negatively charged surface sites	
Kaolinite	1.0	5–30		1–150	Weak H bonding	
Natural zeolite	0.25	0–2	Simulated human digestion solutions	Not reported	Sorption on the external surface	Albayrak et al., 2011
Zeolite	1.5	0.1	Digestion model simulating dynamic gastrointestinal tract of poultry	4.7	Binding mechanisms not determined; possibly electrostatic attractions, EDA attraction, and calcium-bridging linkages	Zavala-Franco et al., 2018
Zeolite	0.5	0.1	Digestion model simulating dynamic gastrointestinal tract of avian species	15.1[d]	Binding mechanisms not determined; possibly electrostatic attractions, EDA attraction, and calcium-bridging linkages	Vázquez-Durán et al., 2021

[a]*Maximum binding capacity (B_{max});* [b]*Maximum adsorption (q_{max});* [c]*Exposure of smectite to aflatoxin repeated twice;* [d]*Maximum adsorption capacity.*

The adsorption capacity and/or the affinity of clays towards mycotoxins and heavy metals can be improved by various treatments. For example, the replacement of inorganic cations with organic cations (usually quaternary ammonium compounds) leads to the formation of organo-clays, which are characterized by weaker interlayer interactions leading to increasing surface area and reduced

hydrophilicity (Baglieri et al., 2013; Liu et al., 2021; Otunola and Ololade, 2020; Wang et al., 2018). Alternatively, calcination (heat treatment at temperature from 600 to 1,000°C) leads to the production of thermally modified clays, characterized by a reduced mass and increased porosity and available surface for ion exchange (Otunola and Ololade, 2020). Moreover, clay minerals can be subjected to acid activation by treatment with hydrochloric, phosphoric, nitric or sulphuric acid, which modifies the material surface and removes cationic impurities, opening pores and edges, increasing available binding sites (Otunola and Ololade, 2020). Smectite minerals can also be exposed to soda activation using sodium carbonate, by which the swelling and adsorption proprieties can be increased (EFSA FEEDAP, 2012).

In dairy ruminants, several clay mineral preparations can be successfully used as mycotoxin adsorbents, as they are effective in reducing aflatoxin M1 (AFM1) excretion in milk (Diaz et al., 2004; Gouda et al., 2019; Maki et al., 2016 a; Maki et al., 2016 b; Maki et al., 2017; Pate et al., 2018; Rodrigues et al., 2019; Smith et al., 1994; Sulzberger et al., 2017). In this view, Na-bentonite seems more effective than Ca-bentonite, probably due to its higher swelling capacity and Na to Ca ratio, that increase the surface area and cation exchange (Diaz et al., 2004). The administration of aluminosilicates to dairy ruminants exposed to aflatoxin containing diets resulted in a corresponding dose-dependent reduction of aflatoxin excretion in milk, urine, and feces (Maki et al., 2016 a; Maki et al., 2016 b; Maki et al., 2017; Pate et al., 2018; Sulzberger et al., 2017).

The improvement of productivity and some pathophysiological indicators can be viewed as indirect evidences of the positive effects of clay administration. For example, clay minerals can promote growth performance in pigs and broilers, and egg production in hens (Liu et al., 2021; Subramaniam and Kim, 2015; Trckova et al., 2004; Valpotić et al., 2017). In pigs, clay administration can successfully counteract the negative effects of AFs, preserving or even improving feed intake, feed efficiency, weight gain, growing performance, and serum clinical chemistry profile (Lindemann et al., 1993; Schell et al., 1993). Different results have been observed after CPL inclusion in the diet of piglets. As reported, CPL caused a significant improvement of feed conversion in the period from weaning to slaughter (Papaioannou et al., 2004). Differently, other authors did not record any improvement of weaned pigs' growth and feed conversion efficiency upon CPL administration with feed (Valpotić et al., 2016). Interestingly, the same authors suggested that CPL was effective as immunomodulatory agent by promoting the recruitment of circulating and intestinal lymphoid cells (Valpotić et al., 2016; Valpotić et al., 2018). Experiments on laying hens reported that the addition of CPL in the diet increased the number of eggs, shell thickness, and the efficiency of feed utilization (Olver M.D., 1997). Other authors observed a significant increase of Al and Si concentrations in serum, possibly related to the improved quality of egg shell and bone development, in hens receiving sodium zeolite A with varying levels of cholecalciferol (Rabon et al., 1995). This may be explained by the partial solubilisation of clays (zeolites) leading to the release of their structural

elements, even if the process should not be feasible at the physiological pH of the animal gastrointestinal tract (Trckova et al., 2004). In broiler chickens, CPL supplementation resulted in increased animal body weight and improved organoleptic meat parameters and ω-3 polyunsaturated fatty acid levels (Mallek et al., 2012). Beneficial effects of aluminosilicate supplementation on performances and health status have been observed also in poultry receiving aflatoxin or ochratoxin contaminated diet, while conflicting results were obtained in case of toxin T-2 contamination (Casarin et al., 2005 a; Casarin et al., 2005 b; Desheng et al., 2005; Goodarzi and Modiri, 2011; Kubena et al., 1993; Kubena et al., 1998). In ruminants, aluminosilicate administration led to improvement of mycotoxin-related health conditions, such as oxidative stress, liver inflammation and damage (Pate et al., 2018; Sulzberger et al., 2017). However, controversial effects on milk yield and quality were observed when Afs were administered with clays, as some authors referred positive effects (Gouda et al., 2019; Pate et al., 2018), while others did not report any effect (Maki et al., 2016 a; Maki et al., 2016 b; Maki et al., 2017; Smith et al., 1994) or even a reduction of milk yield and quality (Sulzberger et al., 2017). In sheep, bentonite administration favored wool growth, which is sensitive to amino acid availability. It is therefore conceivable that bentonite is responsible for a reduction of ruminal protein degradation (Ivan et al., 1992 a). The capability of bentonite to prevent metabolic disorders, increase microbial protein production, and improve rumen pH and fermentation conditions has been considered for explaining the improvement in weight gain and feed consumption efficiency in steers, although high amount of the clay was reported to cause mineral deficiencies due to the high binding capacity (Kianoosh et al., 2012). In dairy heifers, the long-term inclusion of CPL in the diet improved the energy status, milk production, and reproductive parameters, possibly due to beneficial effects on ruminal and/or post-ruminal digestion of starch (Karatzia et al., 2013). In addition, the supplementation of both natural and modified CPL in cow feeding has been observed to improve the energy status and the reproductive performances and to reduce the intramammary infections postpartum (Đuričić et al., 2020 a; Đuričić et al., 2020 b; Đuričić et al., 2020 c; Folnožić et al., 2019 a). Dietary CPL influenced also the blood levels of Ca and P in dairy cows, improving the serum Ca to P ratio during the early post-partum period (Folnožić et al., 2019 b). The reproductive performances were positively affected by the ingestion of mineral adsorbents in cattle (Parmigiani et al., 2013) and sows (Kyriakis et al., 2002) fed with AFB1 contaminated total mixed ration. In addition, clay administration displayed positive effects against some metabolic diseases, such as milk fever and ketosis, due to the ability to reduce Ca availability in the gastrointestinal tract and to improve the energy balance, respectively (Papaioannou et al., 2005). Bentonite was effective in reducing the incidence, but not the severity, of bloat in dairy cows (Carruthers V.R., 1985). Clay administration may also alleviate the effects of subacute ruminal acidosis (SARA), leading to an increase of ruminal and fecal pH and to the modification of rumen volatile fatty acids. In particular, an increase in acetate and a decrease in

propionate and valerate were observed, along with an increased milk yield, milk fat and energy content (Rindig et al., 1969; Sulzberger et al., 2016).

5. Effects of clays on the GI physiology

In the present review, we focused our attention mainly on the potential interactions between clay minerals and cells of the GI system and the GALT.

In this view, the ability of clay minerals to adsorb feed and water contaminants, as well as endogenous produced toxins, may account for most of their beneficial effects. However, there are indications that clays could also improve the production performances of farm animals due to their ability to increase nutrient utilization and exert some positive effects on intestinal physiology, as documented in both humans and animals, even though the underlying mechanisms of action are not fully understood.

Effects of Clays on Nutrient Digestibility

Several mechanisms may account for the positive effects of clay supplementation on the growth performances observed in swine and poultry (Al-Beitawi et al., 2017; Wu et al., 2013; Zhou et al., 2014; Papaioannou et al., 2004). An important feature of clay minerals is represented by the mobility of chemical elements in their interlayers vacancies when they are exposed to environments with different physical-chemical characteristics, such as through the GI tract. Indeed, clays contain different elements, such as Na, K, Mg, Fe, and Ca, which may play particular biochemical roles in improving body weight and feed conversion rate (Al-Beitawi et al., 2017). In this view, clay nanoparticles present chemical and physical properties completely different from those of large-scale (micro) particles, in particular regarding their surface to mass ratio, making ion exchange much more available (Ramìrez-Mella and Hernàndez-Mendo, 2010). As an example, Na^{+}, commonly found in nanoclay minerals, is involved in many fundamental cellular functions, such as acid base balance and the absorption of amino acids (AA) and glucose (Mushtaq et al., 2014).

Thus, clays are potential sources of dietary minerals, either beneficial or potentially dangerous, but the presence of specific minerals in soils or ingested bulk materials does not necessarily guarantee any effect in term of mineral availability. The possible mineral supply obtained by eating clays depends on mineral composition and physicochemical properties, in particular, on CEC of the ingested material (Reilly and Henry, 2000). Specifically, gastric and intestinal environments may play a role in mineral mobility of clays. The effects of chemical leaching during geophagic clay digestion were examined in a series of experiments simulating the gastric and intestinal environments. Although far from being conclusive, results suggested that both beneficial and

dangerous chemical elements could be released from clays during digestion (Tateo and Summa, 2007).

Clay nanoparticles can also increase the intestinal uptake of other nutrients by slowing down the transit rate of the intestinal content through the formation of gels that increase feed viscosity (Ouhida et al., 2000). As a consequence, GI enzymes can be more effective on nutrient digestion (Ouhida et al., 2000). Moreover, an increase of the secretion and/or activity of digestive enzymes were reported thanks to sepiolite or zeolite supplementation (Cabezas et al., 1991; Wu et al., 2013). Different mechanisms were proposed: while zeolite affects intestinal pH, resulting in higher digestive enzyme secretion and activity (Wu et al., 2013), sepiolite forms stable aggregates with pancreatic enzymes, which remain active in a wider range of pH (Cabezas et al., 1991).

The effects of clays on six different kinds of feedstuff were tested in an *in situ* digestion experiment in dairy cows. Clay supplementation altered the degradability of grass hay, wet brewer's grains, soybean meal, and corn silage, and increased nutrient digestible fractions of feedstuffs thanks to their improved degradation, probably due to alteration of rumen microbial population (Hollis et al., 2020).

An increase of the digestibility of dry matter, organic matter, crude protein, ether extract, non-fiber carbohydrates and neutral detergent fibre was observed in lactating goats during clay supplementation when fed a diet naturally contaminated with aflatoxin B1 and zearalenone. However, the study did not establish whether the improved nutrient utilization observed following the administration of clay minerals was an effect of mycotoxin neutralization or a direct effect on the rumen fermentations (Gouda et al., 2019). In sheep, improved nutrient digestibility was observed when bentonite was administered at 4% of the diet (Helal and Abdel-Rahman, 2010; Salem et al., 2001).

Effects of Clays on Gastro-Intestinal Mucosa

Clay administration was observed to influence the intestinal mucosa morphology and function in several animal models.

Morphological modifications and overexpression of proteins involved in lipid metabolism were observed in the enterocyte brush border of rats having free access to kaolinite during refeeding after prolonged fasting (Reichardt et al., 2012). Kaolinite supplemented rats showed an increase in the thickness of the villi with large vacuoles at the base of the mucosal cells and a decrease of the enterocyte microvilli length. Moreover, modifications of the expression level of cytoskeleton proteins were evidenced by proteomic analyses of intestinal mucosa. Few dissociated kaolinite particles and aluminum originating from the ingested clay were observed in the intestinal lumen

and within the mucus barrier. Interestingly, aluminum could directly cross the intestinal mucosa and this should be further investigated, considering the potential neurotoxicity of Al (Reichardt et al., 2009).

Diosmectite, a natural silicate used for the treatment of infectious diarrhea, can adsorb toxins and bacteria and modify the rheological characteristics of gastrointestinal mucus. Diosmectite administration showed anti-inflammatory activity, a general amelioration of the intestinal epithelium morphology, of biomarkers of oxidative stress and a modulatory action of cytokine production by mucosal cells (González et al., 2004). In pigs, in comparison with untreated animals, CPL administration resulted in a higher fraction of lymphoid cell subsets, with exception of CD8+ T cells, and higher recruitment of CD45RA+ (a marker for memory T cells) cells in interfollicular, but not in follicular areas of the ileum Payer's patches (Valpotić et al., 2016).

Clay administration demonstrated protective effects on the intestinal mucosa by physical reinforcing the mucous barrier, the first line of defense during infections, which is possibly responsible for the documented antidiarrheal and anti-inflammatory effects of clays (Dupont and Vernisse, 2009; Song et al., 2012). The slower transit rate of intestinal content, the increase of enzyme activity and morphological changes of the enteric mucosa are among the causes of the reduction of incidence, severity and duration of diarrhea (Dupont C, Vernisse, 2009; Subramaniam and Kim, 2015). A contribution to the anti-diarrheal effects may come from illite and smectite adsorptive properties, which cause a reduction of water and cation enteric excretion leading to the increase in the fecal consistence (Subramian and Kim, 2015). In weaned piglets, dietary supplementation with the hydrous magnesium-aluminum silicate palygorskite (a synonym for attapulgite) improved growth performance and reduced the incidence of diarrhea. Palygorskite administration increased the intestinal villus diameter and lymphocyte number in the jejunum and resulted beneficial to the intestinal integrity (Zhang et al., 2013). Authors suggested that palygorskite might exert its protective action by forming a protective screen on mucosa layer. The protective action of diosmectite on mucus was observed in rats (Leonard et al., 1994; Moré et al., 1987), where diosmectite binds to mucin reducing the pepsin-induced mucolysis due to inflammation. In this case, diosmectite may be included in the adherent intestinal mucus (Dupont and Vernisse, 2009). In addition, an increase in the number and size of mucin-producing goblet cells was observed in pigs fed with smectite (Almeida et al., 2013).

Clay minerals have also been examined as potential tool against SARA. Clay supplementation in cows suffering from SARA increased rumination and pre-stomach pH, reduced blood lactate concentration, and modulated the concentration of liver enzymes (Humer et al., 2019; Sulzberger et al., 2016). These positive effects can be explained by the buffering activity of clays in the GI tract, related to their H^+ adsorption capacity (Jansen van Rensburg et al., 2009), and by the slowing of the transit rate from the rumen. This may influence the rumen and intestinal microbiota, favoring the

production of acetate, and reducing propionate in the rumen, as well as the amount of fermentable carbohydrates in the intestine for post-ruminal fermentations (Rindsig et al., 1969; Sulzberger et al., 2016). Moreover, it was reported that clay supplementation in cattle influenced the concentration of several AA and biogenic amines (BA) depending on the presence of specific ruminal bacteria (Humer et al., 2019). The presence of BA in systemic circulation has deleterious effects on animal health, while AA are essential for the metabolism and immune system. Therefore, clay-dependent modifications in rumen microbiome may improve the maintenance of integrity of nonspecific defenses of the gut wall (Suliman et al., 2005). The enhancement of the defensive capacity of the mucosal immune system has been observed in chickens supplemented with zeolite and attapulgite (palygorskite) by an increase in antioxidant capability and antibacterial activity, as well as in the concentration of secretory immunoglobulin A in jejune mucosa (Zhou et al. 2014).
Finally, effects of clays on the modulation of cytokine production were observed, and anti-inflammatory properties were evidenced (González et al., 2004).
These results suggest that clays can interact with the ruminal and intestinal microbiome (Neubauer et al., 2019), although further investigations are needed to clarify the interconnections.

Effects of Clays on Microbiota

Clays can interact with microorganisms in multiple ways (Cuadros J., 2017), and many of the effects of clays in animals may result from the modulation of the intestinal and/or ruminal microbiota (Chalvatzi et al., 2016; Damato et al., 2021; Damato et al., 2022; Neubauer et al., 2019; Wang et al., 2012). Moreover, clay minerals display antibacterial properties that could be exploited as a potential alternative to the use of antibiotics (Daković et al., 2008; Daković et al., 2012; Liu et al., 2021; Malachová et al, 2009; Merianos J.J., 1991; Slamova et al., 2011). Indeed, montmorillonite can alter the permeability of bacterial cellular membranes allowing the diffusion of intracellular ions and low molecular-weight metabolites (Malachová et al, 2009; Merianos J.J., 1991). As a practical example, the increased growth and elevated organoleptic characteristics of meat in broilers receiving CPL were associated with reduced total gut microbiota and enteric infections (Mallek et al., 2012). Moreover, the antibacterial properties of clays may be selective. Montmorillonite administration can counteract reduced nutrient digestibility, increased oxidative stress, and reduced growth observed in weaning gilts receiving zearalenone. Montmorillonite was beneficial for detoxification of zearalenone, possibly by the selective modification of the intestinal microbiome, resulting in an improved *Lactobacillus* population and a decreased *E. coli* count (Wang et al., 2012). In other syudies, montmorillonite supplementation to pigs promoted the growth of *Lactobacillus* and *Bifidobacterium* at the expense of *Clostridium* and *E. coli* in the small intestine (Subramaniam and Kim, 2015; Song et al., 2012). In sheep, bentonite administration impaired

protozoa viability, leading to the reduction in ruminal degradation of feed protein and bacteria predation in rumen (Ivan et al., 1992 a).

The ion exchange capacity of montmorillonite can be responsible for modification of pH and oxidative state of the intestinal environment, favoring some bacteria over others. Clays could also damage the bacterial cell membrane and adsorb bacterial toxins (Song et al., 2012). The hydrogen bonding between clays (such as diosmectite and kaolin) and enterotoxins of *E. coli*, *V. cholerae*, *Bacteroides fragilis*, and *Clostridium* species prevented the interactions of the toxins to cellular membrane receptors, preventing mucosa damage (Brouillard and Rateau, 1989; Martirosian et al., 1998; Weese et al., 2003). Montmorillonite may also facilitate the flush out of Gram-negative bacteria from the intestine, rather than inhibiting bacteria growth (Malachová et al., 2009). In fact, montmorillonite can adsorb *E. coli* cells by anchoring bacteria to the positively charged sites on its surface (Schiffenbauer and Stotzky, 1982) or by the adhesion of bacteria through the fimbriae located in their cell wall (Malachová et al., 2009).

Clays can modulate the ruminal microbiome also by regulating ammonia availability, as these minerals ammonia as a function of its concentration, functioning as an ammonia buffering system for ruminal microbiome (Gouda et al., 2019; Salem et al., 2001). When rumen microbes are not efficient in capturing ammonia, this molecule is absorbed and converted by the animal liver into urea, which is largely excreted in urine. When urinary urea enters the environment, it breaks down to ammonia and nitrous oxide as environmental pollutants (Ariyarathne et al., 2021). In this context, clay administration could be seen as a feeding management strategy to reduce nitrogen wastage. Thus, mineral adsorbents can be seen as an animal feed and slurry management strategy against the environmental dispersion of nitrogen by reducing ruminal ammonia release, improving its utilization by ruminants, and reducing ammonia volatilization from slurries (Britton et al., 1978; Meisinger et al., 2001; Sulzberger et al., 2016; Trckova et al., 2004).

6. Potential contraindications of the use of clays

So far, the actions of clays against the negative effects and the carryover of mycotoxins and the positive side effects of clays on some production parameters of farm animals have been highlighted. However, it is important to bear in mind that not all studies reported positive effects of clays, which in some cases were ineffective or even responsible of an increase of mycotoxicosis symptoms or other negative side effects (Elliott et al., 2020). For instance, according to Döll and Dänicke (2004) most of the experiments on the ability of sequestering agents to detoxify *Fusarium* toxins carried out in swine and poultry did not demonstrate any preventive effect.

Due to their non-specific mechanism(s) of action, clay minerals can interact with substances different from mycotoxins. Their detoxifying effectiveness depends on the type and concentration

of clay and the specific mycotoxin considered, but is also influenced by other factors, including feed composition, the presence of specific ions and molecules (including proteins, enzymes, and vitamins), and pH (Barrientos-Velázquez et al., 2016; Magnoli et al., 2013). Indeed, clay minerals are able to interact with metabolites and nutrients, such as nucleic acids, AA, and proteins by cation exchange, electrostatic interactions, hydrophobic/hydrophilic interactions, hydrogen bonding, and van der Waals forces (Hedges and Hare, 1987; Yu et al., 2013). These interactions are commonly considered beneficial, as they are related to higher amount of bypass proteins and more efficient utilization of AA in ruminants (Colling D.P., 1975). The possible application of clays as vectors for gene and drug delivery was also suggested (Yu et al., 2013). However, clay minerals can be responsible for imbalances of essential nutrients and can interact with hormones and veterinary drugs, leading to negative effects on animal health and productive parameters (Elliott et al., 2020). *In vitro* experiments simulating the ingestion of geophagic soils/clay minerals demonstrated that these additives can reduce the bioavailability of some essential elements, such as Fe, Cu, and Zn (Hooda et al., 2004; Seim et al., 2013), and can enhance the bioavailability of others, such as Ca, Mg, and Mn (Hooda et al., 2004). A series of experiments in rats fed with a diet supplemented with 2% bentonite showed that this clay improved body weight (BW) gain and stimulated Fe absorption, but caused a moderate persistent decrease in the absorption and organ content of Ca and Se (Grosicki and Kowalski, 2003; Grosicki et al., 2004; Grosicki and Rachubik, 2005). Despite the deficiencies reported in these studies were moderate, it must be considered that Se intake is marginal strongly depend on geographical region (Grosicki and Rachubik, 2005). However, other studies in rats did not find any relevant mineral alteration following the dietary administration of processed calcium montmorillonite (up to 2%), neither in pregnant animals (Wiles et al., 2004) nor after long treatments (28 weeks) (Afriyie-Gyawu et al., 2005). Also the inclusion of up to 2% Na-bentonite in rat diet did not show signs of toxicity even if administered for 3 months (Marroquín-Cardona et al., 2022). Regarding farm animals, FEEDAP EFSA (2011a; 2011b) reported a reduction of Mn availability in poultry treated with 0.5% bentonite and Franciscato et al. (2006) noted that 0.5% sodium montmorillonite was responsible of a decrease in P serum content in broiler chickens. In contrast, diet supplementation with 0.5 and 1.0% hydrated sodium calcium alumino-silicate (HSCAS) in chicks did not compromise the utilization of Mn, phytate, and inorganic P, as well as of vitamin A and riboflavin, but caused a slight decrease of Zn utilization (Chung and Baker, 1990; Chung et al., 1990). Excessive administration of bentonite (≥ 2%) in chicks can induce poor weight gain and overt signs of vitamin A deficiency up to an increase in mortality (by 4 weeks administration of 5% bentonite) (Briggs and Spivey Fox, 1956). Moreover, sodium bentonite affected testosterone and thyroid hormone levels in male broiler chicks (Eraslan et al., 2005). Diet supplementation with zeolite in laying hens could be harmful for the formation of the eggshell, since 1, 2, and 3% administration resulted in increased Zn and Al levels and decreased Mg and Cu

concentrations in serum (Utlu et al., 2007). However, Rizzi et al. (2003) did not observe any considerable alteration of egg quality due to dietary administration of CPL (2%) in laying hens. Excessive administration of montmorillonite can impair growth performance and health in starter pigs: inclusion of more than 1% in the ration had negative effects on liver structure and serum mineral concentrations and 5% montmorillonite caused a decrease in feed intake, aggravation of liver damage, and a reduced antioxidant capacity (Zhao et al., 2017). In female weaned piglets, a diet with 0.4 % aluminosilicate failed to counteract the effects of *Fusarium* toxins, tended to reduce feed intake and feed to gain ratio, decreased serum concentrations of cholesterol and α-tocopherol, increased levels of albumin, aspartate transaminase, and γ-glutamyl transferase, but it did not affect the concentrations of retinol and retinyl esters in liver and serum (Döll et al., 2005).

Undesirable effects of clay mineral administration have also been reported in ruminants. In rams, bentonite decreased the ruminal availability of Cu, Zn, and Mg and liver concentrations of Cu (Ivan et al., 1992 b). However, considering the predisposition of sheep to Cu poisoning, the reduction of dietary Cu bioavailability could be seen as a positive effect of bentonite (Ivan et al., 1992 b). Feeding growing goats with diet supplemented with zeolite at 0.12 and 0.16 % for 3 weeks, Schwaller et al. (2016) recorded slight alterations of Ca homeostasis, signs of increased bone resorption without alterations of bone structure. The treatment led also to the significant decline in plasma concentrations of P, Mg, and 1,25-dihydroxycholecalciferol and in renal excretion of P, warning of possible negative long-term effects. The addition of micronized zeolite up to 2% to lamb feed did not affect animal performance and carcass yield, but affected serum total protein, calcium and phosphorus concentrations. Increasing zeolite dose to 3% led to a decrease of slaughter weight, hot and cold carcass weights (Toprak et al., 2016). Bentonite administration at 5 and 10% in Holstein cows fed with high-grain ration was responsible for a significant decrease of energy and crude protein digestibility and affected Ca, Mg, and P balances with a statistically significant decrease of Mg and P (Rindsig and Schultz, 1970). However, lower concentrations of bentonite (0.125, 0.25, 0.5, and 1%) were sufficient to counteract AFs effects and did not alter dairy cows' health status and production [bodyweight, body condition score (BCS), dry matter intake, milk yield, milk quality and composition, minerals, vitamin A, and riboflavin concentrations in milk] (Maki et al., 2016 a; Maki et al., 2016 b; Maki et al., 2017).

In humans, 2 weeks oral treatment with 1.5 or 3.0 g/die of NovaSil caused in the first 2 days gastrointestinal symptoms (bloating, constipation, diarrhea, flatulence, and abdominal pain) in 24 and 28% volunteers, respectively, and dizziness in two subjects receiving 1.5 g NovaSil. However, no statistical significance was found of these adverse effects between the two groups. After treatment, statistically significant decrease (within the normal range of clinical references) in blood levels of RBCs (red blood cells), hemoglobin, total protein, albumin, ALT (alanine amino-transferase), and S were recorded in the low-dose group, but not in the high-dose group. A

significant dose-dependent increase of serum Sr was observed. No other significant differences in hematology, liver and kidney function, and in electrolyte, mineral, vitamins A and E concentrations were found in both groups. According to authors, results suggest the relative safety of NovaSil clay in humans (Wang et al., 2005).

Regarding the interactions between adsorbing agents and drugs/pharmaceuticals, binding phenomena to clays can enhance or reduce the effects of drugs (Elliott et al., 2020; EFSA FEEDAP, 2011; EFSA FEEDAP, 2011b). For example, tylosin was ineffective in cattle when bentonite was concomitantly administered (Canadian Bureau of Veterinary Drugs, 1992). Similarly, bentonite led to reduced or even canceled efficacy of tilmicosin in poultry (Shryock et al., 1994). Devreese et al. (2012) demonstrated, by administering tylosin in broiler chickens, that bentonite is able to bind the macrolide antibiotic and alter its pharmacokinetics. Moreover, bentonite can reduce the effectiveness of coccidiostats, such as monensin and salinomycin (EFSA FEEDAP, 2011; Gray et al., 1998).

Altogether, findings here reported evidence that further studies are need to investigate on interactions between clays and biological relevant nutrients and drugs. In addition, these findings should suggest the adoption of specific control strategies and animal health practice, modifying animal diet, dosages and withdrawal times of drugs and/or adsorbent additives, in order to prevent cases of toxicity or nutrient deficiency/antibiotic ineffectiveness (related to possible enhancement of microbial resistance development) in animals and to safeguard public health. EFSA has already proposed to ban the simultaneous use of coccidiostats when bentonite is administered above 0.5% and recommended to report the information on the label of bentonite packaging to avoid its oral use concomitantly with certain medicinal substances (e.g., macrolides) (EFSA FEEDAP, 2011). Analogously, the Food and Drug Administration (FDA, 1969, 1971, 1972) has ruled not to use robenidine, ipronidazole, and buquinolate in combination with bentonite.

7. Conclusions and future perspectives

The use of clay minerals in the agri-food sector seems destined to increase for several reasons: the first is the adoption of prevention and decontamination strategies able to minimize food and feed contamination by mycotoxins and, consequently, reduce health risks for humans and animals and economic losses. Likely, foreseen climate changes will affect mycotoxin production and distribution in different world regions and these toxins are predicted to become a major problem in Europe within the next 100 years (Battilani et al., 2016). Indeed, climate will become milder in northern countries, making these areas suitable for fungal growth and mycotoxin production. At the same time, heat and drought in southern countries will cause a decrease in agricultural production up to

desertification and a replacement of currently prevalent mycotoxins with AFs (Medina et al., 2017; Kovalsky Paris et al., 2015; Paterson and Lima, 2011; Pinotti et al., 2016).

Heavy metals are naturally present in soil and atmosphere, fresh and salty waters. Moreover, human activities (e.g., mining, industrial production, waste dumping, etc.) significantly contribute to heavy metal pollution (Babula et al., 2008; Briffa et al., 2020). In addition, certain agricultural and farming activities can be considered either as a source of heavy metals or can contribute to their recirculation (Briffa et al., 2020; Hejna et al., 2018). The administration of clay minerals can be applied for controlling heavy metals absorption by farm animals.

Finally, an increased use of clay minerals can be expected for their putative contribution to fight against various problems linked to intensive farming (e.g., antibiotic resistance, SARA, greenhouse gases and ammonia emission, etc.), which represent a huge economic problem for farmers and involve the consumers' sensitivity toward issues, such as animal wellbeing and environmental sustainability.

National and international authorities set the maximum limits or guide levels for the use of clays and clay minerals as feed additives, which may be an ideal choice given their physical-chemical properties, low cost, low or null toxicity, and eco-compatibility. However, it must be considered that clay minerals are not completely inert additives, and can interfere with intestinal/ruminal metabolism with possible consequences on animal health. Most studies on clay minerals, aimed at evaluating their effectiveness against toxic compounds, paid poor attention toward any possible nonspecific collateral effect. Furthermore, many studies did not report in detail the characteristics of the clay used, making the interpretation of results difficult. Other studies examined mostly productive parameters, which, as well, may not be sufficient to provide an exhaustive picture of the possible unwanted effects of clays on animal health and physiology (Döll and Dänicke, 2004; Elliott et al., 2020). Therefore, further studies are needed, particularly on ruminants, to verify possible interferences of clays with rumen fermentations and metabolite uptake, which may affect animal metabolism and, possibly, milk characteristics. In particular, future studies should consider the effects of long-term administration/accumulation of clays. Finally, the fate of clay particles during their transit within the GI system should be investigated as, although clay micro and nanosize particles seem not to be cytotoxic at the intestinal level, their capacity to stimulate an inflammatory response should be carefully considered and their effects on already diseased subjects should be taken into account.

8. References

Abebe, E., Gugsa, G., Ahmed, M., 2020. Review on major food-borne zoonotic bacterial pathogens. *J Trop Med*. 2020, 4674235. doi: 10.1155/2020/4674235

Abollino, O., Aceto, M., Malandrino, M., Sarzanini, C., Mentasti, E., 2003. Adsorption of heavy metals on Na-montmorillonite. Effect of pH and organ substances. *Water Research*. 37(7), 1619–1627. doi: 10.1016/S0043-1354(02)00524-9

Adamis, Z., Williams, R.B., and International Programme on Chemical Safety, 2005. Bentonite, kaolin and selected clay minerals. World Health Organization. Available online: https://apps.who.int/iris/handle/10665/43102

Adegbeye, M.J., Reddy, P.R.K., Chilaka, C.A., Balogun, O.B., Elghandour, M.M.M.Y., Rivas-Caceres, R.R., Salem, A.Z.M., 2020. Mycotoxin toxicity and residue in animal products: prevalence, consumer exposure and reduction strategies—A review. *Toxicon*. 177, 96–108. doi: 10.1016/j.toxicon.2020.01.007

Afriyie-Gyawu, E., Mackie, J., Dash, B., Wiles, M., Taylor, J., Huebner, H., Tang, L., Guan, H., Wang, J.-S., Phillips, T. Dr., 2005. Chronic toxicological evaluation of dietary NovaSil Clay in Sprague-Dawley rats. *Food Additives & Contaminants*. 22(3), 259–269. doi: 10.1080/02652030500110758

Agriopoulou, S., Stamatelopoulou, E., Varzakas, T., 2020. Advances in occurrence, importance, and mycotoxin control strategies: prevention and detoxification in foods. *Foods*. 9(2), 137. doi: 10.3390/foods9020137

Al-Beitawi, N.A., Shaker, M.M., El-Shuraydeh, K.N., Bláha, J., 2017. Effect of nanoclay minerals on growth performance, internal organs and blood biochemistry of broiler chickens compared to vaccines and antibiotics. *J Appl Anim Res*. 45(1), 543–549. doi: 10.1080/09712119.2016.1221827

Alali, W.Q., Ricke, S.C., 2012. The ecology and control of bacterial pathogens in animal feed. In: Johanna Fink-Gremmels (ed.), *Woodhead Publishing Series in Food Science, Technology and Nutrition, Animal Feed Contamination*. Woodhead Publishing, pp. 35-55. doi: 10.1533/9780857093615.1.35

Albayrak, C.B., Narin, G., Ulku, S., 2011. Adsorption of Aflatoxin B1 on the local natural zeolite. 4th International Conference on Medical Geology, Italy. Available online: http://www.cprm.gov.br/publique/media/gestao_territorial/geologia_medica/bari_italia/4.pdf

Alloway, B.J., 2013. Heavy Metals and Metalloids as Micronutrients for Plants and Animals. In: Alloway, B. (eds), *Heavy Metals in Soils. Environmental Pollution*, 22. Springer, Dordrecht. 195–209. doi: 10.1007/978-94-007-4470-7_7

Almeida, J.A.S., Liu, Y., Song, M., Lee, J.J., Gaskins, R., Maddox, C.W., Osuna, O., Pettigrew J.E., 2013. *Escherichia coli* challenge and one type of smectite alter intestinal barrier of pigs. *J Animal Sci Biotechnol*. 4 (52), 1–8. doi: 10.1186/2049-1891-4-52

Amari, A., Mohammed Alzahrani, F., Mohammedsaleh Katubi, K.; Salem Alsaiari, N., Tahoon, M.A., Ben Rebah, F., 2021. Clay-Polymer Nanocomposites: Preparations and Utilization for Pollutants Removal. *Materials*, 14(6), 1365, 1-21. doi.org/10.3390/ma14061365

Ariyarathne, H.B.P.C., Correa-Luna, M., Blair, H., Garrick, D., Lopez-Villalobos, N., 2021. Can nitrogene excretion of dairy cows be reduced by genetic selection for low milk urea nitrogen concentration? *Animals*, 11(3), 737. doi: 10.3390/ani11030737

Aytas, S., Yurtlua, M., Donat, R., 2009. Adsorption characteristic of U(VI) ion onto thermally activated bentonite. *Journal of Hazardous Materials*, 172(2-3), 667–674. doi: 10.1016/j.jhazmat.2009.07.049

Babula, P., Adam, V., Opatrilova, R., Zehnalek, J., Havel, L., Kizek, R., 2008. Uncommon heavy metals, metalloids and their plant toxicity: a review. *Environ Chem Lett*. 6, 189–213. doi: 10.1007/s10311-008-0159-9

Baek, M., Lee, J.A., Choi, S.J., 2012. Toxicological effects of a cationic clay, montmorillonite *in vitro* and *in vivo*. *Mol Cell Toxicol*. 8, 95–101. doi: 10.1007/s13273-012-0012-x

Baglieri, A., Reyneri, A., Gennari, M., Nègre, M., 2013. Organically modified clays as binders of fumonisins in feedstocks. *J Environ Sci Health,* Part B Pesticides, Food Contaminants, and Agricultural Wastes. 48(9), 776–783. doi: 10.1080/03601234.2013.780941

Barrientos-Velázquez, A.L., Arteaga, S., Dixon, J.B., Deng, Y., 2016. The effects of pH, pepsin, exchange cation, and vitamins on aflatoxin adsorption on smectite in simulated gastric fluids. *Appl Clay Sci*. 120, 17–23. doi: 10.1016/j.clay.2015.11.014

Barton, C.D., Karathanasis, A.D., 2002. Clay minerals. In: Lal, R. (ed.), *Encyclopedia of Soil Science*. Marcel Dekker, New York, USA, pp. 187-192. Available online at: https://www.srs.fs.usda.gov/pubs/ja/ja_barton002.pdf

Battilani, P., Toscano, P., Van Der Fels-Klerx, H.J., Moretti, A., Camardo Leggieri, M., Brera, C., Rortais, A., Goumperis, T., Robinson, T., 2016. Aflatoxin B_1 contamination in maize in Europe increases due to climate change. *Sci Rep*. 6, 24328. doi: 10.1038/srep24328

Becker-Algeri, T.A., Castagnaro, D., de Bortoli, K., de Souza, C., Drunkler, D.A., Badiale-Furlong, E., 2016. Mycotoxins in bovine milk and dairy products: a review. *J Food Sci*. 81(3), R544–R552. doi: 10.1111/1750-3841.13204

Bellmann, S., Carlander, D., Fasano, A., Momcilovic, D., Scimeca, J.A., Waldman, W.J., Gombau, L., Tsytsikova, L., Canady, R., Pereira, D.I.A., Lefebvre, D.E., 2015. Mammalian gastrointestinal tract parameters modulating the integrity, surface properties, and absorption of food-relevant nanomaterials. *WIREs Nanomed Nanobiotechnol*. 7(5), 609–622. doi: 10.1002/wnan.1333

Benne, N., van Duijn, J., Kuiper, J., Jiskoot, W., Slütter, B., 2016. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. *J Controll Release*. 234, 124–134. doi: 10.1016/j.jconrel.2016.05.033

Bennett, J.W., 1987. Mycotoxins, mycotoxicoses, mycotoxicology and Mycopathologia. *Mycopathologia*. 100, 3–5. doi: 10.1007/BF00769561

Bennett, J.W., Klich, M., 2003. Mycotoxins. *Clin Microbiol Rev*. 16(3), 497–516. doi: 10.1128/CMR.16.3.497–516.2003

Briffa, J., Sinagra, E., Blundell, R., 2020. Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon*. 6(9), e04691. doi: 10.1016/j.heliyon.2020.e04691

Brigatti, M.F., Galán, E., Theng, B.K.G., 2013. Structure and mineralogy of clay minerals. *Develop Clay Sci.* 5, 21–81. doi: 10.1016/B978-0-08-098258-8.00002-X

Briggs, G.M., Spivey Fox, M.R., 1956. Vitamin A deficiency in chicks produced by adding high levels of bentonite to synthetic diets. *Poult Sci*. 35(3), 570–576. doi: 10.3382/ps.0350570

Britton, R.A., Colling, D.P., Klopfenstein, T.J., 1978. Effect of complexing sodium bentonite with soybean meal or urea in vitro ruminal ammonia release and nitrogen utilization in ruminants. *J Anim Sci*. 46(6), 1738–1747. doi: 10.2527/jas1978.4661738x

Brouillard, M.Y., Rateau, J.G., 1989. Pouvoir d'adsorption de deux argiles, la smectite et le kaolin sur des entérotoxines bactériennes. Etude in vitro sur culture cellulaire et sur intestin de souriceau nouveau-né [Adsorption potency of 2 clays, smectite and kaolin on bacterial enterotoxins. In vitro study in cell culture and in the intestine of newborn mice]. *Gastroenterol Clin Biol*. 13(1), 18–24.

Cabezas, M.J., Salvador, D., Sinisterra, J.V., 1991. Stabilization-activation of pancreatic enzymes adsorbed on to a sepiolite clay. *J Chem Technol Biotechnol*. 52(2), 265–274. doi: 10.1002/jctb.280520213

Canadian Bureau of Veterinary Drugs, 1992. Suspected drug adverse reactions reported to The Bureau of Veterinary Drugs. *Can Vet J*. 33(4), 237–244. Available online at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1481214/pdf/canvetj00053-0031.pdf

Carr, K.E., Smyth, S.H., McCullough, M.T., Morris, J.F., Moyes, S.M., 2012. Morphological aspects of interactions between microparticles and mammalian cells: intestinal uptake and onward movement. *Prog Histochem Cytochem*. 46(4), 185–252. doi: 10.1016/j.proghi.2011.11.001

Carruthers, V.R., 1985. Effect of bentonite on incidence of bloat, milk production, and mineral status in dairy cows. *N Z J Agricult Res*. 28(2), 221–223. doi: 10.1080/00288233.1985.10420931

Casarin, A., Forat, M., Soto, E., Contreras, M., Zaviezo, D., 2005 a. Evaluation of the efficacy of a commercial hydrated sodium calcium aluminosilicate to reduce the toxicity of aflatoxin and ochratoxin in broiler chicks. In: 4th Annual Meeting abstracts, Poultry Science Association. p. S171.

Casarin A, Forat M, Soto E, Fazekas B, Tanyi J, Zaviezo D., 2005 b. Evaluation of the efficacy of a commercial hydrated sodium calcium aluminosilicate to reduce the toxicity of toxin T-2 in broiler chicks. In: 4th Annual Meeting abstracts, Poultry Science Association. p. S177.

Chalvatzi, S., Kalamaki, M.S., Arsenos, G., Fortomaris, P., 2016. Dietary supplementation with the clay mineral palygorskite affects performance and beneficially modulates caecal microbiota in laying pullets. *Journal of Applied Microbiology*, 120(4), 1033–1040. doi: 10.1111/jam.13041

Chantawong, V., Harvey, N.W., Bashkin, V.N., 2003. Comparison of Heavy Metal Adsorptions by Thai Kaolin and Ballclay. *Water, Air, & Soil Pollution*, 148, 111–125. doi: 10.1023/A:1025401927023

Chen, S., Guo, H., Cui, M., Huang, R., Su, R., Qi, W., He, Z., 2020. Interaction of particles with mucosae and cell membranes. *Colloids Surf B: Biointer*. 186, 110657. doi: 10.1016/j.colsurfb.2019.110657

Chung, T.K., Baker, D.H., 1990. Phosphorus utilization in chicks fed hydrated sodium calcium aluminosilicate. *J Anim Sci*. 68(7), 1992–1998. doi: 10.2527/1990.6871992x

Chung, T.K., Erdman, J.W.JR., Baker, D.H. 1990. Hydrated sodium calcium aluminosilicate: effects on zinc, manganese, vitamin A, and riboflavin utilization. *Poult Sci*. 69(8), 1364–1370. doi: 10.3382/ps.0691364

Colling, D.P., 1975. Sodium Bentonite in Ruminant Rations. Thesis (M.S.), University of Nebraska, Lincoln, Department of Animal Science. Available online at: https://digitalcommons.unl.edu/cgi/viewcontent.cgi?article=1008&context=opentheses

Connolly, M., Zhang, Y., Mahri, S., Brown, D.M., Ortuño, N., Jordá-Beneyto, M., Maciaszek, K., Stone, V., Fernandes, T.F., Johnston, H.J., 2019. The influence of organic modification on the cytotoxicity of clay particles to keratinocytes, hepatocytes and macrophages; an investigation towards the safe use of polymer-clay nanocomposite packaging. *Food Chem Toxicol*. 126, 178–191. doi: 10.1016/j.fct.2019.02.015

Cuadros, J., 2017. Clay minerals interaction with microorganisms: A review. *Clay Miner*. 52(2), 235–261. doi: 10.1180/claymin.2017.052.2.05

Daković, A., Matijašević, S., Rottinghaus, G.E., Ledoux, D.R., Butkeraitis, P., Sekulić, Ž., 2008. Aflatoxin B_1 adsorption by natural and copper modified montmorillonite. *Coll Surf B: Biointerfaces*. 66(1), 20–25. doi: 10.1016/j.colsurfb.2008.05.008

Daković, A., Kragović, M., Rottinghaus, G.E., Ledoux, D.R., Butkeraitis, P., Vojislavljević, D.Z., Zarić, S.D., Stamenić, L., 2012. Preparation and characterization of zinc-exchanged montmorillonite and its effectiveness as aflatoxin B_1 adsorbent. *Mater Chem Phys*. 137(1), 213–220. doi: 10.1016/j.matchemphys.2012.09.010

Damato A, Vanzani P, Giaretta E, Novelli E, Gianesella M, Zennaro L., 2021. Possible interferences of bentonite on rumen metabolome highlighted by 1 H-NMR spectroscopy in dairy Holstein cows. Proceedings of 74th Convegno SISVet, p. 295.

Damato, A., Vanzani, P., Giannuzzi, D., Giaretta, E., Novelli, E., Vianello, F., Tagliapietra, F., Zennaro, L., 2022. Bentonite does not affect *in vitro* ruminal gross fermentations but could modify ruminal metabolome and mineral content. A proof of concept. *Res Vet Sci*. 144, 78–81. doi: 10.1016/j.rvsc.2022.01.012

Deng, Y., Velázquez, A.L.B., Billes, F., Dixon, J.B., 2010. Bonding mechanisms between aflatoxin B_1 and smectite. *Appl Clay Sci*. 50(1), 92–98. doi: 10.1016/j.clay.2010.07.008

Dening, T.J., Joyce, P., Webber, J.L., Beattie, D.A., Prestidge, C.A., 2018. Inorganic surface chemistry and nanostructure controls lipolytic product speciation and partitioning during the digestion of inorganic-lipid hybrid particles. *J Colloid Interface Sci*. 532, 666–679. doi: 10.1016/j.jcis.2018.08.015

Denli, M., 2015. Implications of mycotoxins in livestock feeds. AgroLife Sci J. 4(1), 52–55. Available online at: https://agrolifejournal.usamv.ro/pdf/vol.IV_1/Art6.pdf

Desheng, Q., Fan, L., Yanhu, Y., Niya, Z., 2005. Adsorption of aflatoxin B_1 on montmorillonite. *Poult Sci*. 84(6), 959–961. doi: 10.1093/ps/84.6.959

Devreese, M., Osselaere, A., Goossens, J., Vandenbroucke, V., De Baere, S., De Backer, P., Croubels, S., 2012. Interaction between tylosin and bentonite clay from a pharmacokinetic perspective. *Vet J*. 194(3), 437–439. doi: 10.1016/j.tvjl.2012.05.016

Diaz, D.E., Hagler, W.M., Blackwelder, J.T., Eve, J.A., Hopkins, B.A., Anderson, K.L., Jones, F.T., Whitlow, L.W., 2004. Aflatoxin Binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. *Mycopathologia*. 157, 233–241. doi: 10.1023/B:MYCO.0000020587.93872.59

Diaz, D.E., and Smith, T.K, 2005. Mycotoxin sequestering agents: practical tools for the neutralisation of mycotoxins. In: Diaz, D.E. (ed.), *The Mycotoxin Blue Book*. Nottingham University Press, Nottingham, United Kingdom, pp. 323–339.

Di Gregorio, M.C., de Neeff, D.V., Jager, A.V., Corassin, C.H., de Pinho Carão, Á.C., de Albuquerque, R., de Azevedo, A.C., Oliveira, C.A.F., 2014. Mineral adsorbents for prevention of mycotoxins in animal feeds. *Toxin Rev*. 33(3), 125–135. doi: 10.3109/15569543.2014.905604

Dogan, A.U., Dogan, M., Onal, M., Sarikaya, Y., Aburub, A., Wurster, D.E., 2006. Baseline studies of The Clay Minerals Society source clays: Specific surface area by Brunauer Emmett Teller (BET) method. *Clays Clay Miner*. 54, 62–66. doi: 10.1346/CCMN.2006.0540108

Döll, S., Dänicke, S., 2004. *In vivo* Detoxification of *Fusarium* toxins. *Arch Anim Nutr*. 58(6), 419–441. doi: 10.1080/00039420400020066

Döll, S., Gericke, S., Dänicke, S., Raila, J., Ueberschär, K.-H., Valenta, H., Schnurrbusch, U., Schweigert, F.J., Flachowsky, G., 2005. The efficacy of a modified aluminosilicate as a detoxifying agent in *Fusarium* toxin contaminated maize containing diets for piglets. *J Anim Physiol Anim Nutr*. 89(9-10), 342–358. doi: 10.1111/j.1439-0396.2005.00527.x

Dominy, N.J., Davoust, E., Minekus, M., 2004. Adaptive function of soil consumption: an in vitro study modeling the human stomach and small intestine. *J Exp Biol*, 207(Pt 2), 319-24. doi: 10.1242/jeb.00758.

Dupont, C., and Vernisse, B., 2009. Anti-diarrheal effects of diosmectite in the treatment of acute diarrhea in children. *Pediatric Drugs*. 11, 89–99. doi: 10.2165/00148581-200911020-00001

Đuričić, D., Sukalić, T., Marković, F., Kočila, P., Žura Žaja, I, Menčik, S., Dobranić, T., Benić, M., Samardžija, M., 2020 a. Effects of dietary vibroactivated clinoptilolite supplementation on the intramammary microbiological findings in dairy cows. *Animals*. 10, 202. doi: 10.3390/ani10020202

Đuričić, D., Vince, S., Lojkić, M., Jelušić, S., Turk, R., Valpotić, H., Gračner, D., Maćešić, N., Folnožić, I., Šostar, Z., Samardžija, M., 2020 b. Effects of dietary clinoptilolite on reproductive performance, serum progesterone and insulin-like growth factor-1 concentrations in dairy cows during pregnancy and lactation. *Pol J Vet Sci*. 23(1), 69–75. doi: 10.24425/pjvs.2020.132750

Đuričić, D., Ljubić, B.B., Vince, S., Turk, R., Valpotić, H., Žura Žaja, I., Maćešić, N., Benić, M., Getz, I., Samardžija, M., 2020 c. Effects of dietary clinoptilolite supplementation on β-hydroxybutirate serum level and milk fat to protein ratio during early lactation in Holstein-Friesian cows. *Microporous Mesoporous Mat*. 292, 109766. doi: 10.1016/j.micromeso.2019.109766

EFSA FEEDAP (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011. Scientific Opinion on the safety and efficacy of bentonite (dioctahedral montmorillonite) as feed additive for all species. EFSA J., 9(2), 2007. doi:10.2903/j.efsa.2011.2007

EFSA FEEDAP (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2011b. Scientific Opinion on the efficacy of Bentonite (dioctahedral montmorillonite) for all species. EFSA J., 9(6), 2276. doi:10.2903/j.efsa.2011.2276

EFSA FEEDAP (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2012. Scientific Opinion on the safety and efficacy of bentonite as a technological feed additive for all species. EFSA J., 10(7), 2787. doi:10.2903/j.efsa.2012.2787

EFSA FEEDAP (EFSA Panel on Additives and Products or Substances usedin Animal Feed), Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M.L., Bories, G., Chesson, A., Cocconcelli, P.S., Flachowsky, G., Gropp, J., Kolar, B., Kouba, M., Lopez-Alonso, M., Mantovani, A., Mayo, B., Ramos, F., Saarela, M., Villa, R.E., Wallace, R.J., Wester, P., Martelli, G., Renshaw, D., Lopez-Galvez, G., and Lopez Puente, S., 2017. Scientific Opinion on the Safety and efficacy of bentonite as a feed additive for all animal species. *EFSA J*. 15(12), e05096. doi: 10.2903/j.efsa.2017.5096

Elliott, C.T., Connolly, L., Kolawole, O., 2020. Potential adverse effects on animal health and performance caused by the addition of mineral adsorbents to feeds to reduce mycotoxin exposure. *Mycotoxin Res*. 36(1), 115–126. doi: 10.1007/s12550-019-00375-7

Eraslan, G., Essiz, D., Akdogan, M., Sahindokuyucu, F., Altintas, L., Hismiogullari, S.E., 2005. Effects of dietary aflatoxin and sodium bentonite on some hormones in broiler chickens. *Bull Vet Inst Pulawy*. 49, 93–96. Available online at: https://www.researchgate.net/profile/Sahver-Hismiogullari/publication/228624856_Effects_of_dietary_aflatoxin_and_sodium_bentonite_on_some_ho rmones_in_broiler_chickens/links/5df3751192851c83647940e9/Effects-of- dietary-aflatoxin-and-sodium-bentonite-on-some-hormones-in-broiler-ch ickens.pdf

European Commission, 2020. The Rapid Alert System for Food Feed (RASFF) Annual Report 2019. Luxembourg: Publications Office of the European Union. Available online at: https://op.europa.eu/en/publication-detail/-/publication/2c5c7729-0c31-11eb-bc07-01aa75ed71a1/language-en/format-PDF/source-174742448 (accessed January 15, 2022).

European Commission, 2021. European Union Register of feed Additives. Pursuant to Regulation (EC) no 1831/2003. Annex I: List of Additives, Edition 06/2021 (294). Ed. Publications Office of the European Union, Luxembourg. doi: 10.2875/699878

Faisal, H.M.N., Katti, K.S., Katti, D.R., 2021. Molecular mechanics of the swelling clay tactoid under compression, tension and shear. *Appl Clay Sci*. 200, 105908. doi: 10.1016/j.clay.2020.105908

FDA (Food and Drug Admonistration), 1969. Food additives. Buquinolate. Washington, DC, USA. Federal Register, 34(183), 14733. Available online at: https://upload.wikimedia.org/wikipedia/commons/f/f5/Federal_Register_1969-09-24-_Vol_34_Iss_183_%28IA_sim_federal-register-find_1969-09-24_34_183%29.pdf

FDA (Food and Drug Admonistration), 1971. New animal drugs for use in animal feeds. Ipronidazole. Washington, DC, USA. Federal Register. 36(195), 12901. Available online at: https://www.govinfo.gov/content/pkg/FR-1971-10-07/pdf/FR-1971-10-07.pdf

FDA (Food and Drug Admonistration), 1972. New animal drugs for use in animal feeds. Robenidine hydrochloride. Washington, DC, USA. Federal Register. 37(244), 27621. Available online at: https://www.govinfo.gov/content/pkg/FR-1972-12-19/pdf/FR-1972-12-19.pdf

Foged, C., Brodin, B., Frokjaer, S., Sundblad, A., 2005. Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. *Int J Pharm*. 298(2), 315–322. doi: 10.1016/j.ijpharm.2005.03.035

Folnožić, I., Samardžija, M., Đuričić, D., Vince, S., Perkov, S., Jelušić, S., Valpotić, H., Ljubić, B.B., Lojkić, M., Gračner, D., Žura Žaja, I., Maćešić, N., Grizelj, J., Dobranić, T., Redžepi, G., Šostar, Z., Turk, R., 2019 a. Effects of in-feed clinoptilolite treatment on serum metabolic and antioxidative biomarkers and acute phase response in dairy cows during pregnancy and early lactation. *Res Vet Sci*. 127, 57–64. doi: 10.1016/j.rvsc.2019.10.010

Folnožić, I., Đuričić, D., Žura Žaja, I., Vince, S., Perkov, S., Turk, R., Valpotić, H., Gračner, D., Maćešić, N., Lojkić, M., Kovačić, M., Samardžija, M., 2019 b. The influence of dietary clinoptilolite on blood serum mineral profile in dairy cows. *Vet Arh*. 89(4), 447–462. doi: 10.24099/vet.arhiv.0662

Franciscato, C., Lopes, S.T.d.A., Santurio, J.M., Wolkmer, P., Maciel, R.M., de Paula, M.T., Garmatz, B.C., Costa, M.M., 2006. Seric mineral concentrations and hepatic and renal functions of chickens

intoxicated by aflatoxin and treated with sodic montmorillonite. *Pesquisa Agropecuária Brasileira*. 41(11), 1573–7. doi: 10.1590/S0100-204X2006001100001

Fubini, B., Fenoglio, I., Tomatis, M., Turci, F., 2011. Effect of chemical composition and state of the surface on the toxic response to high aspect ratio nanomaterials. *Nanomedicine*. 6(5), 899–920. doi: 10.2217/nnm.11.80

García-Romero, E., Lorenzo, A., García-Vicente, A., Morales, J., García-Rivas, J., Suárez, M., 2021. On the structural formula of smectites: a review and new data on the influence of exchangeable cations. *J Appl Crystallogr*. 54, 251–262. doi: 10.1107/S1600576720016040

Gerber, P.J., Hristov, A.N., Henderson, B., Makkar, H., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A.T., Yang, W.Z., Tricarico, J.M., Kebreab, E., Waghorn, G., Dijkstra, J., Oosting, S., 2013. Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. *Animal*, 7, Supplement 2, 220–34. doi: 10.1017/S1751731113000876

Gerstner, K., Liesegang, A., 2018. Effect of a montmorillonite-bentonite-based product on faecal parameters of horses. *J Anim Physiol Anim Nutr*. 102(S1), 43–46. doi: 10.1111/jpn.12888

Ghadiri, M., Chrzanowski, W., Rohanizadeh, R., 2015. Biomedical applications of cationic clay minerals. *RSC Adv*. 5(37), 29467–29481. doi: 10.1039/C4RA16945J

González, R., de Medina, F.S., Martínez-Augustin, O., Nieto, A., Gálvez, J., Risco, S., Zarzuelo, A., 2004. Anti-inflammatory effect of diosmectite in hapten-induced colitis in the rat. *Br J Pharmacol*. 141(6), 951–960. doi: 10.1038/sj.bjp.0705710

Goodarzi, M., and Modiri, D. 2011. The use clinoptilolite in broiler diet to decrease of aflatoxin effects. In: International Conference on Asia Agriculture and Animal, IPCBEE, 13. Singapore, IACSIT Press. pp. 38–43.

Gouda, G.A., Khattab, H.M., Abdel-Wahhab, M.A., Abo El-Nor, S.A., El-Sayed, H.M., Kholif, S.M., 2019. Clay minerals as sorbents for mycotoxins in lactating goat's diets: Intake, digestibility, blood chemistry, ruminal fermentation, milk yield and composition, and milk aflatoxin M_1 content. *Small Ruminant Res*. 175, 15–22. doi: 10.1016/j.smallrumres.2019.04.003

Gray, S.J., Ward, T.L., Southern, L.L., Ingram, D.R., 1998. Interactive effects of sodium bentonite and coccidiosis with monensin or salinomycin in chicks. *Poult Sci*. 77(4), 600–604. doi: 10.1093/ps/77.4.600

Grosicki, A., and Kowalski, B., 2003. Influence of bentonite on trace element kinetics in rats. I. Iron. *Bull Vet Inst Pulawy*. 47, 555–558. Available online at: https://www.researchgate.net/publication/228494124_Influence_of_bentonite_on_trace_element_kinetics_in_rats_I_Iron

Grosicki, A., Kowalski, B., Bik, D., 2004. Influence of bentonite on trace element kinetics in rats. II Calcium. *Bull Vet Inst Pulawy*. 48, 337–340. Available online at: https://www.researchgate.net/publication/228494123_Influence_of_bentonite_on_trace_element_kinetics_in_rats_II_Calcium

Grosicki, A., and Rachubik, J., 2005. Influence of bentonite on trace element kinetics in rats. III. Selenium. *Bull Vet Inst Pulawy*. 49, 121–123. Available online at: https://www.researchgate.net/publication/228683083_Influence_of_bentonite_on_trace_element_kinetics_in_rats_III_Selenium

Guggenheim, S., and Martin, R.T., 1995. Definition of clay and clay mineral: Joint report of the AIPEA nomenclature and CMS nomenclature committees. *Clays Clay Miner*. 43(2), 255–256. doi: 10.1346/CCMN.1995.0430213

Hallab, N.J., Jacobs, J.J., 2009. Biologic effects of implant debris. *Bull NYU Hosp Joint Dis*. 67(2), 182–188.

Hassen, A., Jamoussi, F., Saidi, N., Mabrouki, Z., Fakhfakh, E., 2003. Microbial and copper adsorption by smectitic clay--an experimental study. *Environ Technol*, 24(9):1117-27. doi: 10.1080/09593330309385652

He, Y., Park, K., 2016. Effects of the microparticle shape on cellular uptake. *Mol. Pharmaceutics*, 13(7), 2164–2171. doi: 10.1021/acs.molpharmaceut.5b00992

Hedges, J.I., Hare, P.E., 1987. Amino acid adsorption by clay minerals in distilled water. *Geochim Cosmochim Acta*, 51(2), 255–259. doi: 10.1016/0016-7037(87)90237-7

Hejna, M., Gottardo, D., Baldi, A., Dell'Orto, V., Cheli, F., Zaninelli, M., Rossi, L., 2018. Review: Nutritional ecology of heavy metals. *Animal*. 12(10), 2156–2170. doi: 10.1017/S175173111700355X

Helal, F.I.S., Abdel-Rahman, K.A., 2010. Productive performance of lactating ewes fed diets supplementing with dry yeast and/or bentonite as feed additives. *World J Agricult Sci*. 6(5), 489–498. Available online at: http://www.idosi.org/wjas/wjas6(5)/4.pdf

Hollis, M.E., Pate, R.T., Sulzberger, S., Pineda, A., Khidoyatov, Y., Murphy, M.R., Cardoso, F.C., 2020. Improvements of in situ degradability of grass hay, wet brewer's grains, and soybean meal with addition of clay in the diet of Holstein cows. *Anim Feed Sci Technol*. 259, 114331. doi: 10.1016/j.anifeedsci.2019.114331

Hooda, P.S., Henry, C.J.K., Seyoum, T.A., Armstrong, L.D.M., Fowler, M.B., 2004. The potential impact of soil ingestion on human mineral nutrition. *Sci Total Environ*. 333(1–3), 75–87. doi: 10.1016/j.scitotenv.2004.04.023

Howard, B., 2021. Environmental Pathways of Radionuclides to Animal Products in Different Farming and Harvesting Systems. In: Naletoski, I., Luckins, A.G., Viljoen, G. (eds), *Nuclear and Radiological Emergencies in Animal Production Systems, Preparedness, Response and Recovery*. Springer, Berlin, Heidelberg. pp. 53-105. doi: 10.1007/978-3-662-63021-1_5

Hu, L., Mao, Z., Gao, C., 2009. Colloidal particles for cellular uptake and delivery. *J Mater Chem*. 19(20), 3108–3115. doi: 10.1039/b815958k

Humer, E., Kröger, I., Neubauer, V., Reisinger, N., Zebeli, Q., 2019. Supplementation of a clay mineral-based product modulates plasma metabolomic profile and liver enzymes in cattle fed grain-rich diets. *Animal*, 13(6), 1214–1223. doi: 10.1017/S1751731118002665

Iheshiulor, O.O.M., Esonu, B.O., Chuwuka, O.K., Omede, A.A., Okoli, I.C., Ogbuewu, I.P., 2011. Effects of Mycotoxins in Animal Nutrition: A Review. *Asian J Animal Sci*. 5, 19–33. doi: 10.3923/ajas.2011.19.33

Ivan, M., Dayrell, M.D., Mahadevan, S., Hidiroglou, M., 1992 a. Effects of bentonite on wool growth and nitrogen metabolism in fauna-free and faunated sheep. *J Anim Sci*. 70, 3194–3202. doi: 10.2527/1992.70103194x

Ivan, M., Dayrell, M. de S., Hidiroglou, M., 1992 b. Effects of bentonite and monensin on selected elements in the stomach and liver of fauna-free and faunated sheep. *J Dairy Sci*. 75(1), 201–208. doi: 10.3168/jds.S0022-0302(92)77754-6

Jayrajsinh, S., Shankar, G., Agrawal, Y.K., Bakre, L., 2017. Montmorillonite nanoclay as a multifaceted drug-delivery carrier: a review. *J Drug Deliv Sci Technol*. 39, 200–209. doi: 10.1016/j.jddst.2017.03.023

Jansen van Rensburg, H.G., Claassens, A.S., Beukes, D.J., 2009. Relationships between soil buffer capacity and selected soil properties in a resource-poor farming area in the Mpumalanga Province of South Africa. *South African Journal of Plant and Soil*. 26(4), 237–243. doi: 10.1080/02571862.2009.10639961

Kaduk, J.A., Faber, J., 1995. Crystal structure of zeolite Y as a function of ion exchange. *The Rigaku Journal*. 12(2), 14–34. Available online at: https://www.researchgate.net/publication/237475013_Crystal_structure_of_zeolite_Y_as_a_function_of_ion_exchange

Kang, F., Ge, Y., Hu, X., Goikavi, C., Waigi, M.G., Gao, Y., Ling, W., 2016. Understanding the sorption mechanisms of aflatoxin B1 to kaolinite, illite, and smectite clays via a comparative computational study. *J Hazard Mater*. 320, 80–87. doi: 10.1016/j.jhazmat.2016.08.006

Kannewischer, I., Tenorio Arvide, M.G., White, G.N., Dixon, J.B., 2006. Smectite Clays as Adsorbents of Aflatoxin B1: Initial Steps. *Clay Sci*. 12(2), 199–204. Available online at: https://www.jstage.jst.go.jp/article/jcssjclayscience1960/12/Supplement2/12_Supplement2_199/_pdf

Kantesaria, N., Sharma, S., 2020. Exfoliation and extraction of nanoclay from montmorillonite mineral rich bentonite soil. In: Prashant, A., Sachan, A., Desai, C. (eds) Advances in Computer Methods and Geomechanics. Lecture Notes in Civil Engineering, vol 56. Springer, Singapore. doi: 10.1007/978-981-15-0890-5_1

Karatzia, M.A., Katsoulos, P.D., Karatzias, H., 2013. Diet supplementation with clinoptilolite improves energy status, reproductive efficiency and increases milk yield in dairy heifers. *Animal Prod Sci*. 53(3), 234–239. doi: 10.1071/AN11347

Kianoosh S, Shivazad M, Yazdi K., 2012. Bentonite clays effects as animal feed additives on meat quality and health. (Abst.). In: 49th Annual Meeting of the Clay Minerals Society. Colorado School of Mines, Golden, Colorado, USA.

Kovalsky Paris, M.P., Liu, Y.-J., Nahrer, K., Binder, E.M., 2015. Climate change impacts on mycotoxin production. In: Botana, L.M. and Sainz, M.J. (eds) Climate Change and Mycotoxins. De Gruyter, Berlin, München, Boston, pp. 133-152. doi: 10.1515/9783110333619-009

Kubena, L.F., Harvey, R.B., Phillips, T.D., Clement, B.A., 1993. Effect of hydrated sodium calcium aluminosilicates on aflatoxicosis in broiler chicks. *Poult Sci*. 72(4), 651–657. doi: 10.3382/ps.0720651

Kubena, L.F., Harvey, R.B., Bailey, R.H., Buckley, S.A., Rottinghaus, G.E., 1998. Effects of a hydrated sodium calcium aluminosilicate (T-Bind) on mycotoxicosis in young broiler chickens. *Poult Sci*. 77(10), 1502–1509. doi: 10.1093/ps/77.10.1502

Kumari, N., Mohan, C., 2021. Basics of clay minerals and their characteristic properties. *Clay and Clay Minerals*. IntechOpen. doi: 10.5772/intechopen.97672

Kyriakis, S.C., Papaioannou, D.S., Alexopoulos, C., Polizopoulou, Z., Tzika, E.D., Kyriakis, C.S., 2002. Experimental studies on safety and efficacy of the dietary use of a clinoptilolite-rich tuff in sows: a review of recent research in Greece. *Microporous Mesoporous Mat*. 51(1), 65–74. doi: 10.1016/S1387-1811(01)00475-9

Lai, X., Agarwal, M., Lvov, Y.M., Pachpande, C., Varahramyan, K., Witzmann, F.A., 2013. Proteomic profiling of halloysite clay nanotube exposure in intestinal cell co-culture. *J Appl Toxicol*. 33(11), 1316–1329. doi: 10.1002/jat.2858

Leonard, A., Droy-Lefaix, M., Allen A., 1994. Pepsin hydrolysis of the adherent mucus barrier and subsequent gastric mucosal damage in the rat: Effect of diosmectite and 16,16 dimethyl prostaglandin E2. *Gastroenterol Clin Biol*. 18(6-7), 609–616.

Lesniak, A., Salvati, A., Santos-Martinez, M.J., Radomski, M.W., Dawson, K.A., Åberg, C., 2013. Nanoparticle adhesion to the cell membrane and its effect on nanoparticle uptake efficiency. *J Am Chem Soc*. 135(4), 1438–1444. doi: 10.1021/ja309812z

Lindemann, M.D., Blodgett, D.J., Kornegay, E.T., Schurig, G.G., 1993. Potential ameliorators of aflatoxicosis in weanling/growing swine. *J Anim Sci*. 71, 171–178. doi: 10.2527/1993.711171x

Liu, J.H., Cai, W.K., Khatoon, N., Yu, W.H., Zhou, C.H., 2021. On how montmorillonite as an ingredient in animal feed functions. *Appl Clay Sci*. 202, 105963. doi: 10.1016/j.clay.2020.105963

Llonch, P., Haskell, M.J., Dewhurst, R.J., Turner, S.P., 2017. Current available strategies to mitigate greenhouse gas emissions in livestock systems: an animal welfare perspective. *Animal*. 11(2), 274–284. doi: 10.1017/S1751731116001440

Loos, C., Syrovets, T., Musyanovych, A., Mailänder, V., Landfester, K., Nienhaus, G.U., Simmet, T., 2014. Functionalized polystyrene nanoparticles as a platform for studying bio-nano interactions. *Beilstein J Nanotechnol*., 5, 2403-2412. doi: 10.3762/bjnano.5.250

Lu, T., Gou, H., Rao, H., Zhao, G., 2021. Recent progress in nanoclay-based Pickering emulsion and applications. *J Environ Chem Eng*. 9(5), 105941. doi: 10.1016/j.jece.2021.105941

Magnoli, A.P., Alonso, V.A., Cavaglieri, L.R., Dalcero, A.M., Chiacchiera, S.M., 2013. Effect of monogastric and ruminant gastrointestinal conditions on *in vitro* aflatoxin B_1 adsorption ability by a montmorillonite. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 30(4), 743–749. doi: 10.1080/19440049.2013.784398

Magro, M., Vianello, F., 2019. Bare iron oxide nanoparticles: Surface tunability for biomedical, sensing and environmental applications. *Nanomaterials*. 9(11), 1608. doi: 10.3390/nano9111608

Maisanaba, S., Gutiérrez-Praena, D., Pichardo, S., Moreno, F.J., Jordá, M., Cameán, A.M., Aucejo, S., Jos, A., 2013. Toxic effects of a modified montmorillonite clay on the human intestinal cell line Caco-2. *J Appl Toxicol*. 34(6), 714–725. doi: 10.1002/jat.2945

Maisanaba, S., Pichardo, S., Puerto, M., Gutiérrez-Praena, D., Cameán, A.M., Jos, A., 2015. Toxicological evaluation of clay minerals and derived nanocomposites: A review. *Environ Res*. 138, 233–254. doi: 10.1016/j.envres.2014.12.024

Maki, C.R., Monteiro, A.P.A., Elmore, S.E., Tao, S., Bernard, J.K., Harvey, R.B., Romoser, A.A., Phillips, T.D., 2016 a. Calcium montmorillonite clay in dairy feed reduces aflatoxin concentrations in milk

without interfering with milk quality, composition or yield. *Anim Feed Sci Technol*. 214, 130–135. doi: 10.1016/j.anifeedsci.2016.02.014

Maki, C.R., Thomas, A.D., Elmore, S.E., Romoser, A.A., Harvey, R.B., Ramirez-Ramirez, H.A., Phillips, T.D., 2016 b. Effects of calcium montmorillonite clay and aflatoxin exposure on dry matter intake, milk production, and milk composition. *J Dairy Sci*. 99(2), 1039–1046. doi: 10.3168/jds.2015-10242

Maki, C.R., Haney, S., Wang, M., Ward, S.H., Rude, B.J., Bailey, H.R., Harvey, R.B., Phillips T.D., 2017. Calcium montmorillonite clay for the reduction of aflatoxin residues in milk and dairy products. *Dairy and Vet Sci J*. 2(3), 555587. doi: 10.19080/JDVS.2017.02.555587

Makkar, H.P.S., 2016. Animal nutrition in a 360-degree view and a framework for future R&D work: towards sustainable livestock production. *Animal Prod Sci*. 56(10), 1561–1568. doi: 10.1071/AN15265

Malachová, K., Praus, P., Pavlíčková, Z., Turicová, M., 2009. Activity of antibacterial compounds immobilised on montmorillonite. *Appl Clay Sci*. 43(3-4), 364–368. doi: 10.1016/j.clay.2008.11.003

Mallek, Z., Fendri, I., Khannous, L., Ben Hassena, A., Traore, A.I., Ayadi, M.-A., Gdoura, R., 2012. Effect of zeolite (clinoptilolite) as feed additive in Tunisian broilers on the total flora, meat texture and the production of omega 3 polyunsaturated fatty acid. *Lipids Health Dis*. 11, 35. doi: 10.1186/1476-511X-11-35

Marroquín-Cardona, A., Deng, Y., Garcia-Mazcorro, J.F., Johnson, N.M., Mitchell, N.J., Tang, L., Robinson, A., Taylor, J.F., Wang, J.-S., Phillips, T.D., 2011. Characterization and safety of uniform particle size NovaSil clay as a potential aflatoxin enterosorbent. *Appl Clay Sci*. 54(3-4), 248–257. doi: 10.1016/j.clay.2011.09.009

Marroquín-Cardona, A.G., Deng, Y., Garcia-Mazcorro, J.F., Johnson, N.M., Mitchell, N.J., Tang, L., Wang, J.-S., Harvey, R.B., Phillips, T.D., 2022. Aflatoxin B_1 sorption and safety of dietary sodium bentonite in Sprague-Dawley rats. *Clays Clay Min*. 70, 165-181. doi: 10.1007/s42860-022-00173-w

Martirosian, G., Rouyan, G., Zalewski, T., Meisel-Mikolajczyk, F., 1998. Dioctahedral smectite neutralization activity of Clostridium difficile and Bacteroides fragilis toxins in vitro. *Acta Microbiol Pol*. 47(2), 177–183.

Matusik, J., Deng, Y., 2020. Fumonisin B_1 Interaction with Mg-Al and Mg-Fe layered double hydroxides: removal efficiency and mechanisms. *Materials*. 13(19), 4344. doi: 10.3390/ma13194344

Medina, Á., González-Jartín, J.M., Sainz, M.J., 2017. Impact of global warming on mycotoxins. *Curr Opin Food Sci*. 18, 76–81. doi: 10.1016/j.cofs.2017.11.009

Meisinger, J.J., Lefcourt, A.M., Van Kessel, J.A., Wilkerson, V., 2001. Managing ammonia emissions from dairy cows by amending slurry with alum or zeolite or by diet modification. *TheScientificWorld*. 1(S2), 860–865. doi: 10.1100/tsw.2001.100

Mensching, A., Hummel, J., Sharifi, A.R., 2020. Statistical modeling of ruminal pH parameters from dairy cows based on a meta-analysis. *J Dairy Sci*. 103(1), 750–767. doi: 10.3168/jds.2019-16802

Merianos, J.J., 1991. Quaternary ammonium antimicrobial compounds. In: Block, S.S. (ed.), *Disinfection, Sterilization and Preservation*. 4th Edition. Lea & Febiger, Philadelphia, pp. 225-255.

Moré, J., Bénazet, F., Fioramonti, J., Droy-Lefaix, M.-T., 1987. Effects of treatment with smectite on gastric and intestinal glycoproteins in the rat: A histochemical study. *Histochem J*. 19, 665–670. doi: 10.1007/BF01676173

Moreno-Maroto, J.M., Alonso-Azcárate, J., 2018. What is clay? A new definition of “clay” based on plasticity and its impact on the most widespread soil classification systems. *Appl Clay Sci*. 161, 57–63. doi: 10.1016/j.clay.2018.04.011

Mousa, M., Evans, N.D., Oreffo, R.O.C., Dawson, J.I., 2018. Clay nanoparticles for regenerative medicine and biomaterial design: A review of clay bioactivity. *Biomaterials*. 159, 204–214. doi: 10.1016/j.biomaterials.2017.12.024

Moyes, S.M., Morris, J.F., Carr, K.E., 2010. Macrophages increase microparticle uptake by enterocyte-like Caco-2 cell monolayers. *J Anat*. 217(6), 740–754. doi: 10.1111/j.1469-7580.2010.01304.x

Müller, R.H., Rühl, D., Schulze-Forster, K., 1997. Interaction of differentiated HL60 cells with poloxamer and poloxamine surface modified model drug carriers. *Eur J Pharmaceut Sci*. 5(3), 147–153. doi: 10.1016/S0928-0987(97)00271-6

Mundargi, R.C., Babu, V.R., Rangaswamy, V., Patel, P., Aminabhavi, T.M., 2008. Nano/micro technologies for delivering macromolecular therapeutics using poly(D,L-lactide-co-glycolide) and its derivatives. *J Control Release*., 125(3), 193-209. doi: 10.1016/j.jconrel.2007.09.013

Mushtaq, M.M.H., Parvin, R., Kim, J., 2014. Carcass and body organ characteristics of broilers supplemented with dietary sodium and sodium salts under a phase feeding system. *J Anim Sci Technol*. 56, 4. doi: 10.1186/2055-0391-56-4

Nadziakiewicza, M., Kehoe, S., Micek, P., 2019. Physico-chemical properties of clay minerals and their use as a health promoting feed additive. *Animals*. 9(10), 714. doi: 10.3390/ani9100714

Nanzyo, M., Kanno, H., 2018. Secondary Minerals. In: Inorganic Constituents in Soil. Singapore: Springer. p. 37–58. doi: 10.1007/978-981-13-1214-4_3

Neubauer, V., Humer, E., Mann, E., Kröger, I., Reisinger, N., Wagner, M., Zebeli, Q., Petri, R.M., 2019. Effects of clay mineral supplementation on particle-associated and epimural microbiota, and gene expression in the rumen of cows fed high-concentrate diet. *Anaerobe*. 59, 38–48. doi: 10.1016/j.anaerobe.2019.05.003

Nicolete, R., dos Santos, D.F., Faccioli, L.H., 2011. The uptake of PLGA micro or nanoparticles by macrophages provokes distinct in vitro inflammatory response. *Int Immunopharmacol*. 11(10), 1557–1563. doi: 10.1016/j.intimp.2011.05.014

OECD (Organisation for Economic Co-operation and Development), 2015. Dossier on Nanoclays. In: OECD Environment, Health and Safety Publications. Series on the Safety of Manufactured Nanomaterials. No. 47. ENV/JM/MONO(2015)10. Available online at: pdf (oecd.org)

Olver, M.D., 1997. Effect of feeding clinoptilolite (zeolite) on the performance of three strains of laying hens. *Br Poult Sci*. 38(2), 220–222. doi: 10.1080/00071669708417973

Ostry, V., Malir, F., Toman, J., Grosse, Y., 2017. Mycotoxins as human carcinogens—the *IARC Monographs* classification. *Mycotoxin Res*. 33, 65–73. doi: 10.1007/s12550-016-0265-7

Otunola, B.O., Ololade, O.O., 2020. A review on the application of clay minerals as heavy metal adsorbents for remediation purposes. *Environ Technol Innov*. 18, 100692. doi: 10.1016/j.eti.2020.100692

Ouhida, I., Pérez, J.F., Piedrafita, J., Gasa, J., 2000. The effects of sepiolite in broiler chicken diets of high, medium and low viscosity. Productive performance and nutritive value. *Animal Feed Sci Technol*. 85(3-4), 183–194. doi: 10.1016/S0377-8401(00)00148-6

Palmonari, A., Stevenson, D.M., Mertens, D.R., Cruywagen, C.W., Weimer, P.J., 2010. pH dynamics and bacterial community composition in the rumen of lactating dairy cows. J Dairy Sci. 93:279–87. doi: 10.3168/jds.2009-2207

Papaioannou, D.S., Kyriakis, C.S., Alexopoulos, C., Tzika, E.D., Polizopoulou, Z.S., Kyriakis, S.C., 2004. A field study on the effect of the dietary use of a clinoptilolite-rich tuff, alone or in combination with certain antimicrobials, on the health status and performance of weaned, growing and finishing pigs. *Res Vet Sci*. 76(1), 19–29. doi: 10.1016/j.rvsc.2003.08.006

Papaioannou, D., Katsoulos, P.D., Panousis, N., Karatzias, H., 2005. The role of natural and synthetic zeolites as feed additives on the prevention and/or the treatment of certain farm animal diseases: A review. *Microporous Mesoporous Mat*. 84(1-3), 161–170. doi: 10.1016/j.micromeso.2005.05.030

Parmigiani E, Bigliardi E, Di Ianni F, Bresciani C, Morini G., 2013. Aflatoxin B1, M1: Role of mycotoxin sequestering agents on reproductive efficiency and milk production in dairy cows. In: Proceedings of 11th Congress of the Italian Society of Animal Reproduction, June 19-22, 2013, Ustica, Italy. pp. 259–262. doi: 10.4488/SIRA.2013.47

Pate, R.T., Paulus Compart, D.M., Cardoso, F.C., 2018. Aluminosilicate clay improves production responses and reduces inflammation during an aflatoxin challenge in lactating Holstein cows. *J Dairy Sci*. 101(12), P11421–11434. doi: 10.3168/jds.2018-15024

Paterson, R.R.M., Lima, N., 2011. Further mycotoxin effects from climate change. *Food Res Int*. 44(9), 2555–2566. doi: 10.1016/j.foodres.2011.05.038

Paumier, S., Monnet, P., Pantet, A., 2008. Rheological behavior of smectite dispersions: the influence of suspension concentration and exchangeable cation. *Adv Mat Sci Eng*. 2008, 856714. doi: 10.1155/2008/856714

Pavelić, S.K., Medica, J.S., Gumbarević, D., Filošević, A., Pržulj, N., Pavelić, K., 2018. Critical review on zeolite clinoptilolite safety and medical applications *in vivo*. *Front Pharmacol*. 9, 1350. doi: 10.3389/fphar.2018.01350

Phillips, T.D., Sarr, A.B., Grant, P.G., 1995. Selective chemisorption and detoxification of aflatoxins by phyllosilicate clay. *Nat Toxins*. 3(4), 204–213. doi: 10.1002/nt.2620030407

Phillips, T.D., Afriyie-Gyawu, E., Williams, J., Huebner, H., Ankrah, N.-A., Ofori-Adjei, D., Jolly, P., Johnson, N., Taylor, J., Marroquin-Cardona, A., Xu, L., Tang, L., Wang J.-S., 2008. Reducing human exposure to aflatoxin through the use of clay: A review. *Food Addit Contam: Part A Chem Anal Control Expo Risk Assess*. 25(2), 134–145. doi: 10.1080/02652030701567467

Phillips, T.D., Wang, M., Elmore, S.E., Hearon, S., Wang, J.-S., 2019. NovaSil clay for the protection of humans and animals from aflatoxins and other contaminants. *Clays Clay Miner*. 67, 99–110. doi: 10.1007/s42860-019-0008-x

Pinotti, L., Ottoboni, M., Giromini, C., Dell'Orto, V., Cheli, F., 2016. Mycotoxin contamination in the EU feed supply chain: a focus on cereal byproducts. *Toxins*. 8(2), 45. doi: 10.3390/toxins8020045

Powell, J.J., Ainley, C.C., Harvey, R.S.J., Mason, I.M., Kendall, M.D., Sankey, E.A., Dhillon, A.P., Thompson, R.P.H., 1996. Characterisation of inorganic microparticles in pigment cells of human gut associated lymphoid tissue. *Gut*. 38, 390–395. doi: 10.1136/gut.38.3.390

Prasai, T.P., Walsh, K.B., Bhattarai, S.P., Midmore, D.J., Van, T.T.H., Moore, R.J., Stanley, D., 2016. Biochar, bentonite and zeolite supplemented feeding of layer chickens alters intestinal microbiota and reduces campylobacter load. *PLoS ONE*. 11(4), e0154061. doi: 10.1371/journal.pone.0154061

Rabon, H.W.JR., Roland, D.A.SR., Bryant. M.M., Smith, R.C., Barnes, D.G., Laurent, S.M., 1995. Absorption of silicon and aluminum by hens fed sodium zeolite A with various levels of dietary cholecalciferol. *Poult Sci*. 74(2), 352–359. doi: 10.3382/ps.0740352

Ramìrez-Mella, M., Hernàndez-Mendo, O., 2010. Nanotecnology on animal production. *Trop Subtrop Agroecosyst*. 12, 423–429. Available online at: https://www.researchgate.net/publication/48186910_NANOTECHNOLOGY_ON_ANIMAL_PRODUCTION

Reichardt, F., Habold, C., Chaumande, B., Ackermann, A., Ehret-Sabatier, L., Le Maho, Y., Angel, F., Liewig, N., Lignot, J.-H., 2009. Interactions between ingested kaolinite and the intestinal mucosa in rat: proteomic and cellular evidences. *Fundam Clin Pharmacol*. 23(1), 69–79. doi: 10.1111/j.1472-8206.2008.00646.x

Reichardt, F., Chaumande, B., Habold, C., Robin, J.-P-, Ehret-Sabatier, L., Le Maho, Y., Liewig, N., Angel, F., Lignot, J.-H., 2012. Kaolinite ingestion facilitates restoration of body energy reserves during refeeding after prolonged fasting. *Fundam Clin Pharmacol*. 26(5), 577–588. doi: 10.1111/j.1472-8206.2011.00989.x

Reilly, C., Henry, J., 2000. Geophagia: why do humans consume soil? *Nutr Bull*. 25(2), 141–144. doi: 10.1046/j.1467-3010.2000.00032.x

Rindsig, R.B., Schultz, L.H., Shook, G.E., 1969. Effects of the addition of bentonite to high-grain dairy rations which depress milk fat percentage. *J Dairy Sci*. 52(11), 1770–1775. doi: 10.3168/jds.S0022-0302(69)86839-6

Rindsig, R.B., Schultz, L.H., 1970. Effect of Bentonite on Nitrogen and Mineral Balances and Ration Digestibility of High-Grain Rations Fed to Lactating Dairy Cows. *J Dairy Sci*. 53(7), 888–892. doi: 10.3168/jds.S0022-0302(70)86313-5

Rizzi, L., Simioli, M., Roncada, P., Zaghini, A., 2003. Aflatoxin B1 and clinoptilolite in feed for laying hens: effects on egg quality, mycotoxin residues in livers, and hepatic mixed-function oxygenase activities. *J Food Prot*. 66(5), 860–865. doi: 10.4315/0362-028X-66.5.860

Rodrigues, R.O., Rodrigues, R.O., Ledoux, D.R., Rottinghaus, G.E., Borutova, R., Averkieva, O., McFadden, T.B., 2019. Feed additives containing sequestrant clay minerals and inactivated yeast reduce aflatoxin excretion in milk of dairy cows. *J Dairy Sci*. 102(7), 6614–6623. doi: 10.3168/jds.2018-16151

Salem, F.A.F., El-Amary, H., Hassanin, S.H., 2001. Effect of bentonite supplementation on nutrients digestibility; rumen fermentation; some blood physiological parameters and performance of growing lambs. *Egypt J Nutr Feed*. 4, 179–191.

Schell, T.C., Lindemann, M.D., Kornegay, E.T., Blodgett, D.J., Doerr, J.A., 1993. Effectiveness of different types of clay for reducing the detrimental effects of aflatoxin-contaminated diets on performance and serum profiles of weanling pigs. *J Anim Sci*. 71(5), 1226–1231. doi: 10.2527/1993.7151226x

Schiffenbauer, M., Stotzky, G., 1982. Adsorption of coliphages T1 and T7 to clay minerals. *Appl Environ Microbiol*. 43(3), 590–596. doi: 10.1128/aem.43.3.590-596.1982

Schwaller, D., Wilkens, M.R., Liesegang, A., 2016. Zeolite A effect on calcium homeostasis in growing goats. *J Anim Sci*. 94(4), 1576–1586. doi: 10.2527/jas.2015-9690

Segad, M., Hanski, S., Olsson, U., Ruokolainen, J., Åkesson, T., Jönsson, B., 2012 a. Microstructural and swelling properties of Ca and Na Montmorillonite: (In situ) observations with Cryo-TEM and SAXS. *J Phys Chem C*. 116(13), 7596–7601. doi: 10.1021/jp300531y

Segad, M., Jönsson, B., Cabane, B., 2012 b. Tactoid formation in montmorillonite. *J Phys Chem C*. 116(48), 25425–25433. doi: 10.1021/jp3094929

Segad, M., Åkesson, T., Cabane, B., Jönsson, B., 2015. Nature of flocculation and tactoid formation in montmorillonite: the role of pH. *Phys Chem Chem Phys*. 17(44), 29608–29615. doi: 10.1039/C5CP04007H

Seim, G.L., Ahn, C.I., Bodis, M.S., Luwedde, F., Miller, D.D., Hillier, S., Tako, E., Glahn, R.P., Young, S.L., 2013. Bioavailability of iron in geophagic earths and clay minerals, and their effect on dietary iron absorption using an *in vitro* digestion/Caco-2 cell model. *Food Funct*. 4(8), 1263–1270. doi: 10.1039/c3fo30380b

Shainberg, I., Levy, G.J., 2005. Flocculation and Dispersion. In: Hillel, D. (ed) *Encyclopedia of Soils in the Environment,* Elsevier, pp. 27–34. doi: https://doi.org/10.1016/B0-12-348530-4/00363-5

Sharma, A.K., Schmidt, B., Frandsen, H., Jacobsen, N.R., Larsen, E.H., Binderup, M.-L., 2010. Genotoxicity of unmodified and organo-modified montmorillonite. *Mutat Res Genetic Toxicol Environ Mutagen*. 700(1-2), 18–25. doi: 10.1016/j.mrgentox.2010.04.021

Sharma, A.K., Mortensen, A., Schmidt, B., Frandsen, H., Hadrup, N., Larsen, E.H., Binderup, M.-L., 2014. In-vivo study of genotoxic and inflammatory effects of the organo-modified Montmorillonite

Cloisite® 30B. *Mutat Res Genetic Toxicol Environ Mutagenesis*. 770, 66–71. doi: 10.1016/j.mrgentox.2014.04.023

Shen, C.C., Petit, S., Li, C.J., Li, C.S., Khatoon, N., Zhou, C.H., 2020. Interactions between smectites and polyelectrolytes. *Appl Clay Sci*. 198, 105778. doi: 10.1016/j.clay.2020.105778

Shryock, T.R., Klink, P.R., Readnour, R.S., Tonkinson, L.V., 1994. Effect of bentonite incorporated in a feed ration with tilmicosin in the prevention of induced Mycoplasma gallisepticum airsacculitis in broiler chickens. *Avian Dis*. 38(3), 501–505. doi: 10.2307/1592071

Slamova, R., Trckova, M., Vondruskova, H., Zraly, Z., Pavlik, I., 2011. Clay minerals in animal nutrition. *Appl Clay Sci*. 51(4), 395–398. doi: 10.1016/j.clay.2011.01.005

Smith, E.E., Phillips, T.D., Ellis, J.A., Harvey, R.B., Kubena, L.F., Thompson, J., Newton, G., 1994. Dietary hydrated sodium calcium aluminosilicate reduction of aflatoxin M_1 residue in dairy goat milk and effects on milk production and components. *J Anim Sci*. 72(3), 677–682. doi: 10.2527/1994.723677x

Song, M., Liu, Y., Soares, J.A., Che, T.M., Osuna, O., Maddox, C.W., Pettigrew, J.E., 2012. Dietary clays alleviate diarrhea of weaned pigs. *J Anim Sci*. 90(1), 345–360. doi: 10.2527/jas.2010-3662

Sparks, D.L., 2003. Inorganic soils components. In: Sparks, D.L. (ed) Environmental Soil Chemistry (Second Edition), Academic Press, pp. 43–73. doi: 10.1016/B978-012656446-4/50002-5

Subramaniam, M.D., Kim, I.H., 2015. Clays as dietary supplements for swine: A review. *J Anim Sci Biotechnol*. 6, 38. doi: 10.1186/s40104-015-0037-9

Suliman, M.E., Qureshi, A.R., Stenvinkel, P., Pecoits-Filho, R., Bárány, P., Heimbürger, O., Anderstam, B., Rodríguez Ayala, E., Filho, J.C.D., Alvestrand, A., Lindholm, B., 2005. Inflammation contributes to low plasma amino acid concentrations in patients with chronic kidney disease. *The American Journal of Clinical Nutrition.* 82(2), 342–349. doi: 10.1093/ajcn/82.2.342

Sulzberger, S.A., Kalebich, C.C., Melnichenko, S., Cardoso, F.C., 2016. Effects of clay after a grain challenge on milk composition and on ruminal, blood, and fecal pH in Holstein cows. *J Dairy Sci*. 99(10), 8028–8040. doi: 10.3168/jds.2016-11030

Sulzberger, S.A., Melnichenko, S., Cardoso, F.C., 2017. Effects of clay after an aflatoxin challenge on aflatoxin clearance, milk production, and metabolism of Holstein cows. *J Dairy Sci*. 100(3), 1856–1869. doi: 10.3168/jds.2016-11612

Sun, L.-H., Lei, M.-Y., Zhang, N.-Y., Gao, X., Li, C., Krumm, C.S., Qi, D.-S., 2015. Individual and combined cytotoxic effects of aflatoxin B_1, zearalenone, deoxynivalenol and fumonisin B_1 on BRL 3A rat liver cells. *Toxicon*. 95, 6–12. doi: 10.1016/j.toxicon.2014.12.010

Szajewska, H., Dziechciarz, P., Mrukowicz, J., 2006. Meta-analysis: Smectite in the treatment of acute infectious diarrhoea in children. *Aliment Pharmacol Ther*, 23(2), 217-27. doi: 10.1111/j.1365-2036.2006.02760.x

Tan, X., Liu, F., Hu, L., Reed, A.H., Furukawa, Y., Zhang, G., 2017. Evaluation of the particle sizes of four clay minerals. *Appl Clay Sci*. 135, 313–324. doi: 10.1016/j.clay.2016.10.012

Tateo, F., Summa, V., 2007. Element mobility in clays for healing use. *Appl Clay Sci*. 36(1-3), 64–76. doi: 10.1016/j.clay.2006.05.011

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy Metal Toxicity and the Environment. In: Luch, A. (ed) Molecular, clinical and environmental toxicicology. Experimentia Supplementum. Springer, pp. 133–164. doi: 10.1007/978-3-7643-8340-4_6

Tenorio Arvide, M.G., Mulder, I., Barrientos Velazquez, A.L., Dixon, J.B., 2008. Smectite clay adsorption of Aflatoxin *vs*. octahedral composition as indicated by FTIR. *Clays Clay Min*. 56, 571–578. doi: 10.1346/CCMN.2008.0560510

Thumbi, S.M., Kariuki Njenga, M., Marsh, T.L., Noh, S., Otiang, E., Munyua, P., Ochieng, L., Ogola, E., Yoder, J., Audi, A., Montgomery, J.M., Bigogo, G., Breiman, R.F., Palmer, G.H., McElwain, T.F., 2015. Linking human health and livestock health: a “one-health” platform for integrated analysis of human health, livestock health, and economic welfare in livestock dependent communities. *PLoS ONE*. 10(3), e0120761. doi: 10.1371/journal.pone.0120761

Toprak, N.N., Yilmaz, A., Öztürk, E., Yigit, O., Cedden, F., 2016. Effect of micronized zeolite addition to lamb concentrate feeds on growth performance and some blood chemistry and metabolites. *S Afr J Anim Sci*. 46(3), 313–320. doi: 10.4314/sajas.v46i3.11

Trckova, M., Matlova, L., Dvorska, L., Pavlik, I., 2004. Kaolin, bentonite, and zeolites as feed supplements for animals: health advantages and risks. *Vet Med*. 49(10), 389–399. doi: 10.17221/5728-VETMED

Uddin, F., 2008. Clays, nanoclays, and montmorillonite minerals. *Metall Mater Trans A*. 39, 2804–2814. doi: 10.1007/s11661-008-9603-5

Uddin, F., 2018. Montmorillonite: An introduction to properties and utilization. In: Current Topics in the Utilization of Clay in Industrial and Medical Applications. IntechOpen. doi: 10.5772/intechopen.77987

Utlu, N., Celebi, S., Yücel, O., 2007. The effects of natural zeolite supplementation to diet on serum element concentrations in laying hens. *Revue de Médecine Vétérinaire*. 158, 598–602.

Valpotić, H., Terzic, S., Vince, S., Samardzija, M., Turk, R., Lackovic, G., Habrun, B., Djuricic, D., Sadikovic, M., Valpotic, I., 2016. In-feed supplementation of clinoptilolite favourably modulates intestinal and systemic immunity and some production parameters in weaned pigs. *Vet Med*. 61(6), 317–327. doi: 10.17221/175/2015-VETMED

Valpotić, H., Gračner, D., Turk, R., Đuričić, D., Vince, S., Folnožić, I., Lojkić, M., Žura Žaja, I., Bedrica, L., Maćešić, N., Getz, I., Dobranić, T., Samardžija, M., 2017. Zeolite clinoptilolite nanoporous feed additive for animals of veterinary importance: potentials and limitations. *Period Biol*. 119(3), 159–172. doi: 10.18054/pb.v119i3.5434

Valpotić, H., Žura Žaja, I., Samardžija, M., Habrun, B., Ostović, M., Duričić, D., Maćesić N, Mikulec Z, Kocila P, Sobiech P, Valpotić I, Vince S., 2018. Dietary supplementation with mannan oligosaccharide and clinoptilolite modulates innate and adaptive immune parameters of weaned pigs. *Pol J Vet Sci*. 21(1), 83–93. doi: 10.24425/119025

Vázquez-Durán, A., Nava-Ramírez, M. de J., Hernández-Patlán, D., Solís-Cruz, B., Hernández-Gómez, V., Téllez-Isaías, G., Méndez-Albores, A., 2021. Potential of kale and lettuce residues as natural adsorbents of the carcinogen aflatoxin B_1 in a dynamic gastrointestinal tract-simulated model. *Toxins*. 13(11), 771. doi: 10.3390/toxins13110771

Verma, N.K., Moore, E., Blau, W., Volkov, Y., Babu, P.R., 2012. Cytotoxicity evaluation of nanoclays in human epithelial cell line A549 using high content screening and real-time impedance analysis. *J Nanopart Res*. 14, 1137. doi: 10.1007/s11051-012-1137-5

Vianello, F., Cecconello, A., Magro, M., 2021. Toward the specificity of bare nanomaterial surfaces for protein corona formation. *Int J Mol Sci*. 22(14), 7625. doi: 10.3390/ijms22147625

Vietti, A.J., Dunn, F., 2006. Another dimension to slump. In: R Jewell, S Lawson & P Newman (eds), *Paste 2006: Proceedings of the Ninth International Seminar on Paste and Thickened Tailings*. Australian Centre for Geomechanics, Perth, pp. 25–36. doi: 10.36487/ACG_repo/663_3

Völkel, I., Schröer-Merker, E., Czerny C.-P., 2011. The carry-over of mycotoxins in products of animal origin with special regard to its implications for the european food safety legislation. *Food Nutr Sci*. 2(8), 852–867. doi: 10.4236/fns.2011.28117

Wang, G., Miao, Y., Sun, Z., Zheng, S., 2018. Simultaneous adsorption of aflatoxin B_1 and zearalenone by mono- and di-alkyl cationic surfactants modified montmorillonites. *J Colloid Interface Sci*. 511, 67–76. doi: 10.1016/j.jcis.2017.09.074

Wang, J.-S., Luo, H., Billam, M., Wang, Z., Guan, H., Tang, L., Goldston, T., Afriyie-Gyawu, E., Lovett, C., Griswold, J., Brattin, B., Taylor, R.J., Huebner, H.J., Phillips, T.D., 2005. Short-term safety evaluation of processed calcium montmorillonite clay (NovaSil) in humans. *Food Addit Contam*. 22(3), 270–279. doi: 10.1080/02652030500111129

Wang, J.P., Chi, F., Kim, I.H., 2012. Effects of montmorillonite clay on growth performance, nutrient digestibility, vulva size, faecal microflora, and oxidative stress in weaning gilts challenged with zearalenone. *Anim Feed Sci Technol*. 178(3-4), 158–166. doi: 10.1016/j.anifeedsci.2012.09.004

Weese, J.S., Cote, N.M., Degannes, R.V.G., 2003. Evaluation of in vitro properties of di-tri-octahedral smectite on clostridial toxins and growth. *Equine Vet J*. 35(7), 638–641. doi: 10.2746/042516403775696384

Wiles, M.C., Huebner, H.J., Afriyie-Gyawu, E., Taylor, R.J., Bratton, G.R., Phillips, T.D., 2004. Toxicological evaluation and metal bioavailability in pregnant rats following exposure to clay minerals in the diet. *J Toxicol Environ Health,* Part A. 67(11), 863–874. doi: 10.1080/15287390490425777

Wiley, A.S., Katz, S.H., 1998. Geophagy in pregnancy: a test of a hypothesis. *Current Anthropology*. *The University of Chicago Press,* 39(4), 532–545. doi: 10.1086/204769

Wu, Q.J., Zhou, Y.M., Wu, Y.N., Wang, T., 2013. Intestinal development and function of broiler chickens on diets supplemented with clinoptilolite. *Asian Australas J Anim Sci*. 26(7), 987–994. doi: 10.5713/ajas.2012.12545

Yu, W.H., Li, N., Tong, D.S., Zhou, C.H., Lin, C.X., Xu, C.Y., 2013. Adsorption of proteins and nucleic acids on clay minerals and their interactions: A review. *Appl Clay Sci*. 80–81, 443–452. doi: 10.1016/j.clay.2013.06.003

Zain, M.E., 2011. Impact of mycotoxins on humans and animals. *J Saudi Chem Soc*. 15(2), 129–144. doi: 10.1016/j.jscs.2010.06.006

Zavala-Franco, A., Hernández-Patlán, D., Solís-Cruz, B., López-Arellano, R., Tellez-Isaias, G., Vázquez-Durán, A., Méndez-Albores, A., 2018. Assessing the aflatoxin B_1 adsorption capacity between biosorbents using an in vitro multicompartmental model simulating the dynamic conditions in the gastrointestinal tract of poultry. *Toxins*. 10(11), 484. doi: 10.3390/toxins10110484

Zhang, J., Lv, Y., Tang, C., Wang, X., 2013. Effects of dietary supplementation with palygorskite on intestinal integrity in weaned piglets. *Appl Clay Sci*. 86, 185–189. doi: 10.1016/j.clay.2013.10.009

Zhao, H.Y., Mao, X.B., Yu, B., He, J., Zheng, P., Yu, J., Luo, J.Q., Wang, Q.Y., Chen, D.W., 2017. Excess of dietary montmorillonite impairs growth performance, liver function, and antioxidant capacity in starter pigs. *J Anim Sci*. 95(7), 2943–2951. doi: 10.2527/jas.2016.1277

Zhou, P., Tan, Y.Q., Zhang, L., Zhou, Y.M., Gao, F., Zhou, G.H., 2014. Effects of dietary supplementation with the combination of zeolite and attapulgite on growth performance, nutrient digestibility, secretion of digestive enzymes and intestinal health in broiler chickens. *Asian-Australas J Anim Sci*. 27(9), 1311–1318. doi: 10.5713/ajas.2014.14241

Zhu, S., Gong, L., Li, Y., Xu, H., Gu, Z., Zhao, Y., 2019. Safety assessment of nanomaterials to eyes: an important but neglected issue. *Adv Sci*. 6(16), 1802289. doi: 10.1002/advs.201802289

SECTION 2 – Experimental

1. Pilot *in vitro* study on the putative effects of bentonite on ruminal fermentations, metabolome, and mineral content

This chapter presents the data published as a short communication in the journal Research in Veterinary Science: Damato A, Vanzani P, Giannuzzi D, Giaretta E, Novelli E, Vianello F, Tagliapietra F, Zennaro L (2022) Bentonite does not affect *in vitro* ruminal gross fermentations but could modify ruminal metabolome and mineral content. A proof of concept. *Research in Veterinary Science*, 144, 78-81. https://doi.org/10.1016/j.rvsc.2022.01.012.

1.1. Introduction

Bentonite is a clay mineral belonging to the phyllosilicate family used as feed additive in animals (European Commission, 2021). In fact, clay minerals act as binders to produce pelleted feed and as adsorbents for mycotoxins and heavy metals, and exert mostly beneficial effects on animal physiology (Ghadiri et al., 2015; Nadziakiewicza et al., 2019; Slamova et al., 2011). However, some studies reported on possible undesirable effects due to clay administration both *in vitro* and *in vivo*, such as mineral and vitamin unbalances, interactions with veterinary drugs, intestinal toxicity, hepatic damage, and decreased growth performance (Elliott et al., 2020). As clay minerals can interact with microorganisms (Cuadros, 2017), their administration could acquire relevance for the animal physiology due to the possible effect on intestinal and/or ruminal microbiota, thus affecting the fermentative processes and metabolite availability (Chalvatzi et al., 2016; Neubauer et al., 2019; Wang et al., 2012). An increased use of clay minerals could be expected in the future due to their ability to adsorb mycotoxins and to modulate the intestinal and ruminal microbiota, but, for an effective and safe application of these additives, further studies are needed to clarify the clays-microbiota-animal physiology interplay. The present *in vitro* experiment has been designed to test if the bentonite administration in absence of mycotoxins may influence ruminal fermentations, the ruminal metabolome and the ruminal mineral content.

1.2. Materials and methods

The ruminal fluid for the *in vitro* fermentation was collected by oesophageal probe from a healthy Holstein lactating cow (OPBA, Prot. n. 0197903 of 16 May 2019) fed with a total mixed ration (TMR) based on corn and grass silages, cereals, soybean meal and a vitamin-mineral premix. The fermentation bottles were prepared with 50 mL of the ruminal fluid filtered with a cheesecloth, 100

mL of Menke medium (Menke and Steingass, 1988), and 1 g of the dried TMR used *in vivo*. Five treatment groups (B0, B50, B100, B200, B1000) were set up by adding 0, 2.5, 5, 10 and 50 mg of bentonite (GLOBALFEED®T1). Five different replicates were prepared for each treatment group. The samples were incubated at 39.0 °C for 24h, monitoring in continuous the kinetics of gas production (GP) with an Ankom RF Gas Production System equipment (Ankom Technology, NY, USA) (Tagliapietra et al., 2010).

At the end of the incubation period, an exploratory study of the potential effect of bentonite on metabolome and minerals of the rumen *in vitro* system was performed by ^{1}H NMR Spectroscopy and Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), respectively. For this purpose, a 15 mL sample from each treatment group was obtained by mixing an aliquot of each replicate. Sodium azide (NaN_3) was added as antibacterial agent (5 mM final concentration) and then samples were centrifuged at 5000 × *g* for 15 min at 4 °C. The supernatants were passed through 0.22 µm syringe filters and divided into 1.5 mL aliquots before storage at -80 °C.

For the ^{1}H NMR measurements an aliquot of each treatment group was thawed at room temperature, centrifuged at 1800 × *g* for 30 min at 22 °C, and then passed through a 0.22 µm syringe filter to eliminate eventual residual particulate. Each filtered sample (390 µL) was diluted with 150 µL of potassium phosphate buffer (1.5 M, pH 7.4) containing 5 mM NaN_3. Then, 60 µL of deuterium oxide (D_2O) containing 4 mM TSP (trimethyl-silyl-propionic acid) were added. The final 600 µL samples were transferred into 5 mm NMR tubes for analysis, which was performed by a Bruker Avance III spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 300 MHz (Larmor frequency of ^{1}H) and equipped with a Z-gradient probe. All the spectra were acquired at the constant temperature of 300 ± 0.1 K. Three different pulse sequences were used for each sample: 1D-Noesy, 1D Carr Purcell Meiboom Gill (CPMG) spin echo sequence, and 1D diffusion-edited sequence. Before acquiring the spectra, the large and intense water signal was suppressed for all sequences by means of the zgpr pre-saturation pulse sequence. For both the acquisition and processing of the spectra the Topspin 3.6.1 software (Bruker) was used. The acquisition parameters used were 64 and 512 scans, 64 K data points, 30 ppm spectral width, 4 dummy scans and 4 seconds as relaxation delay. A 0.3 Hz line broadening was applied to all the spectra before performing the Fourier Transform.

The recognition of metabolites in the ^{1}H NMR spectra was carried out by comparing the multiplicity and position of the peaks with the data reported in the databases available in the literature (Bovine Metabolome Database, BMDB: https://bovinedb.ca/; Human Metabolome Database, HMDB: https://hmdb.ca/) and by adding known quantities of the single pure metabolites (Sigma-Aldrich) to the samples.

The acquired ^{1}H NMR spectra were processed for the analysis of the principal components of variance (PCA) to highlight any differences in the metabolic profile induced by bentonite. The

analysis was carried out by using the AMIX 4.0.1 software (Bruker Biospin), applied on the spectral range 0.5–10.0 ppm, divided into constant rectangular intervals (buckets) of 0.05 ppm. The spectral range containing the signals of water and contaminants were excluded. The values of the original variables were normalized to the total intensity of the spectra and subsequently subjected to a Pareto Scaling procedure to minimize the *mask* effect of the more intense variables on the less intense ones (Yang et al., 2015).

For the evaluation of the mineral content by ICP-OES, two 1.5 mL aliquots of each treatment group were thawed at room temperature and centrifuged at 17,000 × *g* for 5 min at room temperature. Each supernatant (1 mL) was added with a 5% solution of nitric acid to the final volume of 4 mL, and incubated overnight at room temperature in this oxidizing environment. Subsequently, an ICP-OES 5110 (Agilent) spectrometer, operating with Argon plasma, was used to investigate the content of Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Se, Sr, Zn. For the calibration curves, certified single-element standards (Agilent) were used to prepare six standard solutions at different concentrations (0, 0.300, 0.600, 1.000, 3.000, 6.000 ppm for Na and P; 0, 0.010, 0.020, 0.050, 0.100, 0.200 ppm for Fe, Ca, and Mg; 0, 0.050, 0.100, 0.500, 1.000, 2.000 ppm for K; 0, 0.005, 0.010, 0.050, 0.100, 0.200 ppm for the other elements). To quantify the elements present at higher concentrations (Ca, K, Mg, Na, P) the samples were further diluted 1: 300 (v/v) with a 5% aqueous solution of HNO_3.

1.3. Results and Discussion

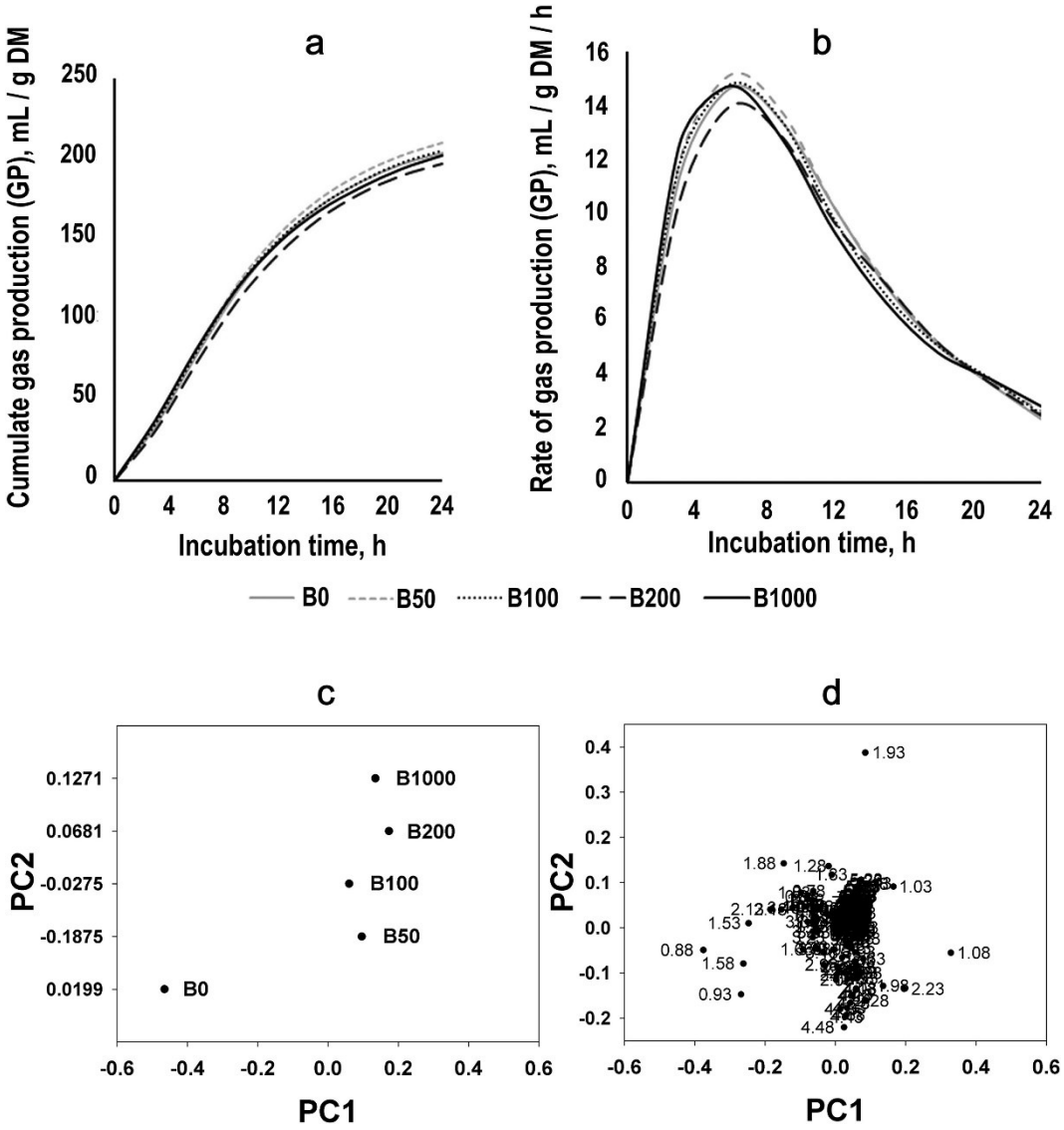
The measured cumulative GP (mL/g DM) and GP rate (mL/g DM/h) (**Figure 1.1**) did not reveal any significant differences between the treatment groups.

The metabolites recognized in the metabolic profiles of the *in vitro* rumen samples were: among organic acids, acetate, acetoacetate, butyrate, formate, isovalerate, propionate, succinate, valerate, 3-hydroxybutyrate and 3-phenylpropionate; among amino acids, glutamine, glycine, proline, sarcosine, tyrosine and valine; among alcohols, ethanol and glycerol; among amines, methylamine and trimethylamine.

The PCA analysis revealed a clear separation between the sample without bentonite and the samples added with bentonite, with PC1 and PC2 explaining 83.92% of the total variance (69.59% and 14.33%, respectively), and identified as responsible for such a variance the spectral ranges that include the signals of butyrate and propionate, providing the indication of a decreasing concentration of butyrate and increasing concentration of propionate for the samples added with bentonite.

Measured average concentrations of Ba, Co, Cr, Cu, Mo and Se were in the nanomolar range. Differently Al, Fe, Li, Mn and Sr were in the micromolar range, while Ca, K, Mg, Na and P were in

Fig. 1.1. Cumulate gas production (a) and Rate of gas production (b) obtained incubating increasing dosages (mg/g DM) of bentonite (B0, B50, B100, B200, B1000) with 1 g of diet for lactating cow. Each curve is the mean of five replicates (*n* = 5). Scores plot (c) and Loadings plot of the two first principal components (PC1 and PC2) of the PCA analysis: each point in the Scores plot represent a sample obtained by a pooled mixture of five replicates, with the point label indicating the bentonite treatment. Each point in the loadings plot represent a 1H NMR spectral region, with the point label indicating the ppm value of the region's centre.



the millimolar range. Values obtained for As, Cd and Pb were not considered, because their concentrations were lower than the detection limit. At the same time Zn was affected by a marked matrix effect and, therefore, the measurement was considered unreliable. To evaluate any relationship between bentonite additions and mineral concentrations a regression analysis was performed by SPSS software, which highlighted that increasing dosages of bentonite were responsible for a significant decrease of Ba, Ca, and Mn and for a significant increase of Al, Cr, Mo and Sr (**Table 1.1**). No significant changes of the concentration of the other analyzed elements were found.

Table 1.1. Regression analysis between the dose of bentonite and mineral concentrations. Only minerals that showed a significant Standardised Regression Coefficient (Beta) with increasing dosage of bentonite are reported.

Minerals	Fitting	Beta	$Beta^2$	F	b0 (μM)	b1
Al	Linear	0.894	0.799	11.951*	1.610 ± 0.058	$4.000*10^{-4}$
Ba	Exponential	-0.964	0.900	38.992**	0.197 ± 0.008	-0.001
Ca	Exponential	-0.895	0.802	12.139*	982.730 ± 85.699	-0.001
Cr	Linear	0.958	0.917	33.246**	0.216 ± 0.001	$1.781*10^{-5}$
Mn	Exponential	-0.945	0.893	25.163*	6.262 ± 0.303	-0.001
Mo	Linear	0.906	0.821	13.771*	0.048 ± 0.004	$3.316*10^{-3}$
Sr	Linear	0.987	0.975	115.523**	1.317 ± 0.030	0.001

Linear model: $Y = b0 + b1 \cdot X$; Exponential model: $Y = b0 \cdot e^{(b1 \cdot X)}$; * $P < 0.05$; ** $P < 0.01$.

1.4. Conclusion

These preliminary results, here reported as a proof of concept, suggest that bentonite administration does not affect ruminal gross fermentations *in vitro*, while it could modify the ruminal metabolome (altering in particular butyrate and propionate) and concentrations of few minerals (Al, Ba, Ca, Cr, Mn, Mo and Sr) in ruminal fluids.

1.5. References

Chalvatzi, S., Kalamaki, M.S., Arsenos, G., Fortomaris, P., 2016. Dietary supplementation with the clay mineral palygorskite affects performance and beneficially modulates caecal microbiota in laying pullets. *J. Appl. Microbiol*. 120(4), 1033–1040. doi: 10.1111/jam.13041.

Cuadros, J., 2017. Clay minerals interaction with microorganisms: a review. *Clay Miner*. 52(2), 235–261. doi: 10.1180/claymin.2017.052.2.05.

Elliott, C.T., Connolly, L., Kolawole, O., 2020. Potential adverse effects on animal health and performance caused by the addition of mineral adsorbents to feeds to reduce mycotoxin exposure. *Mycotoxin Res*. 36(1), 115–126. doi: 10.1007/s12550-019-00375-7.

European Commission, 2021. European Union Register of feed Additives. Pursuant to Regulation (EC) no 1831/2003. Annex I: List of Additives, Edition 06/2021 (294). Ed. Publications Office of the European Union, Luxembourg. doi: 10.2875/699878.

Ghadiri, M., Chrzanowski, W., Rohanizadeh, R., 2015. Biomedical applications of cationic clay minerals. *RSC Adv*. 5(37), 29467–29481. doi: 10.1039/c4ra16945j.

Menke, K.H., and Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim. Res*. *Dev*. 28,7–55.

Nadziakiewicza, M., Kehoe, S., Micek, P., 2019. Physico-chemical properties of clay minerals and their use as a health promoting feed additive. *Animals*, 9(10), 714. doi: 10.3390/ani9100714.

Neubauer, V., Humer, E., Mann, E., Kröger, I., Reisinger, N., Wagner, M., Zebeli, Q., Petri, R.M., 2019. Effects of clay mineral supplementation on particle-associated and epimural microbiota, and gene expression in the rumen of cows fed high-concentrate diet. *Anaerobe* 59, 38–48. doi: 10.1016/j.anaerobe.2019.05.003.

Slamova, R., Trckova, M., Vondruskova, H., Zraly, Z., Pavlik, I., 2011. Clay minerals in animal nutrition. *Appl. Clay Sci*. 51(4), 395–398. doi: 10.1016/j.clay.2011.01.005.

Tagliapietra, F., Cattani, M., Bailoni, L., Schiavon, S., 2010. *In vitro* rumen fermentation: Effect of headspace pressure on the gas production kinetics of corn meal and meadow hay. *Anim. Feed Sci. Technol*. 158(3-4), 197–201. doi: 10.1016/j.anifeedsci.2010.04.003.

Wang, J.P., Chi, F., Kim, I.H., 2012. Effects of montmorillonite clay on growth performance, nutrient digestibility, vulva size, faecal microflora, and oxidative stress in weaning gilts challenged with zearalenone. *Anim. Feed Sci. Technol*. 178(3-4), 158–166. doi: 10.1016/j.anifeedsci.2012.09.004.

Yang, J., Zhao, X., Lu, X., Lin, X., Xu, G., 2015. A data preprocessing strategy for metabolomics to reduce the mask effect in data analysis. *Front. Mol. Biosci*. 2, 4. doi: 10.3389/fmolb.2015.00004.

2. Further *in vitro* investigation on the effects of bentonite on ruminal fermentations, metabolome, and mineral content

2.1. Introduction

The *in vitro* experiment reported in the previous chapter represented a pilot study to produce initial insights into possible alterations on rumen fermentations, metabolome, and mineral content due to bentonite administration in dairy cows (Damato et al., 2022). As reported, bentonite did not interfere with gas production, but appeared to modify ruminal metabolome and the concentrations of some minerals (Al, Ba, Ca, Cr, Mn, Mo, and Sr). In order to confirm the results obtained, a second *in vitro* experiment was performed, refining the experimental design. In particular, in addition to technical replicates, biological replicates were also considered and all the replicates were analyzed separately (not pooled). Moreover, two different techniques, i.e., liquid chromatography/direct flow injection-tandem mass spectrometry (LC/DFI-MS/MS) and 700 MHz ^{1}H NMR spectroscopy, were applied for quantitative metabolomics study and inductively coupled plasma-mass spectrometry (ICP-MS) was used for the analyses of trace elemental content.

2.2. Materials and methods

2.2.1. Experimental design and incubation

The procedure for the *in vitro* fermentations was the same used in the previous experiment (Damato et al., 2022), but with some modifications. Briefly, the rumen fluid was collected from three different healthy lactating cows by esophageal probe (OPBA, Prot. n. 0197903 of 16 May 2019) in order to obtain three different biological replicates. The *in vitro* incubations were performed at the laboratories of the Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE) of the University of Padua (Legnaro, Padua, Italy), using Ankom RF Gas Production System (Ankom Technology, NY, USA), consisting of fermentation bottles and RF pressure sensor modules wirelessly connected to a computer and equipped with an electromechanical valve to control the release of gas, as previously described by Tagliapietra et al. (2010). In particular, after pH measurement, the three rumen fluids were filtered through 3 layers of sterilized cheesecloths. For each biological replicate, 17 fermentation bottles were prepared. First, 50 mL of rumen fluid filtrate and 100 mL of Menke medium (Menke and Steingass, 1988) were combined in all the 17 bottles. Then, 2 bottles were used as blank without adding feed or bentonite, while the remaining 15 bottles were added with 1 g of dried feed and different amounts (0, 5, and 50 mg) of bentonite GLOBALFEED® T1 (Laviosa SpA), in order to obtain three different treatment groups (B0 or control, B100, and B1000, respectively) with five technical replicates each.

Information about the properties and characteristics of the bentonite used were provided by the manufacturer through the Technical Data Sheet of the product, which is available online (https://www.laviosa.com/wp-content/uploads/2017/01/LAVIOSA_AnimalFeed_GLOBALFEED-T1_TDS_ENG-1.pdf). The dried feed used was a mycotoxin-free total mixed ration (TMR) [corn silage 32.98% of DM, grass silage 11.01% of DM, Multimix 5 (Consorzio Agrario Del Nordest; composition: corn, barley, sugarcane molasses) 20.38% of DM, Protein mix 36 (Consorzio Agrario Del Nordest; composition: dehulled sunflower seed flour, roasted soybean seeds, dehulled soybean flour feeds, corn, sugarcane molasses) 16.19% of DM, corn semolina 7.84% of DM, alfalfa hay 7.35% of DM, Unilac Smc 500 Vitamin® (Nutristar; composition: calcium carbonate, sodium bicarbonate, sodium chloride, monocalcium phosphate, magnesium oxide, algae calcium (Lithotamnion), magnesium sulphate, monoammonium phosphate, dried algae Ascophyllum nodosum (TASCO) 1.8%, calcium sulphate dihydrate, wheat middlings, potassium carbonate, sulphur flower, wheat) 2.73% of DM, Germolino® (Nutristar; composition: expanded and blanched linseed, expanded and blanched corn germ, corn) 1.54% of DM].

The procedures described above were conducted under anaerobic conditions thanks to continuous flushing of the headspace of the bottles with CO_2. Once ready, all bottles were immediately closed with the RF sensor modules and placed in a ventilated incubator at 39.0 °C for 24 h.

2.2.2. Pressure recording and gas production computation

As previously described (Tagliapietra et al., 2010), gas pressure in the headspace of each bottle was monitored continuously by the RF sensor module, which sent the information to the computer every 5 min. During the 24 h-incubation, the bottles were vented through the electromechanical valve of the modules whenever the headspace pressure reached a threshold value of 1 psi, corresponding to 6.895 kPa. The pressure data recorded every 3 h were converted in terms of cumulative gas production (GP, mL/g DM), which in turn was used to calculate gas production rate (GP rate, mL/g DM/h).

2.2.3. Sample collection and pre-treatments

At the end of the 24 h-incubation, all the bottles were removed from the incubator and kept on ice to interrupt fermentations. After pH measurement, from each bottle, 25 ml were collected in a Falcon® tube with NaN_3 at final concentration of 5 mM. Samples were centrifuged at 17,500 × *g* for 15 min at 4 °C under vacuum. The supernatant obtained was transferred in another Falcon® tube and then aliquoted into 2 ml cryopreservation tubes. The aliquots were frozen at -80 °C and subsequently sent in dry ice to TMIC (The Metabolomics Innovation Center), located at the University of Alberta (Edmonton, Alberta, Canada), where they were stored at -80 °C. On the day

of analyses, an aliquot of each sample (about 1.5 mL) was thawed on ice and vortexed before taking the volumes needed for each analysis.

2.2.4. Metabolomic analysis by LC/DFI-MS/MS

A targeted quantitative metabolite profiling was performed, using the TMIC Prime assay, based on multiple reaction monitoring (MRM) and combining direct flow injection−mass spectrometry (DFI-MS/MS) with reverse-phase liquid chromatography-mass spectrometry (RPLC-MS/MS) (Foroutan et al. 2019; Foroutan et al., 2020; Zheng et al., 2020). This assay has the potential to identify and quantify up to 143 different endogenous compounds, including amino acids, biogenic amines, sugars, organic acids, triglycerides (TGs), acylcarnitines (ACs), phosphatidylcholines (PCs), lysophosphatidylcholines (LysoPCs), sphingomyelins (SMs) etc. (complete list available online: https://metabolomicscentre.ca/service/tmic-prime/).

Sample preparation

Each thawed and vortexed sample was split to be subjected to two separate derivatization reactions: the phenyl-isothiocyanate (PITC) derivatization for amine-containing compounds (such as amino acids and their derivatives, biogenic amines, hexose, lipids, and acylcarnitines) and the 3-nitrophenylhydrazine (3-NPH) derivatization for keto- and carboxyl-containing compounds, such as organic acids (OA).

For the analysis of both PITC-derivatized metabolites and OA, seven calibration solutions of the analytes considered and internal standard (ISTD) mixture solutions of isotope-labeled were prepared by mixing and diluting standard stock solutions. Three quality control solutions (QCs) were also prepared for each analysis.

The analysis of PITC-derivatized metabolites started pipetting a total of 20 µL of the specific ISTD mixture solutions onto the center of all the wells of a 96-well filter plate applied on a 96 deep-well plate. Then, 10 µL of PBS blank, 10 µL of the seven calibration solutions, 10 µL of the QCs, and 10 µL of each sample were pipetted into a dedicated well. After keeping the plate under gentle nitrogen stream for 30 min to dry, the derivatization was obtained by adding 50 µL of 5% PITC derivatization solution (prepared with PITC reagent and a mixture of ethanol, water, and pyridine) to each well and keeping the plate at room temperature for 20 min. The excess PITC solution was removed using an evaporator. For metabolite extraction, 300 µL of solvent (methanol with 5 mM ammonium acetate) were added to each well and the plate was covered, shaken at 330 rpm for 30 min at room temperature, and then centrifuged at 500 rpm for 5 min at room temperature (Eppendorf™ Centrifuge 5810) in order to collect the extracts from the wells of the filter plate into the corresponding wells of the 96 deep-well plate. The extracts were splits into two aliquots: 150

µL were diluted with 150 µL HPLC water to perform LC-MS/MS analysis for the quantification of amino acids, biogenic amines, and their derivatives, while the remaining 150 µL were diluted with 400 µL of MS running solvent (60 µL of formic acid, 10 mL of water, and 290 mL of methanol) to perform DFI-MS/MS analysis for the quantification of lipids, acylcarnitines, and glucose/hexose.
For the analysis of organic acids, 50 µL of each sample were transferred in an Eppendorf tube with 10 µL of ISTD mixture solution and 150 µL of ice-cold methanol. Blanks, calibration solutions, and QCs were also prepared. After vortex, the tubes were left overnight at -20 °C for protein precipitation. The following day, tubes were centrifuged at 13,000 rpm for 15 min at 4 °C (Eppendorf™ Centrifuge 5810 R) and 50 µL of the obtained supernatants were transferred into the corresponding wells of a Nunc 96 DeepWell plate, followed by the addition of 25 µL of 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (150 mM in methanol), 25 µL of 3-NPH (250 mM in 50% aqueous methanol), and 25 µL of pyridine (7.5% in 75% aqueous methanol). The plate was shaken at 330 rpm for 2 h at room temperature to favour the derivatization reaction. Then, 350 µL of water and 50 µL of BHT solution (butylated hydroxytoluene in methanol) were added to each well.

LC/DFI-MS/MS analysis

All the analyses were performed with an AB Sciex QTRAP® 4000 tandem mass spectrometer (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA), using Analyst 1.6.2 as controlling software. For the LC separations of PITC- and 3-NPH-derivatized compounds, two separate Agilent reversed-phase Zorbax Eclipse XDB C18 column and different solvents (solvent A 0.2% v/v formic acid in water and solvent B 0.2% v/v formic acid in acetonitrile for PITC-derivatized metabolites; solvent A 0.01% v/v formic acid in water and solvent B 0.01% v/v formic acid in methanol for 3-NPH-derivatized compounds) were used. On the other hand, sugars and lipids were detected by column-free DFI-MS/MS. The mobile phase consisted in the DFI buffer mentioned in the description of sample preparation.
The parameters set for the analyses were the same described in details by Zheng et al. (2020).

2.2.5. Metabolomic analysis by ^{1}H NMR spectroscopy

^{1}H NMR spectroscopy was applied in order to detect and quantify metabolites undetectable or unquantifiable (e.g., due to saturation signals) through the LC/DFI-MS/MS assay described above.

Preparation of samples for ^{1}H NMR spectroscopy

For each sample, 220 µl were transferred in a 1.5 mL Eppendorf tube and centrifuged at 14,000 rpm for 15 min at 4 °C (Eppendorf™ Centrifuge 5810 R) to remove any residual particulate. Then, 200 µl of the supernatant were mixed with 50 µl of a standard NMR buffer solution [750 mM potassium

phosphate (pH 7.0), 5 mM DSS-d6 (3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt), 5.84 mM 2-chloropyrimidine-5-carboxylic acid, and 54% v/v D_2O in H_2O], according to the procedure described by Foroutan et al. (2020) for ruminal fluid samples, slightly modified. The final 250 µl NMR samples contained 150 mM potassium phosphate, 1 mM DSS-d6 as calibrate internal standard, and 10% D_2O. In addition to the samples, a blank, consisting in 50 µl of buffer solution and 200 µl of aqueous solution with 5 mM NaN_3, and a quality control (QC) sample, consisting in 50 µl of buffer solution and 200 µl of a standard mixture of four different amino acids (Glycine, L-Alanine, L-Threonine, and L-Aspartate) at known concentration (1.25 mM), were prepared. The purpose of analyzing blank and QC sample was to check the presence of any contaminants in the buffer and possible interferences due to NaN_3 present in the samples and to verify the accuracy of the NMR analysis for the concentration measurement.

All the prepared Eppendorf tubes were vortexed and centrifuged at 13,000 rpm for 10 min at 4 °C (Eppendorf™ Centrifuge 5810 R). Then, all the samples, the blank, and the QC were transferred into 3 mm NMR tubes by means of glass Pasteur pipettes.

Acquisition and processing of ^{1}H NMR spectra

The ^{1}H NMR spectra were acquired by using a Bruker Avance III Ascend™ 700 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a 5 mm cryo-probe and an autosampler (SampleJet, Bruker Biospin). Each spectrum was collected at the constant temperature of 298 K and with the first transient of a nuclear Overhauser effect spectroscopy (NOESY)-presaturation pulse sequence, which allows quantitative accuracy (Saude et al., 2006). IconNMR™ Automation Software (Bruker BioSpin) was used for the acquisition and the processing of spectra, using 128 scans, 4 s acquisition time, 1 s recycle delay, 0.5 Hz line broadening, and the DSS singlet as chemical shift reference standard at 0.00 ppm.

^{1}H NMR spectra compound identification and quantification

The acquired spectra were then imported into Chenomx NMR Suite 8.2 software (Chenomx Inc., Edmonton, AB, Canada). After improving phase correction, line broadening, CSI calibration, and baseline correction by means of the Processor module, data were sent to the Profiler module to identify and quantify the metabolites in the samples. Briefly, the spectral signals of each sample were compared manually with the spectra of pure compounds included in Spectral Reference Libraries. Then, the concentration of the identified metabolites was computed by the software using DSS-d6 1 mM as internal standard for quantification.

2.2.6. Trace element analysis by ICP-MS

The ICP-MS protocol applied allows to identify and quantify up to 35 different elements in samples. In particular, 300 µl of each sample were transferred in an Eppendorf tube and centrifuged at 10,000 rpm for 5 minutes (Eppendorf™ Centrifuge 5810 R) to remove eventual particles. Then, the supernatant was diluted by a factor of 5 with a solvent containing 1% HNO_3 and 5% H_2O_2 in Milli-Q water and added with Indium (In) at the final concentration of 25 ppb as internal.

The trace element concentrations were measured on a Perkin-Elmer NexION 350x ICP–MS (Perkin-Elmer, Woodbridge, ON, Canada) operating with an Argon plasma. Helium was used to minimize the contamination. The analysis was performed in kinetic energy discrimination (KED) mode using the following parameters: nebulizing gas flow of 0.9 mL/min, auxiliary gas flow of 1.2 mL/min, plasma gas flow of 16 mL/min, dwell time for each metal ion of 50 ms, and total integration time of 500 ms (10 sweeps per reading and three replicates).

Before analysing the samples, a performance check of the ICP-MS was conducted using a commercial Nexion Solution (Perkin Elmer).

In addition to the samples, calibration solutions and quality control samples (QCs) were also submitted to the same analysis protocol. In particular, seven calibration solutions containing known concentrations of investigated elements were used to create the calibration curves. QCs, consisting in diluted standard reference materials with known trace elements at known concentrations, were analyzed to evaluate the accuracy of the analysis.

Moreover, every 15 samples analyzed, a continuing calibration verification (CCV) was run to monitor the validity of the calibration curves.

2.2.7. Statistical analysis

Before performing the statistical analysis of the data, datasets were created applying the following strategy to manage dependent variables with values below the limits of detection (LODs): *i)* exclusion (NA, not available) of the values < LOD if present in smaller numbers than 5; *ii)* substitution of such values with half of the LOD (LOD/2) if they were present in a number between 5 and 15; *iii)* exclusion of the dependent variable from the dataset if the number of values < LOD was more than 15. Then, for each treatment group, the means of the data of the five technical replicates were calculated and used for the statistical analysis.

The statistical analysis was performed using linear mixed models in R statistical environment v. 3.6.2 (The R Foundation for Statistical Computing, Vienna, Austria). In particular, for the analysis of pH data, metabolites and trace elements concentrations, the fixed effect was the treatment (B0, B100, or B1000) and the random effect was the biological replicate (rumen fluid 1, 2, or 3), while for the analysis of cumulative GP and GP rate data, the fixed effect of the incubation time (measurement

Figure 2.1. Plot of means of (**A**) cumulative gas production (cumulative GP, mL/g DM) and (**B**) gas production rate (GP rate, mL/g DM/h) over incubation time (h). Points represent mean values and bars the associated standard errors. Letters indicate statistically significant differences ($P < 0.05$) between means.

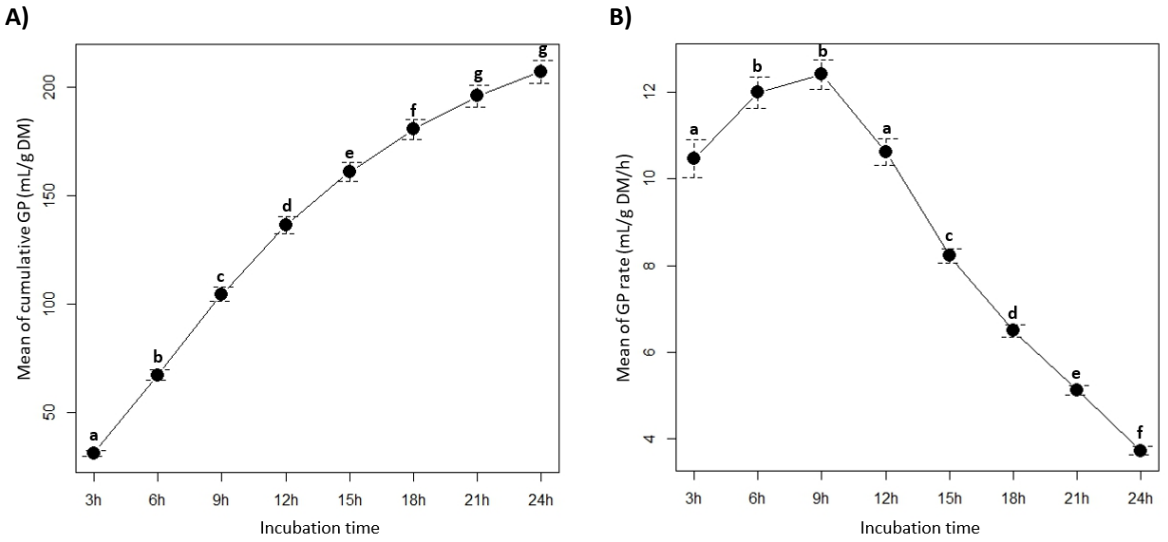
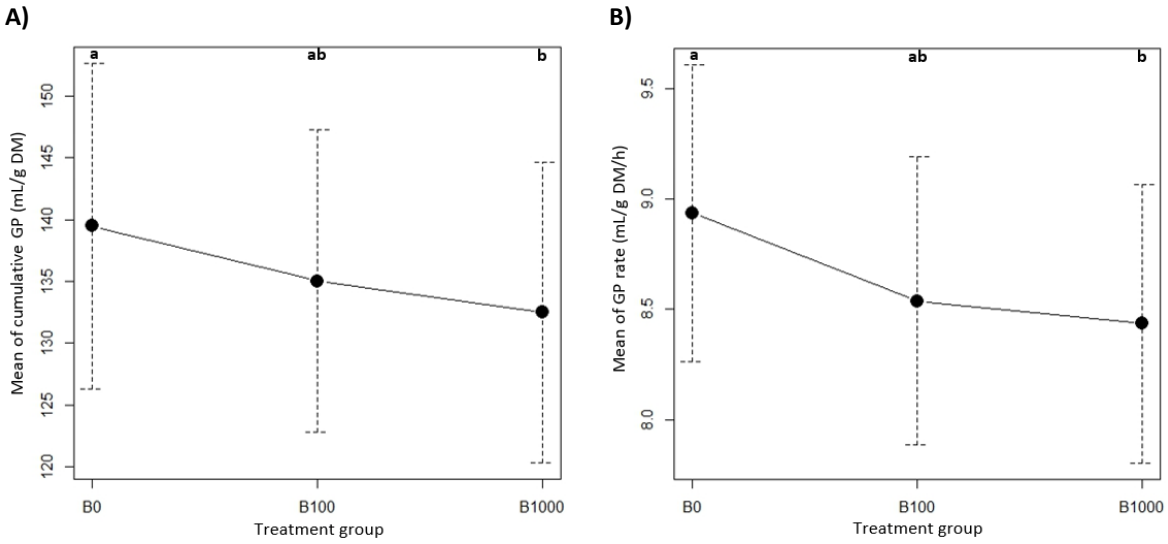


Figure 2.2. Plot of means of (**A**) cumulative gas production (cumulative GP, mL/g DM) and (**B**) gas production rate (GP rate, mL/g DM/h) in the three different treatment groups (B0 = 0 mg of bentonite, B100 = 5 mg of bentonite, and B1000 = 50 mg of bentonite). Points represent mean values and bars the associated standard errors. Letters indicate statistically significant differences ($P < 0.05$) between means.



at 3, 6, 9, 12, 15, 18, or 24 h of incubation) was also considered. Results were considered statistically significant when $P < 0.05$.

2.3. Results

2.3.1. Gas production

The cumulative GP (mL/g DM) and GP rate (mL/g DM/h) were affected by both time and treatment. In particular, cumulative GP (mL/g DM) significantly increased from 3 to 21 h of incubation (**Figure**

Table 2.1. Mean concentration ± standard deviation (µM) of metabolites detected and measured by LC/DFI-MS/MS after 24 h-*in vitro* rumen fermentations with three different bentonite treatments (B0 = 0 mg, B100 = 5 mg, and B1000 = 50 mg of bentonite). Superscript letters indicate statistically significant differences ($P < 0.05$) between the means obtained for a given metabolite in the different treatments.

Metabolites (µM)	Bentonite level		
	B0	B100	B1000
Amino acids, biogenic amines and derivatives			
Aspartic acid	3.723 ± 0.286	3.529 ± 0.095	3.515 ± 0.186
Glutamic acid	5.975 ± 0.323	6.042 ± 0.394	5.714 ± 0.295
Kynurenine	0.245 ± 0.007	0.267 ± 0.020	0.262 ± 0.015
Methionine-sulfoxide	0.137 ± 0.015	0.121 ± 0.037	0.124 ± 0.016
Phenylethylamine	4.640 ± 1.576	4.291 ± 0.773	4.047 ± 0.711
Putrescine	0.768 ± 0.167	0.657 ± 0.140	0.596 ± 0.038
Serotonin	0.043 ± 0.003	0.043 ± 0.005	0.044 ± 0.004
Spermidine	1.092 ± 0.600 [a]	0.597 ± 0.244 [ab]	0.234 ± 0.059 [b]
Spermine	0.576 ± 0.329	0.219 ± 0.069	0.171 ± 0.003
Threonine	5.006 ± 6.965	4.692 ± 6.420	0.994 ± 0.038
Tyramine	6.055 ± 0.491	5.915 ± 1.033	5.833 ± 0.950
Carbohydrates			
Glucose	86.594 ± 16.222	91.291 ± 8.399	95.456 ± 12.507
Organic acids and derivatives			
β-Hydroxybutyric acid	0.938 ± 0.304	0.876 ± 0.358	0.849 ± 0.285
Fumaric acid	0.224 ± 0.046 [a]	0.184 ± 0.046 [b]	0.214 ± 0.051 [a]
Indole acetic acid	11.421 ± 3.262	11.223 ± 0.724	11.081 ± 2.549
Methylmalonic acid	0.044 ± 0.006 [a]	0.042 ± 0.006 [ab]	0.039 ± 0.005 [b]
para-Hydroxyhippuric acid	0.068 ± 0.007	0.068 ± 0.012	0.066 ± 0.011
Succinic acid	1.672 ± 0.020	1.631 ± 0.181	1.577 ± 0.052
Acylcarnitines			
C3-OH (hydroxypropionylcarnitine)	0.041 ± 0.006	0.042 ± 0.006	0.041 ± 0.010
C4-OH (Hydroxybutyrylcarnitine)	0.478 ± 0.138	0.403 ± 0.072	0.301 ± 0.045
C5 (Valerylcarnitine)	0.026 ± 0.009	0.026 ± 0.007	0.028 ± 0.005
C5-M-DC (Methylglutarylcarnitine)	0.022 ± 0.004	0.023 ± 0.004	0.022 ± 0.005
C6:1 (Hexenoylcarnitine)	0.032 ± 0.005	0.033 ± 0.003	0.028 ± 0.004
C16:1 (Hexadecenoylcarnitine)	0.038 ± 0.001	0.036 ± 0.02	0.037 ± 0.003
Lysophosphatidylcholines, acyl C			
LysoPC a C14:0	0.763 ± 0.146 [a]	0.650 ± 0.124 [b]	0.656 ± 0.116 [b]
LysoPC a C16:0	0.077 ± 0.013	0.066 ± 0.008	0.070 ± 0.004

2.1 A), while GP rate (mL/g DM/h) increased during the first 6-9 h of incubation and then progressively decreased (**Figure 2.1 B)**. Bentonite treatment reduced both cumulative GP (mL/g DM) (**Figure 2.2 A**) and GP rate (mL/g DM/h) (**Figure 2.2 B**), with a statistically significant difference ($P < 0.05$) between B0 and B1000. No time x treatment interaction was observed.

Table 2.2. Mean concentration ± standard deviation (µM) of metabolites detected and measured by ^{1}H NMR after 24 h-*in vitro* rumen fermentations with three different bentonite treatments (B0 = 0 mg, B100 = 5 mg, and B1000 = 50 mg of bentonite). Superscript letters indicate statistically significant differences ($P < 0.05$) between the means obtained for a given metabolite in the different treatments.

Metabolites (µM)	Bentonite level		
	B0	B100	B1000
3-Hydroxyphenylacetate	6.9 ± 1.6	6.7 ± 2.0	6.8 ± 1.7
3-Phenylpropionate	346.1 ± 16.2	337.4 ± 21.2	343.5 ± 23.6
4-Hydroxybutyrate	5.7 ± 1.2 [a]	6.1 ± 0.6 [a]	4.7 ± 1.2 [b]
4-Hydroxyphenylacetate	113.9 ± 33.4 [a]	109.6 ± 16.9 [a]	82.6 ± 20.9 [b]
Acetate	47,326.4 ± 3,516.5	46,738.0 ± 3,267.8	45,909.3 ± 4,450.7
Acetone	9.5 ± 1.2	9.6 ± 0.7	9.5 ± 1.2
Adenine	2.1 ± 0.5	1.8 ± 0.3	1.3 ± 0.7
Benzoate	11.2 ± 1.4	11.6 ± 1.0	11.4 ± 1.4
Butyrate	13,506.8 ± 1,479.8	13,151.4 ± 1,295.6	12,826.4 ± 1,624.7
Dimethylamine	15.0 ± 3.2	13.3 ± 1.3	12.6 ± 1.6
Dimethyl sulfone	44.4 ± 0.6	44.9 ± 0.6	44.2 ± 1.3
Ethanol	31.3 ± 7.4	30.0 ± 5.6	31.1 ± 6.4
Formate	23.4 ± 1.6	23.2 ± 1.1	23.8 ± 3.0
Isobutyrate	658.1 ± 104.1	622.7 ± 35.7	591.2 ± 53.0
Isopropanol	12.5 ± 0.5	12.7 ± 0.8	12.4 ± 1.2
Isovalerate	721.4 ± 151.1	670.9 ± 35.6	622.5 ± 61.8
Lactate	18.1 ± 1.6	17.4 ± 1.2	17.4 ± 2.8
Malonate	4.2 ± 0.9	3.6 ± 0.1	3.6 ± 0.5
Methanol	235.2 ± 178.3	229.0 ± 173.7	208.7 ± 166.0
Methylamine	7.1 ± 2.4	5.8 ± 0.8	6.1 ± 0.6
para-Cresol	22.7 ± 8.1	19.1 ± 2.4	22.7 ± 2.1
Phenylacetate	233.0 ± 70.2	210.1 ± 12.4	189.8 ± 22.8
Propionate	18,456.8 ± 1,538.6	18,193.8 ± 1,844.8	17,772.3 ± 2,046.9
Trimethylamine	1.5 ± 0.6 [a]	1.1 ± 0.3 [a]	0.6 ± 0.1 [b]
Valerate	1,619.9 ± 246.7	1,553.6 ± 203.2	1,508.0 ± 258.4

2.3.2. Metabolic profiles and pH

A total of 26 metabolites were identified and quantified by means of LC/DFI-MS/MS (**Table 2.1**) and a further 26 different metabolites by means of ^{1}H NMR spectroscopy (**Table 2.2**). A significant bentonite treatment effect ($P < 0.05$) was observed on the concentrations of seven metabolites in total. In particular, the concentration of the lysophosphatidylcholine LysoPC a C14:0 was significantly reduced in both B100 and B1000 treatments compared with B0. Methylmalonic acid and spermidine decreased as bentonite addition increased, with a significant difference between B0 and B1000. The concentrations of 4-hydroxybutyrate, 4-hydroxyphenylacetate, and trimethylamine were significantly lower in B1000 than in B0 and B100. Lastly, fumaric acid was more concentrated in B0 and B1000 compared with B100.

The pH was not influenced by bentonite treatments ($P = 0.4$).

Table 2.3. Mean concentration ± standard deviation (µg/L) of trace elements detected and measured by ICP-MS after 24 h-*in vitro* rumen fermentations with three different bentonite treatments (B0 = 0 mg, B100 = 5 mg, and B1000 = 50 mg of bentonite). Superscript letters indicate statistically significant differences ($P < 0.05$) between the means obtained for a given element in the different treatments.

Trace elements (µg/L)	Bentonite level		
	B0	B100	B1000
Al	5.40 ± 0.85 [a]	5.25 ± 0.46 [a]	6.63 ± 0.80 [b]
Ba	0.22 ± 0.02	0.21 ± 0.03	0.21 ± 0.02
Ca	1,008.69 ± 103.04	983.83 ± 111.22	1,022.99 ± 97.51
Co	0.11 ± 0.01 [a]	0.09 ± 0.01 [b]	0.06 ± 0.01 [c]
Cu	0.75 ± 0.01	0.74 ± 0.10	0.65 ± 0.10
Fe	8.22 ± 0.87	7.10 ± 1.20	7.45 ± 1.20
K	16,743.49 ± 1,121.94	16,543.04 ± 1,405.72	16,762.48 ± 1,046.38
Li	0.90 ± 0.09	0.93 ± 0.13	0.96 ± 0.16
Mg	430.65 ± 163.74	402.18 ± 108.18	403.90 ± 187.48
Mn	1.97 ± 0.79	1.72 ± 0.37	1.83 ± 0.62
Na	148,323.90 ± 2,418.17	147,191.50 ± 5,615.13	149,169.10 ± 3,695.33
P	16,330.71 ± 856.71	16,065.77 ± 1,213.66	16,153.03 ± 902.17
Rb	2.01 ± 0.15	1.97 ± 0.18	2.03 ± 0.14
Sr	1.32 ± 0.07 [a]	1.35 ± 0.10 [b]	2.21 ±0.05 [b]
Ti	0.81 ± 0.03	0.76 ± 0.04	0.78 ± 0.02
V	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Zn	2.02 ± 0.06 [a]	1.90 ± 0.13 [a]	1.37 ± 0.15 [b]
Zr	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01

2.3.3. Trace element content

A total of 18 trace elements were detected and quantified by ICP-MS analysis (**Table 2.3)**. Among them, four elements were significantly affected ($P < 0.05$) by bentonite treatment. In particular, Al resulted significantly highly concentrated in B1000 than in B0 and B100 and Sr in B100 and B1000 compared with B0. Conversely, Zn concentration was significantly lower in B1000 in comparison with B0 and B100 and Co concentration decreased as bentonite addition increased.

2.4. Discussion and conclusions

The aim of this *in vitro* experiment was to confirm the results obtained from a previous pilot study, which suggested the capacity of bentonite to modify ruminal metabolome and mineral content without altering ruminal gross fermentation (Damato et al., 2022). In this second study, biological and technical replicates were used to consider the effect of random biological variations and limit the effect of random measurement errors, respectively.

In contrast to the above pilot study, the results of the present experiment suggested that bentonite is able to affect ruminal gas production, as both cumulative GP (mL/g DM) and GP rate (mL/g DM/h)

decreased as bentonite addition increased, with a statistically significant difference between B0 and B1000.

The compounds identified by ^{1}H NMR spectroscopy and LC/DFI-MS/MS were found to be metabolites previously reported in bovine rumen fluid samples and in samples from *in vitro* ruminal fermentations (Bovine metabolome database, BMDB: https://bovinedb.ca/; Livestock metabolome database, LMDB: https://lmdb.ca/; Foroutan et al., 2020; Saleem et al., 2013; Eom et al., 2020; Ametaj et al., 2010; Malheiros et al., 2021; Kheirandish et al., 2022), with the exceptions (to the best of our knowledge) of indole acetic acid and p-hydroxyhippuric acid. The detection of indole acetic acid can be explained by the fact that it is a plant hormone and can also be synthetized by microorganisms present in rumen and in mammalian gut (Livestock metabolome database, LMDB; Attwood et al., 2006). Regarding p-hydroxyhippuric acid, also known as 4-hydroxyhippuric acid, it is a secondary metabolite of hippuric acid, which is reported in animal and human blood, urine, and milk and typical increases with the consumption of phenolic compounds (Human Metabolome Database, HMDB: https://hmdb.ca/; Livestock Metabolome Database, LMDB). Tang et al. (2022) reported the production of 4-hydroxyhippuric acid by lactic acid bacteria as an intermediate metabolite of hippuric acid during *in vitro* anaerobic fermentations of the polyphenols of citrus fruits.

The slight bentonite-induced modifications in rumen fermentations were not accompanied by significant pH changes. In this regard, it should be considered that: *i)* no statistically significant variation in the highest produced Volatile Fatty Acids (VFAs) (i.e. acetate, butyrate, propionate, valerate, and isovalerate) between treatments and no lactic acid production due to the microbial fermentations, which are the major source of pH changes in rumen (Sjaastad et al., 2010), were observed, *ii)* the medium used in the fermentation systems contained high concentration of sodium bicarbonate (Menke and Steingass, 1988), which conferred high buffering capacity, *iii)* bentonite has also been proven to exert buffering activity that it can be used in dairy cows to alleviate adverse effects of SARA (subacute ruminal acidosis) (Sulzberger et al., 2016).

The present study seems to confirm the capacity of bentonite treatments (mainly B1000) to affect the ruminal metabolome and the ruminal concentration of some trace elements (mainly microelements), as suggested by the previous pilot study (Damato et al., 2022). However, some differences can be noticed between the two studies considering the metabolic profiles of the fermentation fluids and the metabolites affected by bentonite treatments. Such differences may be associated to: *i)* differences in the ruminal fluid used for the fermentations, depending on different factors (cow, feed intake, diet composition, distance from feed intake, environment etc.), and *ii)* different metabolomics approaches applied. Indeed, for the first *in vitro* experiment only one cow was used as donor and the rumen fluid was collected a few weeks after the cow arrived at "L. Toniolo" experimental farm (Legnaro, Padova, Italy), when probably the animal was still

acclimatizing to the new farming conditions and diet. In addition, for the metabolomic analysis, only ^{1}H NMR spectroscopy (300 MHz spectrometer) was used. In contrast, the present experiment was performed after months, using three cows, different from the previous one, as donors and applying both ^{1}H NMR spectroscopy (700 MHz spectrometer) and LC/DFI-MS/MS for the study of the metabolome.

In conclusion, the capacity of bentonite to interfere with ruminal metabolome and mineral content seems to be confirmed, while, in contrast with previous results, the present study suggested that bentonite is able to affect rumen gas production.

2.5. References

Ametaj, B.N., Zebeli, Q., Saleem, F., Psychogios, N., Lewis, M.J., Dunn, S.M., Xia, J. and Wishart, D.S. 2010. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics*, 6, 583–594. doi: 10.1007/s11306-010-0227-6

Attwood, G., Li, D., Pacheco, D. and Tavendale, M. 2006. Production of indolic compounds by rumen bacteria isolated from grazing ruminants. *Journal of Applied Microbiology*, 100(6), 1261–1271. doi: 10.1111/j.1365-2672.2006.02896.x

Damato, A., Vanzani, P., Giannuzzi, D., Giaretta, E., Novelli, E., Vianello, F., Tagliapietra, F. and Zennaro, L. 2022. Bentonite does not affect *in vitro* ruminal gross fermentations but could modify ruminal metabolome and mineral content. A proof of concept. *Research in Veterinary Science*, 144, 78-81. doi: 10.1016/j.rvsc.2022.01.012

Eom, J.S., Lee, S.J., Kim, H.S., Choi, Y.Y., Kim, S.H., Lee, Y.G. and Lee, S.S. 2020. Metabolomics Comparison of Hanwoo (*Bos taurus coreanae*) Biofluids Using Proton Nuclear Magnetic Resonance Spectroscopy. *Metabolites*. 10(8), 333. doi: 10.3390/metabo10080333

Foroutan, A., Guo, A.C., Vazquez-Fresno, R., Lipfert, M., Zhang, L., Zheng, J., Badran, H., Budinski, Z., Mandal, R., Ametaj, B.N. and Wishart, D.S. 2019. Chemical Composition of Commercial Cow's Milk. *J. Agric. Food Chem*. 67, 4897-4914. doi: 10.1021/acs.jafc.9b00204

Foroutan, A., Fitzsimmons, C., Mandal, R., Piri-Moghadam, H., Zheng, J., Guo. A.C., Li. C., Guan, L.L., and Wishart, D.S. 2020. The Bovine Metabolome. *Metabolites*, 10(6), 233. doi: 10.3390/metabo10060233

Kheirandish, P., Petri, R.M., Sener-Aydemir, A., Schwartz-Zimmermann, H.E., Berthiller, F., Zebeli, Q. and Pacífico, C. 2022. Characterization of microbial intolerances and ruminal dysbiosis towards

different dietary carbohydrate sources using an in vitro model. *J Appl Microbiol*,133(2), 458–476. doi: 10.1111/jam.15573

Malheiros, J.M., Barbosa Correia, B.S., Ceribeli, C., Rodrigues Cardoso, D., Colnago, L.A., Bogusz Junior, S., Reecy, J.M., Gerson Barreto Mourão, G., Lehmann Coutinho, L., Pascale Palhares, J.C., Berndt, A. and Correia de Almeida Regitano, L. 2021. Comparative untargeted metabolome analysis of ruminal fluid and feces of Nelore steers (*Bos indicus*). *Scientific Reports*, 11, 12752. doi: 10.1038/s41598-021-92179-y

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim. Res. Dev*. 28, 7–55.

Saleem, F., Bouatra, S., Guo, A.C., Psychogios, N., Mandal, R., Dunn, S.M., Ametaj, B.N. and Wishart, D.S. 2013. The Bovine Ruminal Fluid Metabolome. *Metabolomics*, 9, 360–378. doi: 10.1007/s11306-012-0458-9

Saude, E., Slupsky, C., Sykes, B., 2006. Optimization of NMR analysis of biological fluids for quantitative accuracy. *Metabolomics*, 2, 113−123. doi: 10.1007/s11306-006-0023-5

Sjaastad, O.V., Sand, O., Hove, K., 2010. *Physiology of Domestic Animals*, Editor: Scandinavian Veterinary Press, Oslo.

Sulzberger, SA, Kalebich, CC, Melnichenko, S, Cardoso, FC., 2016. Effects of clay after a grain challenge on milk composition and on ruminal, blood, and fecal pH in Holstein cows. *J Dairy Sci*. 99(10), 8028–8040. doi: 10.3168/jds.2016-11030

Tagliapietra, F., Cattani, M., Bailoni, L. and Schiavon, S. 2010. *In vitro* rumen fermentation: Effect of headspace pressure on the gas production kinetics of corn meal and meadow hay. *Animal Feed Science and Technology*, 158(3-4), 197–201. doi: 10.1016/j.anifeedsci.2010.04.003

Tang, R., Yu, H., Qi, M., Yuan, X., Ruan, Z., Hu, C., Xiao, M., Xue, Y., Yao, Y. And Liu, Q. 2022. Biotransformation of citrus fruits phenolic profiles by mixed probiotics in vitro anaerobic fermentation. *LWT*, 160, 113087. doi: 10.1016/j.lwt.2022.113087

Zheng, J., Zhang, L., Johnson, M., Mandal, R., and Wishart, D.S. 2020. Comprehensive Targeted Metabolomic Assay for Urine Analysis. *Anal. Chem*., 92(15), 10627−10634. doi: 10.1021/acs.analchem.0c01682

3. *In vivo* evaluation of putative side effects of bentonite administration in dairy cows

3.1. Introduction

The *in vitro* studies object of the previous chapters suggested that zootechnical bentonite is not completely inert at ruminal level. Although sequestering agents used as feed additives are not absorbed by the gastrointestinal tract of animals, their possible interaction with rumen metabolome and mineral content could be reflected on the systemic and mammary metabolism of ruminants, with possible consequences on both animal health and the quality of milk for human consumption and food industry. While most *in vivo* experiments performed in farm animals aimed to evaluate the effectiveness of clay minerals as mycotoxin sequestering agents, and for this reason mycotoxins were usually administered together with clay minerals (Damato et al., 2022; Elliott et al., 2020), in this *in vivo* study no mycotoxins were added to the animal diet in order to evaluate potential side effects of the bentonite on rumen, blood, and mammary gland physiology, and milk characteristics in lactating dairy cows. To this purpose, the same bentonite product tested in our *in vitro* experiments was administered to multiparous lactating Holstein cows. To simulate the actual use of the additive on farms, bentonite was used complying with the maximum doses prescribed by the manufacturer.

3.2. Materials and methods

3.2.1. Animals, animal care and housing

The experiment was approved by the "Organismo Preposto al Benessere degli Animali" (OPBA, Prot. n. 0197903 of 16 May 2019). Six multiparous lactating Italian Holstein cows were purchased from an agricultural company located in the province of Padua and transported to "Lucio Toniolo" experimental farm of the University of Padua (Legnaro, Padua, Italy) to conduct the *in vivo* trial. Cows were identified thanks to their ear-tag numbers as cow 88, 313, 437, 578, 632, and 988. Upon their arrival at the experimental farm on 8 November 2019, cows were housed in individual boxes underneath a dedicated shed, but without preventing them from mutual visual and physical contact. For the entire duration of the experiment, cows were watered *ad libitum* and fed with a basal diet, consisting of a total mixed ration (TMR) formulated for lactating cattle with a milk production of 35 kg/d. The TMR was distributed in the morning between 6:30 and 7:00 a.m. The amount distributed in the individual feeders was determined on the basis of the individual intake, so that each cow always left a residue in the feeder. Thanks to proper barriers, each cow could not have access to the feeder of the neighboring cow. **Table 3.1** reports the ingredient composition of

Table 3.1. Ingredient composition (kg/cow/d as fed) of the TMR ingested by the six cows for the duration of the experiment, considering a mean feed ingestion of 42 kg/cow/d as-fed.

Ingredients	kg/cow/d as-fed
Alfalfa	1.67
Corn semolina	1.90
Protein mix 36 (Consorzio Agrario Del Nordest)[1]	3.79
Multimix 5 (Consorzio Agrario Del Nordest)[2]	4.02
Unilac Smc 500 Vitamin® (Nutristar)[3]	0.56
Germolino® (Nutristar)[4]	0.45
Dried yeast	0.06
Corn silage	17.85
Grass silage	10.04
Molasses	1.67

[1] Complementary feed for dairy cows. Composition: dehulled sunflower seed flour, roasted soybean seeds, dehulled soybean flour feeds, corn, sugarcane molasses.
[2] Complementary feed for ruminants. Composition: corn, barley, sugarcane molasses.
[3] Mineral complementary feed for lactating cows. Composition: calcium carbonate, sodium bicarbonate, sodium chloride, monocalcium phosphate, magnesium oxide, algae calcium (*Lithotamnion*), magnesium sulphate, monoammonium phosphate, dried algae *Ascophyllum nodosum* (TASCO) 1.8%, calcium sulphate dihydrate, wheat middlings, potassium carbonate, sulphur flower, wheat.
[4] Complementary feed for dairy cows. Composition: expanded and blanched linseed, expanded and blanched corn germ, corn.

the TMR, while **Table 3.2** describes the mean chemical composition, with associated standard deviation (SD), of the TMR during over the experiment. Moreover, a tri-weekly monitoring of mycotoxin contamination of the TMR by LC-MS/MS analysis (Veneto Agricoltura, Thiene, Vicenza, Italy) revealed that, throughout the duration of the experiment, aflatoxin concentrations were under the limits of detection (AFB1 < 0.3 µg/kg, AFB2 < 0.5 µg/kg, AFG1 < 0.5 µg/kg, and AFG2 < 1 µg/kg) and fumonisin levels (FB1 = 529-747 µg/kg and FB2 = 185-238 µg/kg) progressively increased over time, but remaining well below the guidance value recommended for adult ruminants feed by the European Commission (2006). Milking was carried out twice a day (at 6:00 a.m. and 5:00 p.m.) with individual bucket system.

Table 3.2. Means and associated standard deviations (SD) of the chemical components of the TMR ingested by the six cows for the duration of the experiment. DM = dry matter, CP = crude protein, CF = crude fat, aNDFom = amylase neutral detergent fiber organic matter, ADF = acid detergent fiber, ADL = acid detergent lignin, NDFD = neutral detergent fiber digestibility, NFC = non-fiber carbohydrates.

Chemical components	Mean	SD
Humidity (%)	43.06	1.54
DM (%)	56.94	1.54
CP (% as-fed)	15.67	0.65
CF (% as-fed)	3.77	0.11
Ash (% as-fed)	8.61	0.19
aNDFom (% as-fed)	33.31	2.20
ADF (% as-fed)	21.09	1.75
ADL (% as-fed)	3.28	0.32
Hemicelluloses (% as-fed)	12.25	0.69
NDFD (% total NDF)	56.60	1.64
Starch (% as-fed)	23.27	1.77
NFC (% as-fed)	38.62	2.11
Pectins (% as-fed)	9.33	0.58
Sugars (% as-fed)	6.02	0.83
Ca (% as-fed)	0.79	0.16
P (% as-fed)	0.42	0.02
Mg (% as-fed)	0.27	0.03
K (% as-fed)	1.56	0.053
Cl (% as-fed)	0.56	0.063
Na (% as-fed)	0.20	0.07
S (% as-fed)	0.21	0.01
Zn (mg/kg)	93.50	16.01
Cu (mg/kg)	13.00	2.16
Fe (mg/kg)	226.00	42.15
Mn (mg/kg)	65.50	6.61
Si (% as-fed)	1.69	0.14

The health status of cows was constantly monitored, with a focus on mammary gland health, by clinical examinations, inspection of the first jets of milk at each milking, and somatic cell count (SCC) monitoring twice a week. Upon arrival at the experimental farm, all subjects were in good health.

In one cow (ID 313), congenital absence of one teat and unproductivity of the corresponding quarter were found. During the following months, three episodes of mastitis were diagnosed in cow ID 437 and successfully treated with intramammary antibiotics.

Four cows (ID 88, 313, 578, and 632) were pregnant on their arrival (51, 105, 32, and 98 d of pregnancy, respectively). The remaining two subjects were inseminated during their stay in the experimental farm (13 December 2019). One of them (ID 437) was successfully fertilized, while the other (ID 988) remained nonpregnant for the entire duration of the experiment.

3.2.2. Experimental design

The trial lasted from November 2019 to February 2020. After a 24-d acclimatization period (t0), the six cows [body weight (BW) = 658,50 ± 67,70 kg; days in milk (DIM) = 192,50 ± 22,72 d; mean ± SD] were randomly assigned to one of three treatments (B0 or control, B50, and B100, corresponding to 0, 50, and 100 g/animal/d of bentonite, respectively) in a 3 x 3 Latin square crossover design with 19-d treatment periods (t1, t2, and t3), separated by 9-d washout periods (**Table 3.3**). Treatment periods were divided into an adaptation phase (d 1 to 14) and a measurement phase (d 15 to 19). The bentonite used was GLOBALFEED® T1 (Laviosa SpA) and the quantities administered correspond to the maximum dosages recommended by the manufacturer for prevention (50 g) and for existing mycotoxin contamination (100 g) in ruminants, as indicated in the Technical Data Sheet, available online, which also describes in detail the properties and characteristics of the product (https://www.laviosa.com/wp-content/uploads/2017/01/LAVIOSA_AnimalFeed_GLOBALFEED-T1_TDS_ENG-1.pdf). Bentonite was administered to fasted animals in the morning, top dressed on about 1 kg of TMR, before distributing the rest of the ration.

Table 3.3. Assignment of the six cows to one of three bentonite treatments (B0 or control = 0 g/animal/d, B50 = 50 g/animal/d, and B100 = 100 g/animal/d) over three different treatment periods (t1, t2, and t3) in a 3 x 3 Latin square crossover design. Cows are identified with their ear-tag numbers: 88, 313, 437, 578, 632,.and 988.

	Treatment periods					
Treatments	t1		t2		t3	
B0	88	578	632	437	313	988
B50	313	437	88	988	632	578
B100	632	988	313	578	88	437

3.2.3. Data collection

BW (kg) was measured at the beginning of t1 and at the end of t3. Body Condition Score (BCS) was evaluated according to Edmonson et al. (1989) on the last day of each treatment period. The TMR offered (kg) and the residual in the feeders (kg) were recorded once a week to determine the feed intake (kg) of each cow. Rumination activity was monitored daily, by means of ruminometers SCR Heatime® HR model. Milk weights (kg) were recorded at each milking and daily milk yield (kg/d) was calculated for each animal by summing the weight of milk produced at the morning and evening milking.

3.2.4. Sampling procedures

Samples of milk, blood, and ruminal fluid were collected at the end of t0 and during the measurement phase of t1, t2, and t3. Due to the large number of planned analyses, two different sampling days were set:

a) Milk, blood, and ruminal fluid samples for the evaluation of potential physiological alterations and metabolic responses were collected on d 18 of t0 and d 15 of t1, t2, and t3. More precisely:

- Milk samples were taken at the morning milking from the individual milking buckets with sterile 25 mL serological pipettes and collected in 50 mL Falcon® tubes: 40 mL added with 100 µL azidiol for milk quality assessment, 50 mL for the study of leukocyte subpopulations by flow cytometry, and 50 mL added with sodium azide (NaN_3) at the final concentration of 5 mM for oxidative stress evaluation, proteomics and peptidomics, metabolomics/metabonomics by ^1H NMR spectroscopy, and mineral content analysis by ICP-OES (Coupled Plasma-Optical Emission Spectroscopy).
- Blood samples were collected in the middle of the morning, 3-4 h after the distribution of the TMR, by jugular venous sampling with animals fixed at the feeding fence. In particular, three different samples were collected from each cow, using three different 10 mL vacuum blood collection tubes (Vacutainer®): one for serum, one for EDTA-plasma, and one for heparin-plasma. Blood samples were intended for oxidative stress evaluation, metabolomics/metabonomics by ^1H NMR spectroscopy, and mineral content analysis by ICP-OES.
- Ruminal fluid samples (10-20 mL) were collected by ruminocenthesis right after blood sampling. The pH was immediately measured using a portable pH meter, before transferring samples into 15 mL Falcon® tubes pre-filled with NaN_3 to obtain the final concentration of 5 mM. Rumen fluid samples were intended for metabolomics/metabonomics by ^1H NMR spectroscopy and mineral content analysis by ICP-OES.

Immediately after collection, all samples were kept refrigerated, with the exception of those for blood serum in order to promote the coagulation process, and underwent a complex sorting, pre-treatment, and transportation procedure.

In particular, milk samples for qualitative assessment were transported to the laboratory of ARAV (Associazione Regionale Allevatori Veneto, Padua, Italy) to be processed within 24 h of collection.

Milk samples for the study of leukocyte subpopulations were transported to the flow cytometry laboratory of the Department of Comparative Biomedicine and Food Science (BCA) of the University of Padua (Legnaro, Padua, Italy) to be processed within 24 h of collection.

Milk samples for proteomic and peptidomic analyses were obtained by taking aliquots (one 15 mL Falcon® tube and three 2 mL cryopreservation tubes) from each milk sample with 5 mM NaN_3. The aliquots were frozen at -80 °C and shipped in dry ice. The analyses were performed by the Department of Health Sciences of the University "Magna Græcia" of Catanzaro in collaboration with the Department of Basic Biotechnological Sciences, Intensive Care and Perioperative Clinics of the Catholic University of the Sacred Heart, School of Medicine Gemelli Hospital (Rome, Italy).

The remaining volume of milk samples with 5 mM NaN_3 and all blood and rumen fluid samples were brought to the laboratory of Veterinary Physiology at the BCA Department of the University of Padua (Legnaro, Padua, Italy) to be pre-treated and aliquoted. In particular, milk was centrifuged at 1500 × *g* for 15 min at 4 °C and the surfaced fat layer was removed. The obtained skimmed milk was transferred into 5 mL tubes (Beckman Coulter™, US) for ultra-centrifugation at 25,000 rpm for 30 min at 4 °C under vacuum till 750 mmHg by means of Optima™ L-90K ultracentrifuge (Beckman Coulter™, US). The supernatant (milk whey) was aliquoted in 2 mL cryopreservation tubes.

Blood samples were pre-treated as follows: after clot formation (in around 30 minutes at room temperature), tubes for serum were centrifuged at 2500 × *g* for 10 min at 20 °C, while tubes for plasma were centrifuged at 1500 × *g* for 15 min at 4 °C. The supernatants obtained (serum or plasma) were aliquoted in 2 mL cryopreservation tubes.

Ruminal fluid samples were centrifuged at 5000 × *g* for 15 minutes at 4 °C and the resulting supernatants were further centrifuged at 8000 × *g* for 15 minutes at 4 °C. The supernatants obtained from the second centrifugation were passed through 0.22 µm syringe filters. This pretreatment, similar to those reported by other authors (Saleem et al., 2012; Saleem et al., 2013; Zhao et al., 2014), was applied to remove the gradually finer particulate matter, such as feed particles, most of the microorganisms and cellular debris. The filtrates were then divided into aliquots in 2 mL cryopreservation tubes.

The aliquots of milk whey and blood plasma for oxidative stress evaluation remained at the BCA Department, while the aliquots of milk whey, blood serum, and ruminal fluid for metabolomic/metabonomic study and mineral content analysis were frozen and then transported in dry ice to the Department of Molecular Medicine (DMM) of the University of Padua (Padua, Italy).

All the aliquots were stored at -80 °C until they were analyzed.

b) Milk samples for cheesemaking tests and milk and rumen fluid samples for the study of microbial populations were collected on d 21 of t0 and d 17 of t1, t2, and t3. More precisely:

- Milk samples were collected at the morning milking. Before attaching milking clusters, from each cow, 50 mL of pooled milk from all quarters were collected in duplicate in 50 mL Falcon® tubes by sterile sampling for the study of microbial populations. Then, after milking was finished, from each individual bucket, 2 L of milk were collected in duplicate for micro cheesemaking tests and the remaining milk was combined in a 30 L plastic jerrycan with the milk from the previous evening's milking (stored at refrigeration temperature) of the same cow to obtain an adequate volume for macro cheesemaking tests. Lastly, from each jerry can, 40 mL were collected by means of a sterile 25 mL serological pipette into a 50 mL Falcon® tube containing 100 µL of azidiol for milk quality assessment.
- Rumen fluid samples were collected 3 h after TMR distribution, fixing animals at the feeding fence and using an oro-esophageal probe. Sampling was performed after feeding animals in accordance with other studies evaluating eventual changes in the metabolic activity of ruminal microbial populations induced by feed additive administration in ruminants (Neubauer et al., 2019; Nur Atikah et al., 2018). The liquid obtained from each cow was immediately filtered through a sterilized dairy cloth with 250 µm mesh size and folded into 4 layers. The obtained filtrate, collected in a beaker, was then transferred into two 50 mL Falcon® tubes. These samples were intended for the study of microbial populations.

  Immediately after collection, all samples were kept refrigerated and transported to different laboratories: milk samples for micro cheesemaking tests to the laboratories of the Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE) of the University of Padua (Legnaro, Padua, Italy), milk samples for macro cheesemaking tests to Veneto Agricoltura (Thiene, Vicenza, Italy), while milk and rumen fluid samples for the study of metabolically active microbial populations to the laboratories of the BCA Department of Comparative Biomedicine and Food Science of the University of Padua (Legnaro, Padua, Italy). In particular, micro cheesemaking and macro cheesemaking tests were conducted according to the procedures reported by Bittante et al. (2022) and Cosentino et al. (2022), respectively.

For the analysis of the metabolically-active rumen and milk bacterial community structure and rumen protozoal community structure, the RNA-based amplicon sequencing method previously described by Bailoni et al. (2021) was applied.

These analyses, although part of the original project, are not a specific topic of this dissertation. However, data on ruminal and milk microbial populations will be used for the discussion of the results.

3.2.5. Qualitative analyses of milk

Qualitative analyses of milk samples were performed on the same day of milk sampling by ARAV laboratory by means of FT6000 MilkoScan infrared analyzer (Foss A/S) and Fossomatic 7 DC analyzer (Foss A/S). The milk quality parameters considered included: *i)* composition (% of fat, lactose, protein, and casein), *ii)* pH, *iii)* content in BHB (β-hydroxybutyrate), urea, and chlorides, *iv)* SSC (somatic cell count) and TBC (total bacterial count), and *v)* MCP (milk coagulation properties), such as a_{30} (curd firmness), k_{20} (curd firming time), RCT (rennet coagulation time), and IAC (index of milk aptitude to coagulate).

3.2.6. Flow cytometry of milk

Flow cytometric analysis was performed on the same day of milk sampling, as the storage of samples by refrigeration or freezing induces cell death and lysis, which may negatively affect the results of the analysis (Li et al., 2015). The applied protocol began with the parallel preparation of cell suspensions from milk samples and antibody solutions for the characterization of leukocyte (WBC) subpopulations. To obtain cell suspensions, each 50 mL milk sample was diluted 1:2 by splitting the sample in two 25 mL aliquots and adding an equal volume of PBS 1X (Phosphate-Buffered Saline 1X), useful for cell preservation. Samples were then centrifuged at 450 × *g* for 15 min at 4 °C. After discarding surfaced fat and supernatant, 50 mL of PBS 1X were added inside each tube and a further centrifugation was performed as described above. The supernatant was discarded again and the two pellets obtained from each sample were combined in the same 15 mL tube and washed by adding 14 mL of PBS and repeating the centrifugation. After removal of the supernatant, the cell pellet was resuspended in 500 µL or 1 mL of RPMI+NaN_3+FBS (Roswell Park Memorial Institute medium + sodium azide + Fetal Bovine Serum) solution, considering the cellularity of the original milk sample, assessed by the SCC results. In particular, 500 µL were added to samples with low cellularity and 1 mL to samples with high cellularity. The functions of the RPMI+NaN_3+FBS solution are: *i)* RPMI medium supports cells, *ii)* Azide acts as a preservative and prevents internalization, polarization, or detachment of Ag-Ab complexes, and *iii)* FBS, in addition

Table 3.4. Fluorophore-conjugated monoclonal antibodies against bovine leukocyte antigens and their dilutions used for flow cytometric analysis of milk samples.

Antibody	Clone	Host	Conjugated fluorophore	Isotype	Company	Dilution
Anti-CD11b	CC126	Mouse	FITC	IgG2b	GeneTex	1:100
Anti-CD14	Tuk4	Mouse	PE	IgG2a	Abcam	1:25
Anti-CD44	IM7	Rat	APC	IgG2b, kappa	eBioscience™	1:25
Anti-CD21	CC21	Mouse	PE	IgG1	GeneTex	1:50
Anti-CD4	CC8	Mouse	PE	IgG2a	GeneTex	1:50
Anti-CD8	CC63	Mouse	AF647	IgG2a	Bio-Rad (Formerly AbD Serotec)	1:50
Anti-CD18	CA1.4E9	Mouse	AF647	IgG1	Bio-Rad (Formerly AbD Serotec)	1:25
Anti-CD45	1.11.32	Mouse	FITC	IgG1	GeneTex	1:200

to supporting cells, exerts a "blocking effect" as it provides proteins to which antibodies can aspecifically bind, reducing nonspecific bindings between Ab and cells.

For the preparation of the antibody solutions, nine different fluorophore-conjugated monoclonal antibodies against bovine WBC antigens were diluted separately with PBS+NaN_3 solution in 1.5 mL Eppendorf® Safe-Lock microtubes (**Table 3.4)**. During the procedure, both the original antibodies and their diluted solutions were kept in ice.

Once both cell suspensions and diluted antibodies solutions were ready, four different tubes were prepared for each milk sample: one tube without antibodies, as negative control, and other three tubes with different combinations of diluted antibodies (50 µL each) for the study of different WBC subpopulations. The antibody combinations chosen were: *1)* Anti-CD11b plus Anti-CD14, *2)* Anti-CD4 plus Anti-CD8, and *3)* Anti-CD45 plus Anti-CD21 plus Anti-CD18. Then, 50 µL of the cell suspension of each sample were added in each corresponding tube. All tubes were incubated for 30 min at 4˚C in the dark, ensuring cell viability and avoiding fluorophore photobleaching. After incubation, the excess antibodies were removed from each tube by adding 500 µL of PBS 1X and centrifuging at 244 × *g* for 10 min at 4 °C. Then, the supernatant was discarded and 900 µL of PBS 1X were added to each tube.

The flow cytometric analysis was performed with the CyFlow Space flow cytometer (Sysmex Partec, Sysmex Europe GmbH), equipped with a red laser (638 nm wavelength), a blue laser (488 nm wavelength), and a UV laser (375 nm wavelength). For data acquisition and processing, FlowMax software version 2.82 (Sysmex Partec, Sysmex Europe GmbH) was used.

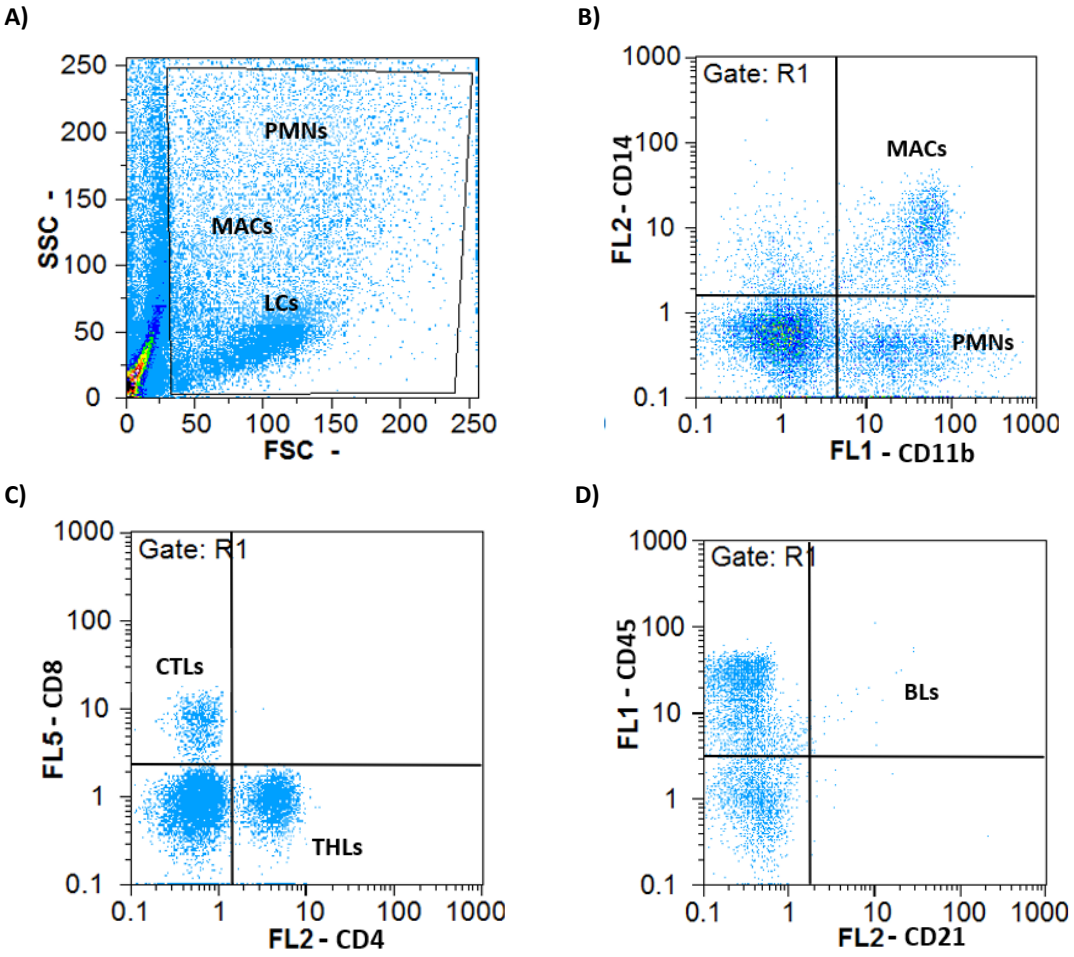
For data display, the first plot set up was the Forward Scatter vs Side Scatter (FSC vs SSC) dot plot. In this plot, the so-called R1 gate was defined so as to circumscribe the events of interest (i.e.,

WBCs) and exclude signals from cellular debris. It was thus possible to set the acquisition to stop automatically once 20,000 events were counted in R1 gate. Also, the other plots were set to represent the fluorescence emitted only by the events included in R1 gate.

The morphological evaluation of sample cells was performed in the FSC vs SSC dot plots, representing the combination of the scattering phenomena generated by each sample particles (cells and debris) intercepted by light rays: scatter in forward direction (Forward Scatter or FSC) and scatter at 90° (Side Scatter or SSC), which give information on particle size and particle internal complexity, respectively (Brown and Wittwer, 2000; McKinnon K.M., 2018). Indeed, FSC (x-axis) increases as cell size increases, and SSC (y-axis) increases as internal complexity of cell increases. In the FSC vs SSC dot plots of milk samples (**Figure 3.1 A**), it is generally possible to sharply distinguish lymphocytes (LCs), with medium-small size and lower internal complexity then neutrophils (PMNs)/macrophages (MACs). In contrast, PMNs and MACs cannot be clearly distinguished from each other in milk because of their morphological similarity, especially along the SSC axis (Taylor et al., 1997).

The identification of different WBC subpopulations and the measurement of their relative percentages were carried out by applying an appropriate gating strategy. For this purpose, in dot plots showing the different wavelengths detected by the fluorescence channels (FL1 for FITC = 518 nm; FL2 for PE = 578 nm; FL5 for APC = 660 nm and AF647 = 668 nm), the WBC subpopulations were recognised based on the different cell distribution of CD antigens (UniProt website: https://www.uniprot.org/; Wilkerson M. J., 2012; Wilkerson and Springer, 2020) bound by the specific fluorochrome-conjugated antibodies. Thus, PMNs were identified as CD11b+ CD14− events, MACs as CD11b+ CD14+ events, T-helper lymphocytes (THLs) as CD4+ CD8− events, cytotoxic T lymphocytes (CTLs) as CD8+ CD4− events, and B lymphocytes (BLs) CD45+ CD21+ CD18+ events (**Figure 3.1 B, C, D**).

Figure 3.1. Typical dot plots obtained by the flow cytometric analysis of milk samples. **A)** FSC vs SSC: the quadrilateral within the plot corresponds to R1 gate, enclosing all the events attributable to cells and excluding the events due to cellular debris. Labels indicate approximately the areas (within R1) where the signals of different WBC subpopulations are visible: PMNs = neutrophils, MACs = macrophages, and LCs = lymphocytes; **B)** FL1-CD11b vs FL2-CD14: plot used to measure PMNs, identified as CD11b+ CD14− with fluorescence in FL1 and not in FL2 (bottom right gate), and MACs, identified as CD11b+ CD14+ with fluorescence in both FL1 and FL2 (top right gate); **C)** FL2-CD4 vs FL5-CD8: plot used to measure THLs = T-helper lymphocytes, identified as CD4+ CD8− with fluorescence in FL2 and not in FL5 (bottom right gate), and CTLs = cytotoxic T lymphocytes, identified as CD8+ CD4− with fluorescence in FL5 and not in FL2 (top left gate); **D)** FL2-CD21 vs FL1-CD45: plot used to measure BLs = B lymphocytes, here identified as CD21+ CD45+ with fluorescence in both FL2 and FL1 (top right gate).



3.2.7. Oxidative stress evaluation in blood plasma and milk whey

Four different biomarkers of oxidative stress in blood EDTA plasma and milk whey samples were considered: three for protein oxidation, i.e., AOPP (Advanced Oxidation Protein Products), thiol groups, and CO (carbonyl groups), and one for lipid peroxidation, i.e., MDA (malondialdehyde). In order to normalize the concentration of protein oxidation biomarkers, the total protein concentration was also measured.

Determination of total proteins (TP)

Total protein concentration (TP) was measured in blood plasma and in milk whey samples by means of the Bradford's method and the bicinchoninic acid (BCA) method, respectively, using the specific assay kits (Bradford Reagent, Sigma-Aldrich Co., St, Louis, MO and BCA Protein Assay Kit, Pierce Biotechnology, Rockford, IL, USA), strictly following manufacturers' instructions.

Determination of Advanced Oxidation Protein Products (AOPP)

AOPP concentration in blood plasma and milk whey was estimated by a spectrometric method, similar to that described by other authors (Bordignon et al., 2014; Talukder et al., 2015; Witko-Sarsat et al., 1996; Witko-Sarsat et al., 1998), using a microplate reader at 340 nm (2030 Multilabel Reader Victor X4, PerkinElmer, Waltham, MA, USA). Briefly, plasma and whey milk were diluted 1:5 and 1:2, respectively, with PBS (v/v, 5 mM, pH 7.2) and 200 µL of diluted samples were placed into the "sample wells" of a microtiter plate with 96 flat-bottom wells (SARSTEDT AG & Co. KG, Germany) and added with 20 µL of glacial acetic acid (Sigma–Aldrich, St. Louis, MO, USA). The calibration curve (range from 0 to 200 µmol/L) was prepared with a 10 mM solution of Chloramine-T (N-Chloro-p-toluenesulfonamide sodium salt, Sigma–Aldrich, St. Louis, MO, USA) by serial dilutions with PBS 20 mM, pH 7.4 (PBS 20X). In the same plates of the samples, 200 µL of the prepared chloramine-T solutions were placed in the "standard wells" and added with 10 µL of 1.16 M solution of potassium iodide (Reagent Plus; Sigma–Aldrich, St. Louis, MO, USA) and 20 µL of acetic acid. The "blank wells" of the plates were filled with 200 µL of PBS 20X, 10 µL of potassium iodide, and 20 µL of acetic acid. After 5 minutes, the absorbance of the reaction mixtures was read against the blank and the AOPP concentrations were expressed as nmol/mL and subsequently normalized on the TP concentration (nmol/mg).

Determination of thiol group

Thiol group concentration was determined using the thiol/disulfide reaction of thiol and Ellman's reagent (5.5'-dithiobisnitrobenzoic acid) described by Hu M.L. (1994). After mixing 20 µL of blood plasma or 50 µL of milk whey with 180 µL of PBS 0.1 M EDTA 1 mM pH 8 and 3.5 µL DTNB (Ellman's reagent, Sigma-Aldrich, Milan, Italy), sulfhydryl groups were estimated in the samples by spectrophotometry at 412 nm (2023 Multilabel Reader VictorX4, PerkinElmer, Waltham, MA, USA), comparing to a standard curve obtained with known concentrations of a sulfhydryl-containing compound, i.e., cysteine (0.25-1.5 mM). The results were expressed in nmol/mL and in nmol/mg of TP.

Determination of carbonyl groups (CO)

Carbonyl groups were determined using the derivatization with 2,4-dinitrophenylhydrazine (DNPH) (Bordignon et al., 2014; Reznick and Packer, 1994). First, all samples were divided into two aliquots, one for the reaction with DNPH and the other for the negative control. Both aliquots of each sample (50 µL of blood plasma or 100 µL of milk whey) were added with 1 mL of 10% trichloroacetic acid (TCA) at 4°C to induce protein precipitation. After 10 min, samples were centrifuged at 5000 × *g* for 10 min at 4°C. The supernatants were discarded and 0.5 mL of DNPH 10 mM in HCl 2.5 N were added to the protein pellets in the tubes intended for the derivatization, while 0.5 mL of HCl 2.5 N were added to the negative controls. Samples resuspended were placed in the dark for 1 h at room temperature and shaken every 10 min. Then, all the aliquots were added with 0.5 mL of TCA 20%, vortexed, and kept in ice for 10 min, before being centrifuged at 5000 × *g* for 15 min at 4 °C. After supernatant was removed, the protein pellets were washed three times with 1 ml ethanol/ethyl acetate and centrifugation at 5000 × *g* for 10 min at 4°C to remove excess DNPH. Lastly, proteins were solubilized in 1 mL of potassium phosphate 20 mM pH 2.3, left at 37 °C for 15 min and centrifuged again. The carbonyl content was determined by means of the spectrophotometer JASCO V-630 with an absorbance of 380 nm using the molar extinction coefficient of DNPH (ε_{380} = 22,000 M^{-1} cm^{-1}) (Levine et al., 1990). The results were expressed both in nmol/mL and in nmol/mg of TP.

Determination of malondialdheyde (MDA)

MDA concentration was measured using the thiobarbituric acid reactive substances (TBARS) method (Yoshida et al., 2005; Vinagre et al. 2012; Wasowicz et al., 1993; Gabai et al., 2004). Briefly, 25 µL of sodium dodecyl sulfate solution (8.1% w/v; Sigma-Aldrich, Milan, Italy), 187.5 µL of acetic acid buffer (20% v/v, pH 3.5; Sigma-Aldrich), and 187.5 µL of thiobarbituric acid (1% v/v; Sigma-Aldrich) were mixed with 100 µL of blood plasma or 200 µL of milk whey. The reaction mixtures were incubated at 100 °C for 10 min and then cooled in ice. After adding 100 µL of water and 625 µL of N-butyl alcohol : pyridine (15 : 1 v/v) (Fluka, Milan, Italy), the mixtures were centrifuged at 2000 × *g* for 10 min at 4 °C. The supernatants were transferred in a microtiter plate with 96 flat-bottom wells (SARSTEDT AG & Co. KG, Germany) and analyzed by spectrophotometry on a microplate reader at 535 nm (2023 Multilabel Reader VictorX4, PerkinElmer, Waltham, MA, USA). A calibration curve (range from 0.154 to 5 mM) was obtained using TMOP (tetramethoxypropane; Sigma-Aldrich). The MDA concentrations were expressed in nmol/mL.

3.2.8. Proteomics and peptidomics of milk

The analyses here reported were performed by the research team of the Department of Health Sciences of the University "Magna Græcia" of Catanzaro in collaboration with the Department of Basic Biotechnological Sciences, Intensive Care and Perioperative Clinics of the Catholic University of the Sacred Heart, School of Medicine Gemelli Hospital (Rome, Italy).

Proteomic analysis

For the proteomic analysis a label-free quantitative approach was used. In order to enhance the separation and characterization of milk proteins, it is generally necessary to apply prefractionation methods (Roncada et al., 2012). In this case, whey proteins were collected from raw milk samples thanks to two steps: a mild centrifugation to discard lipids and proteins present in milk fat globule membrane (MFGM) of the upper layer and the cellular component of the bottom, followed by an ultracentrifugation of the middle layer (skimmed milk) to precipitate and remove the main protein fraction, represented by caseins. The obtained whey proteins were pre-fractionated by electrophoresis. Then, an in-gel digestion of the migration profiles was performed through appropriate protein reduction and alkylation, addition of trypsin protease, and incubation at the optimal temperature (37 °C) and pH (8.5). The obtained tryptic peptides were subsequently eluted from the polyacrylamide gel and purified using stop-and-go-extraction tips (StageTips) (Rappsilber et al., 2007), before being measured, in triplicate, by Ultra-High-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (UHPLC-MS/MS) (Orbitrap Elite™). Bioinformatic analysis of the resulting MS/MS data was performed with MaxQuant software package for identification, by database dependent search analysis, and subsequent quantification of proteins detected. More precisely, protein identification was obtained by compering the MS/MS spectra of the samples with the protein database UniProtKB/Swiss-Prot Protein Knowledgebase, restricted to 'Bos taurus' taxonomy. The search parameters set were: automatic tolerance for precursor ions and for product ions, minimum 1 fragment ion matched per peptide, minimum 3 fragment ions matched per protein, minimum 2 peptides matched per protein, 1 missed cleavage, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification, and false discovery rate (FDR) < 1%.

Peptidomic analysis

Peptidomic analysis was performed by top-down approach. Briefly, after casein depletion as described above, milk samples were analyzed by High-Performance Liquid Chromatography-Electrospray Ionization coupled with high resolution Tandem Mass Spectrometry (HPLC-ESI-MS/MS) (LTQ-Orbitrap XL™). MS/MS raw data were generated by Xcalibur and then analyzed by

Proteome Discoverer 2.2 software using 'Bos taurus' database of UniProtKB and applying "high confidence" filters and FDR equal to 0.01 for peptide matching. Through manual inspection, it was verified that only spectra with an acceptable number of fragment ions were considered.

3.2.9. Metabolomics of ruminal fluid, blood serum, and milk whey by ^{1}H NMR spectroscopy

Preparation of samples for ^{1}H NMR spectroscopy

Several preliminary tests to find the optimal sample preparation procedures and experimental conditions were performed before developing specific protocols for each biofluid.

Milk whey aliquots were thawed at room temperature and vortexed. Then 500 µL of each sample were filtered through 10 kDa filters (Vivapsin 500, Sartorius) at 10,000 × *g* for 30 min at 22 °C. In order to remove the residues of glycerol and NaN_3, the 10 kDa filters were previously washed 3 times with MilliQ water and centrifugation at 10,000 × *g* for 10 min at room temperature. Then, the NMR samples were obtained by mixing 460 µL of each filtrate with 170 µL of potassium phosphate (K/Pi) buffer (1 M, pH 6.8) containing 5 mM NaN_3 and 70 µL of D_2O+TSP solution, consisting of deuterium oxide 99.99% with 40 mM trimethyl-silyl-propionic acid (Sigma-Aldrich).

Blood serum aliquots were thawed at room temperature and vortexed. If any fluff was present in the thawed sample, a 2-5 min centrifugation at 10,000 × *g* at room temperature was performed in order to precipitate the unwanted material and to recover the supernatant only. For each aliquot, the NMR sample was prepared with 400 µL of serum, 320 µL of K/Pi buffer (1.5 M, pH 7.4) containing 5 mM NaN_3, and 80 µL of D_2O+TSP solution.

Rumen fluid aliquots were thawed at room temperature, vortexed, centrifuged at 17,500 × *g* for 30 min at 22 °C, and then passed through 0.22 µm syringe filters with cellulose membrane to eliminate eventual residual particulate. To prepare NMR samples, 450 µL of each filtrate were diluted with 180 µL of K/Pi buffer (1.5 M, pH 7.4) containing 5 mM NaN_3 and 70 µL of D_2O+TSP solution.

Once each NMR sample was ready, the pH was measured, given its importance in ^{1}H NMR spectroscopy in determining the chemical shift of the signals. Then, 600 µL of each sample were transferred into an NMR 535-PP tube (Wilmad®).

Acquisition and processing of 1H NMR spectra

The ^{1}H NMR spectra were acquired for all samples by using a Bruker Avance™ III NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a 5 mm Z-gradient probe and operating at the Larmor frequency for ^{1}H of 300.13 MHz. The temperature was set at 300 K ± 0.1 K for the acquisition of rumen fluid spectra and 310 K ± 0.1 K for blood serum and milk whey spectra.

As soon as each sample was ready, the external side of the NMR tube was degreased with acetone to minimize any interference by external contaminants and the sample was inserted in the magnet. Within 5 minutes the temperature of the sample stabilized and then the suppression of the large and intense water signal was carried out by means of the zgpr pre-saturation pulse sequence. Next, three different one-dimensional spectra were acquired by means of three different types of pulse sequences:

- 1D-Noesy: it is the acquisition sequence which uses a single 90° radio frequency pulse, optimized with the superposition of a magnetic field gradient along the z axis. No digital filter is used in this acquisition sequence and for this reason it is the one classically used for quantitative measurements of the concentrations of nuclear species identified along the NMR spectrum;
- 1D-CPMG (Carr-Purcell-Meiboom-Gill) spin echo sequence: thanks to a digital filter which attenuates the responses of molecules with high T2 relaxation time, this sequence reduces the large spectral signals produced by macromolecules eventually present in the samples (e.g., proteins), allowing a better identification of the peaks of low molecular weight species, i.e., metabolites. For this reason, in metabolomics it is the most commonly used sequence for the qualitative analysis of the NMR spectra;
- 1D-DOSY diffusion-edited sequence: this sequence uses a digital filter which minimizes the signals of small molecules, allowing a better evaluation of the signals produced by macromolecules, which generally appear as broad bands underlying the narrow peaks of small molecules in NMR spectra.

For all the above pulse sequences, 64 scans were acquired. Only for CPMG sequence, a supplementary acquisition with 512 scans was performed, in order to increase the signals of low concentrated metabolites and favor their visualization and identification. The other acquisition parameters were 64 K data points of FID, 30 ppm (corresponding to 9014 Hz) spectral width (SW), 4 dummy scans, 3.635 s acquisition time (AQ), and 4 s relaxation delay (RD). Before performing the Fourier Transform, a 0.3 Hz line broadening correction was applied using a Lorentzian function. Then, the position of the peaks along the spectral scale was internally calibrated by setting the TSP singlet as internal standard for chemical shift referencing to 0 ppm. For both the acquisition and the processing of the spectra, TopSpin 3.5 software (Bruker) was used.
Moreover, thanks to the collaboration with The Metabolomics Innovation Centre (TMIC) located at the University of Alberta (Edmonton, Alberta, Canada), some representative 700 MHz ^{1}H NMR spectra were acquired from a subset of milk whey, rumen fluid, and blood serum samples (see Appendix I).

Qualitative analysis of ^{1}H NMR spectra

A preliminary identification of the metabolites present in milk whey, blood serum, and rumen fluid samples was carried out by evaluating the multiplicity and the position of the peaks in the 300 MHz ^{1}H NMR spectra and comparing them with data and spectra available in online databases (Milk Composition Database: https://mcdb.ca/; Livestock Metabolome Database: https://lmdb.ca/; Bovine Metabolome Database: https://bovinedb.ca/; Human Metabolome Database: https://hmdb.ca/; ChemicalBook: https://www.chemicalbook.com/) and articles (Boiani et al., 2019; Foroutan et al., 2019; Conde-Báez et al., 2017; Rysova et al., 2021; Klein et al., 2010; Sundekilde et al., 2013; Yanibada et al., 2018; Xu et al., 2016; López Radcenco et al., 2021; Chen et al., 2013; Sun et al., 2014; Tiziani et al., 2008; Ametaj et al., 2010; Saleem et al., 2013; Eom et al., 2020; Malheiros et al., 2021; Zhao et al., 2014). Then, standard solutions at known concentration of pure metabolites (Sigma-Aldrich) were prepared and added to the samples for a number of compounds suspected to be present in the NMR spectral profiles: repeating the acquisition of the ^{1}H NMR spectra, it was possible to confirm the presence of the metabolites by observing the intensity increase of the corresponding spectral signals. A further confirmation was obtained thanks to the 700 MHz ^{1}H NMR spectra, which are characterized by a better spectral separation of the peaks and were analyzed by Chenomx NMR Suite software version 8.2 (Chenomx Inc., Edmonton, AB, Canada) and MagMet web system (https://magmet.ca) (see Appendix I).

3.2.10. Mineral content analyses in rumen fluid, blood serum, and milk whey by ICP-OES

For the evaluation of the mineral content by ICP-OES, 1.5 mL aliquots of blood serum, rumen fluid, and milk whey (one per each treatment group per each cow) was thawed at room temperature and then centrifuged at 17,000 × *g* for 5 min at 22 °C. Then, due to the different complexity of the biofluids, samples were treated differently before undergoing ICP analysis. For rumen fluid and milk whey, 1 mL of each supernatant was transferred in a 14 mL Falcon® tube and added with a 5% HNO_3 solution to the final volume of 4 mL and kept overnight at room temperature for a complete oxidation. Following this oxidising treatment, each sample was filtered through a disposable 0.45 µm PTFE syringe filter. Blood serum, on the other hand, were subjected to a more stringent oxidizing treatment, obtained by acid digestion at high temperature and pressure in a microwave digestion unit (UltraWave, Milestone Srl, Sorisole, Bergamo, Italy). Specifically, 1 mL of each supernatant was transferred in a 15 mL quartz tube, added with 1 mL of HNO_3 (68%) and 150 µL of HCl (37%), then inserted in the microwave unit. The digestion program included a ramp of 5 min to reach 110 °C, 15 min to reach 180 °C, 15 min to reach 260 °C, and then a constant temperature

period at 260 °C for 20 min. The microwave power set for the heating successive ramps were, respectively, 800 W, 1200 W, 1500 W, and 1500 W. After digestion, the samples were diluted with distilled H_2O to the final volume of 4 mL and then filtered through a disposable 0.45 µm PTFE syringe filter.

All the filtered solutions were analyzed by an ICP- OES 5110 spectrometer (Agilent, Milan, Italy), operating with an Argon plasma, equipped with a PTFE nebulizer (Sea Spray, Agilent, Milan, Italy) inserted in a standard cyclonic spray chamber, and set in axial view mode. The operating parameters were RF power 1.30 (kW), plasma gas flow rate 12.0 L/min, auxiliary gas flow 1.0 L/min, nebulizer flow 0.70 L/min, nebulizer pump speed 12 rpm, read time 30 s, reading replicates 3. The elements investigated were 5 macrominerals (Ca, K, Mg, Na, P), 10 microminerals (Ba, Co, Cu, Fe, Li, Mn, Mo, Se, Sr, Zn), and 6 heavy metals (Al, As, Cd, Cr, Ni, Pb). To quantify macrominerals, because of their high concentration in the biofluids considered, an aliquot (50 µL) from the filtered sample solutions was further diluted with 5% HNO_3 solution, leading to a final dilution ratio of 1:400 (v/v) for rumen fluid and blood serum samples and 1:100 (v/v) for milk whey samples. Then, several calibration solutions at different concentration ranges were prepared with certified multi element and single element standard solutions (Agilent, Milan, Italy) to obtain the calibration curves for each element investigated. In particular, the concentration ranges of the calibration solutions were 0.005 to 0.200 ppm for Al, As, Ba, Cd, Co, Cu, Cr, Li, Mn, Mo, Ni, Pb, Se, Sr, and Zn, 0.010 to 0.200 for Fe, Ca, and Mg, 0.05 to 2.00 ppm for K, and 0.300 to 6.000 ppm for Na and P.

3.2.11. Statistical analysis

Except for the results obtained from omics, all the other data were analysed using the mixed model procedure of SAS version 9.4 (SAS Institute Inc.) by the following model:

$$y_{ijklm} = \mu + Treatment_i + Time_j + Cow_k + Cell\ (Treatment\ x\ Period)_l + e_{ijklm}$$

where y_{ijklm} corresponds to the observations for dependent variables; µ is the general mean; $Treatment_i$ is the fixed effect of the ith treatment (3 levels = B0, B50, and B100); $Time_j$ is the fixed effect of the jth treatment period (3 levels = t1, t2, and t3); Cow_k is the random effect of the kth cow (6 levels = cow ID 88, 313, 437, 578, 632 and 988); $Cell\ (Treatment\ x\ Period)_l$ is the random effect of the lth Latin square's cell (9 levels = from 1 to 9); e_{ijklm} is the random error.

The dataset for SAS analysis was prepared as follows: *i)* for the daily measured dependent variables (daily rumination time and daily milk yield), the means of the observations recorded during the last 5 days of each measurement phase were calculated, while *ii)* for the dependent variables measured only once during the measurement phase of each period (feed intake, BCS, milk quality parameters,

relative percentages of milk WBC subpopulations, oxidative stress biomarkers of blood plasma and milk whey, and ruminal pH), the single available observation was included.

Proteomic and peptidomic data were analysed by using PRIMER v. 6 software (Primer-e). The analyses were performed by the research team of the Department of Health Sciences of the University of Magna Græcia of Catanzaro in collaboration with the Department of Basic Biotechnological Sciences, Intensive Care and Perioperative Clinics of the Catholic University of the Sacred Heart, School of Medicine Gemelli Hospital (Rome, Italy). For protein abundance data, two different datasets were used: the first one corresponding to the entire list of identified protein profiles, assigning abundance value equal to zero when a certain protein was not detected in the sample, and the second one including only the proteins detected in all samples (so-called "consensus" list). Regarding peptidomic data, the statistical analysis was performed on the abundance of each peptide normalized on the total peptide abundance. Thanks to these procedures, both qualitative and quantitative aspects of protein and peptide profiles could be considered in the statistical evaluation.

On ^{1}H NMR spectra of ruminal fluid, serum, and milk samples, PCA analysis of variance was performed by AMIX v. 4.0.1 software (Bruker Biospin, Milano, Italy), considering the entire spectral range (0.5 – 10.0 ppm) divided into constant rectangular buckets of 0.05 ppm. The spectral range containing the signal of water (4.65-5.00 ppm) was excluded for all the biofluids. In addition, for ruminal fluid only, the spectral ranges corresponding to glycerol peaks (3.28-3.36, 3.39-3.46, 3.50-3.86 ppm) were also excluded because of a contamination by a lot of filters used for a subset of samples. Before calculating the bucket tables, the values of the spectral original variables were normalized to the total intensity of the spectra and, then, a Pareto Scaling was applied to the bucket tables to minimize the mask effect by the more intense variables on the less intense ones (Yang et al., 2015). A maximum number of 10 Principal Components were calculated for all the PCA analyses, explaining 99.5% of the total variance. For all biofluids, the resulting scores and loadings plots were used to evaluate potential clustering of the metabolic profiles due to treatment or period effects.

3.3. Results

3.3.1. General parameters of animal health and production

Body weight (BW) of animals at the end of the trial (713.83 ± 77.91) was higher than the BW measured before starting bentonite treatments (658.50 ± 67.70).

BCS, daily feed intake (kg), daily rumination time (min/d), rumen pH, and daily milk yield (kg/d) from the measurement phase of each treatment period are presented in **Table 3.5** No treatment effect was observed on the parameters considered, while some significant differences have been

Table 3.5. Least squares means (LS Means) and associated standard errors (SE) for daily feed intake (FI), body condition score (BCS), daily rumination time (RT), rumen pH, and daily milk yield (MY) of six cows receiving three bentonite treatments (B0 = 0 g/cow/d, B50 = 50 g/cow/d, and B100 = 100 g/cow/d) during three treatment periods (t1, t2, and t3).

	Treatment, LS Means			Period, LS Means				Treatment effect, *P*-value			Period effect, *P*-value		
									Contrast			Contrast	
Item	B0	B50	B100	t1	t2	t3	SE	ANOVA	Linear	Quadratic	ANOVA	Linear	Quadratic
FI (kg/d)	44.80	43.92	46.60	44.08	42.82	48.42	1.90	0.631	0.540	0.486	0.208	0.182	0.214
BCS	3.21	3.17	3.25	3.08	3.21	3.33	0.13	0.553	0.589	0.367	0.059	0.024	1.000
RT (min/d)	472.10	494.07	499.40	528.27	488.33	448.97	11.42	0.147	0.075	0.447	0.006	0.002	0.979
Rumen pH	6.13	5.94	5.94	6.04	6.10	5.87	0.09	0.27	0.17	0.39	0.22	0.20	0.21
MY (kg/d)	26.51	27.38	25.48	30.11	25.87	23.39	2.33	0.694	0.652	0.494	0.078	0.034	0.656

registered due to the period effect. In particular, there were a linear increase in BCS (*P*=0.024) and a linear reduction of both daily rumination time (*P*=0.002) and daily milk yield (*P*=0.34) from t1 to t3.

3.3.2. Ruminal fluid metabolome

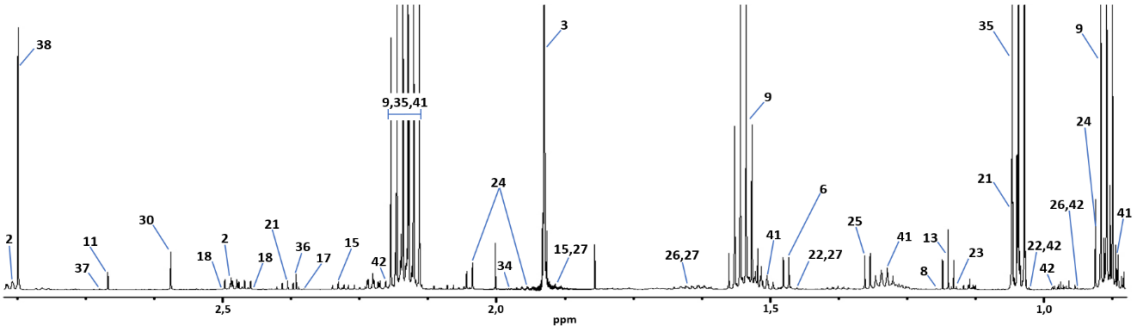
The pH of the NMR samples prepared with rumen fluid ranged from 7.25 to 7.35.

A total of 42 metabolites were identified in the ^{1}H NMR metabolic profile of rumen fluid samples **(Figure 3.2)**: 3-hydroxyphenylacetate, 3-phenylpropionate, acetate, acetoacetate, adenine, alanine, benzoate, BHBA (β-hydroxybutyric acid), butyrate, choline, dimethylamine, dimethyl sulfone, ethanol, formate, GABA (γ-aminobutyric acid), glucose, glutamate, glutamine, glycerol, glycine, isobutyrate, isoleucine, isopropanol, isovalerate, lactate, leucine, lysine, malonate, methanol, methylamine, ornithine, phenylacetate, phenylalanine, proline, propionate, pyruvate, sarcosine, trimethylamine, tyrosine, uracil, valerate, and valine.

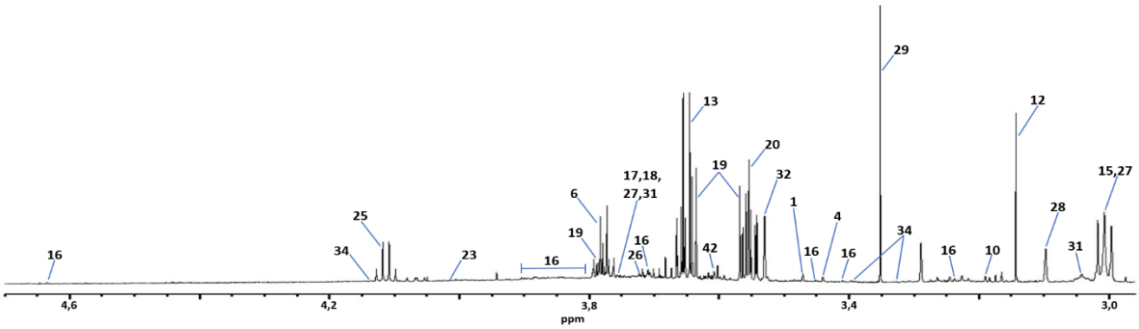
The results of the PCA analysis performed on the 300 MHz ^{1}H NMR spectra of rumen fluid evidenced a clear separation of samples into different clusters, with the first four PC explaining the 86.94% of the total variance (PC1 = 48.37%, PC2 = 20.99%, PC3 0 11.67%, PC4 = 5.91%). In particular, in the scores plot of the first two Principal Components of the variance (**Figure 3.3 A),** the metabolic profiles of rumen fluid samples clustered into three distinct groups according to the experimental periods t0, t1, and t2+t3. Moreover, in the t2+t3 cluster, the two periods tend to separate into two subgroups. However, the metabolic profile from cow ID 313 treated B100 in t2 does not meet the above clustering, falling in t0 group, and the metabolic profile of the same animal treated B50 in t1 lies in the t1+t2 group separate from the others. From the associated loadings plot (**Figure 3.3 B**), the spectral intervals mainly responsible for the above clusterization resulted those including the ^{1}H NMR signals of acetate, alanine, butyrate, dimethylamine, ethanol, GABA, isopropanol, lactate, lysine, methylamine, trimethylamine, propionate, and valerate.

Figure 3.2. Typical 700 MHz ^{1}H NMR spectrum obtained from rumen fluid samples: **A)** 0.85 - 2.90 ppm region, **B)** 2.96 - 4.70 ppm region, and **C)** 5.10 - 8.60 ppm region. A convenient vertical zoom was chosen for each spectral region. Identification numbers: (1) 3-hydroxyphenylacetate, (2) 3-phenylpropionate, (3) acetate, (4) acetoacetate, (5) adenine, (6) alanine, (7) benzoate, (8) BHBA (β-hydroxybutyric acid), (9) butyrate, (10) choline, (11) dimethylamine, (12) dimethyl sulfone, (13) ethanol, (14) formate, (15) GABA (γ-aminobutyric acid), (16) glucose, (17) glutamate, (18) glutamine, (19) glycerol, (20) glycine, (21) isobutyrate, (22) isoleucine, (23) isopropanol, (24) isovalerate, (25) lactate, (26) leucine, (27) lysine, (28) malonate, (29) methanol, (30) methylamine, (31) ornithine, (32) phenylacetate, (33) phenylalanine, (34) proline, (35) propionate, (36) pyruvate, (37) sarcosine, (38) trimethylamine, (39) tyrosine, (40) uracil, (41) valerate, and (42) valine.

A)



B)



C)

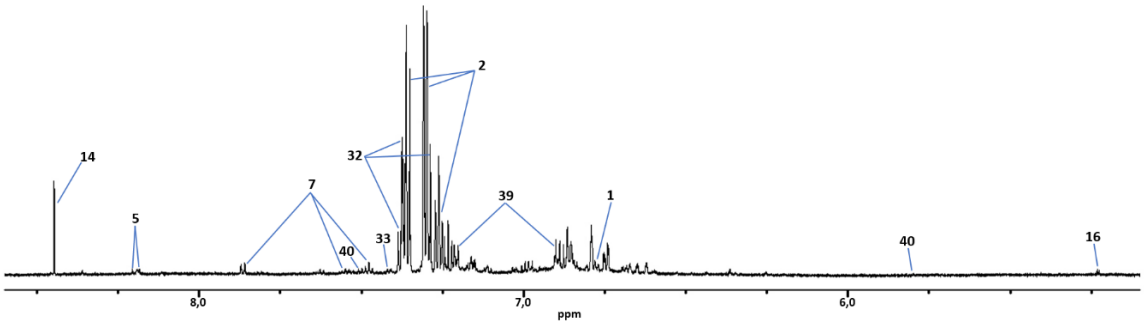


Figure 3.3. PCA analysis of the 300 MHz ^{1}H-NMR spectra of the rumen fluid samples: **A)** scores plot and **B)** corresponding loadings plot of the first two Principal Components of the variance (PC1 vs PC2). Each point in the score plot represents a ruminal fluid sample: black = t0, blue = t1, red = t2, and green = t3. The ellipses represent the clusters identified: black = t0, blue = t1, and red = t2+t3. Each point in the loadings plot represent a ^{1}H-NMR spectral region, with the numbers indicating the ppm value of the region (centre of a spectral region 0.05 ppm wide).

A) **B)**

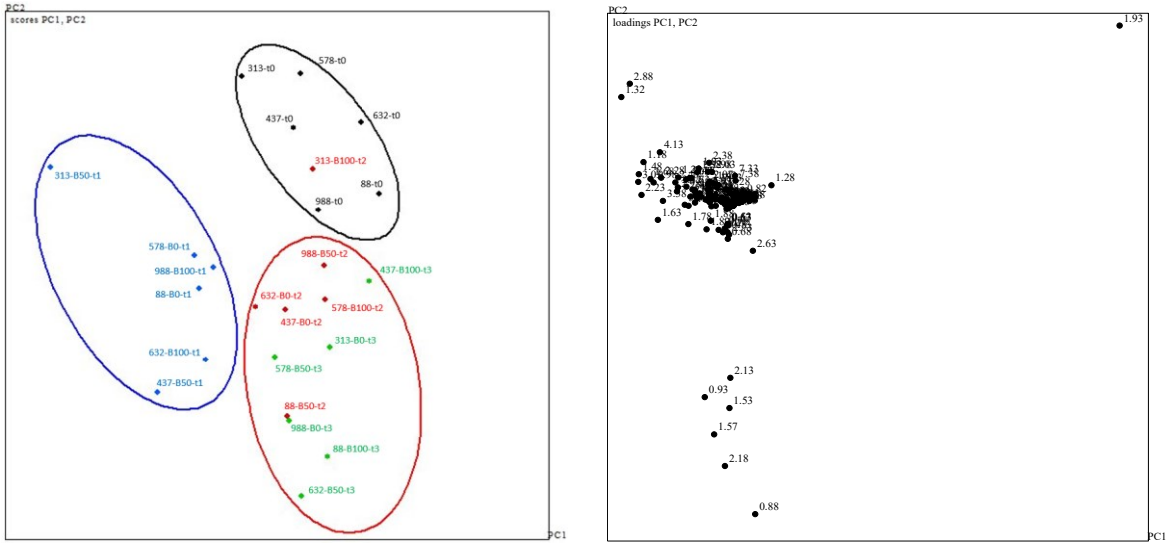


Figure 3.4. PCA analysis of the 300 MHz ^{1}H-NMR spectra of the rumen fluid samples: **A)** scores plot and **B)** corresponding loadings plot of the second and the fourth Principal Components of the variance (PC2 vs PC4). Each point in the score plot represents a ruminal fluid sample, coloured by treatment (black = t0, blue = B0, green = B50, and red = B100). The ellipses represent the clusters identified (black = t0, blue = B0, and red = B50+B100). Each point in the loadings plot represent a ^{1}H-NMR spectral region, with the numbers indicating the ppm value of the region (centre of a spectral region 0.05 ppm wide).

A) **B)**

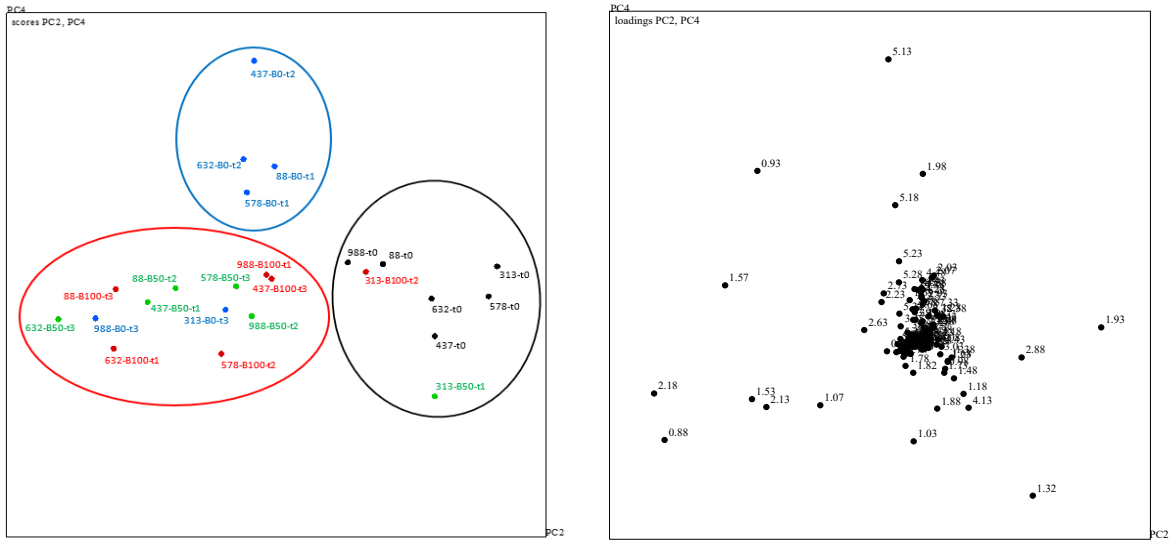


Table 3.6. Least squares means (LS Means) and associated standard errors (SE) for rumen fluid mineral content of six cows receiving three bentonite treatments (B0 = 0 g/cow/d, B50 = 50 g/cow/d, and B100 = 100 g/cow/d) during three treatment periods (t1, t2, and t3).

	Treatment, LS Means			Period, LS Means				Treatment effect, *P*-value			Period effect, *P*-value		
									Contrast			Contrast	
Minerals (mg/L)	B0	B50	B100	t1	t2	t3	SE	ANOVA	Linear	Quadratic	ANOVA	Linear	Quadratic
Macrominerals													
Ca	199.66	229.19	214.52	253.66	195.80	193.91	20.54	0.584	0.606	0.391	0.144	0.088	0.291
K	911,99	924.96	894.16	1018.30	841.19	871.61	53.08	0.915	0.817	0.745	0.136	0.113	0.173
Mg	196.64	222.08	206.33	237.67	200.00	187.38	15.82	0.529	0.668	0.320	0.153	0.074	0.528
Na	2380.58	2325.32	2320,07	2425,85	2318,52	2281,58	96.02	0.866	0.648	0.825	0.530	0.305	0.757
P	343.10	352.98	369.08	393.76	330.54	340.85	20.30	0.643	0.380	0.898	0.142	0.115	0.183
Microminerals and heavy metals													
Al	0.210	0.222	0.228	0.222	0.210	0.228	0.015	0.531	0.295	0.852	0.505	0.666	0.301
As	0.017	0.020	0.015	0.020	0.015	0.016	0.002	0.313	0.541	0.176	0.352	0.271	0.350
Ba	0.070	0.079	0.097	0.094	0.064	0.087	0.015	0.403	0.211	0.791	0.349	0.743	0.179
Cr	0.019	0.015	0.015	0.018	0.015	0.016	0.001	0.235	0.152	0.347	0.502	0.433	0.399
Cu	0.104	0.107	0.093	0.157	0.094	0.053	0.019	0.848	0.675	0.727	0.035	0.014	0.625
Fe	0.824	0.868	0.962	0.952	0.701	0.999	0.133	0.749	0.483	0.882	0.308	0.805	0.151
Li	0.037	0.038	0.038	0.034	0.047	0.032	0.002	0.656	0.453	0.643	0.002	0.269	0.001
Mn	1.190	1.429	1.557	1.743	1.055	1.378	0.204	0.415	0.217	0.810	0.120	0.218	0.080
Ni	0.054	0.067	0.056	0.083	0.048	0.047	0.005	0.250	0.777	0.119	0.012	0.008	0.051
Se	0.052	0.040	0.037	0.049	0.038	0.041	0.006	0.276	0.145	0.596	0.491	0.418	0.397
Sr	0.559	0.627	0.654	0.658	0.589	0.593	0.054	0.330	0.169	0.707	0.467	0.317	0.501
Zn	0.203	0.387	0.275	0.511	0.205	0.148	0.043	0.092	0.306	0.049	0.008	0.004	0.079

A clusterization by bentonite treatment can also be appreciated considering the second and the fourth PC. Indeed, in PC2 vs PC4 scores plot (**Figure 3.4 A**) the following clusters were identified: t0, B0, and B50+B100. However, there were some exceptions: the metabolic profiles of cow ID 313 in B50 and B100 fell into the group of untreated animals (B0) and those of cows ID 313 and 988 in B0 fell into the group of treated animals (B50+B100).

3.3.3. Rumen fluid mineral content

Mineral concentrations measured by ICP-OES in ruminal fluid samples from the measurement phase of each treatment period are shown in **Table 3.6**. Table does not report minerals below the instrument detection limit (Cd, Co, Mo, and Pb). Macrominerals were not significantly affected by both the bentonite treatment and the experimental period. A nonsignificant tendency to a linear decrease was observed in Mg concentration (P = 0.074) over time. Regarding the other minerals considered, only Zn concentration was affected by the treatment (quadratic effect, P=0.049). On the other hand, a significant linear decrease in Cu (P=0.014), Ni (P=0.008), and Zn (P=0.004) content was observed from t1 to t3. Moreover, a significant quadratic period effect was found on Li content (P=0.001).

Table 3.7. Least squares means (LS Means) and associated standard errors (SE) for oxidative stress parameters in blood plasma of six cows receiving three bentonite treatments (B0 = 0 g/cow/d, B50 = 50 g/cow/d, and B100 = 100 g/cow/d) during three treatment periods (t1, t2, and t3). TP = total proteins, AOPP = advanced oxidation protein products, MDA = malondialdehyde, and CO = carbonyl groups.

	Treatment, LS Means			Period, LS Means				Treatment effect, *P*-value			Period effect, *P*-value		
									Contrast			Contrast	
Item	B0	B50	B100	t1	t2	t3	SE	ANOVA	Linear	Quadratic	ANOVA	Linear	Quadratic
TP (mg/mL)	74.49	95.39	67.72	80.13	80.01	77.46	9.73	0.185	0.617	0.089	0.972	0.842	0.917
AOPP (nmol/mL)	51.50	47.73	45.81	45.80	54.94	44.30	4.11	0.642	0.383	0.863	0.254	0.809	0.121
Thiols (nmol/mL)	558.99	557.15	511.23	534.04	573.60	519.74	24.30	0.372	0.231	0.494	0.355	0.694	0.186
MDA (nmol/mL)	0.54	0.20	0.19	0.40	0.33	0.20	0.12	0.186	0.115	0.328	0.567	0.323	0.850
CO (nmol/mL)	1.45	1.60	1.72	1.47	1.23	2.16	0.12	0.528	0.294	0.832	0.007	0.010	0.010

3.3.4. Blood plasma oxidative stress biomarkers

Oxidative stress biomarkers evaluated in blood plasma samples from the measurement phase of each treatment period are showed in **Table 3.7**. No significant modifications due to bentonite administration were reported, while a significant period effect on CO (*P*=0.007) was found.

3.3.5. Blood serum metabolome

The pH of the NMR samples prepared with blood serum ranged from 7.40 to 7.45.

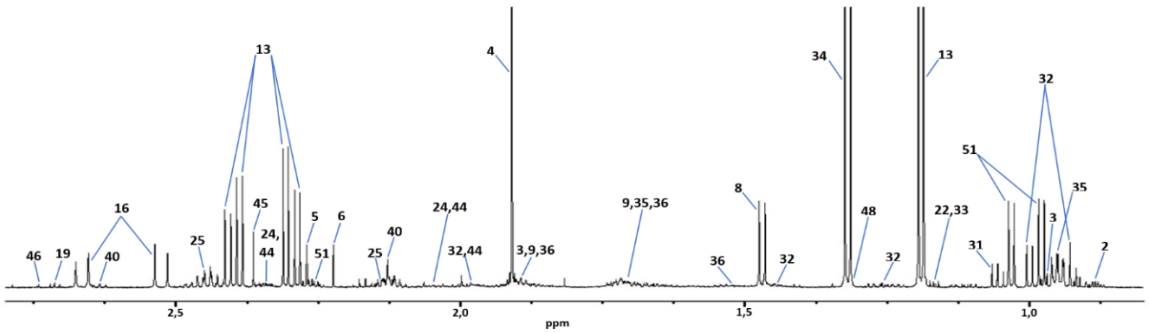
A total of 51 metabolites were recognised in the metabolic profile of blood serum samples **(Figure 3.5)**: 1-methylhistidine, 2-hydroxybutyrate, L-α-aminobutyrate, acetate, acetoacetate, acetone, acetylcarnitine, alanine, arginine, asparagine, aspartate, betaine, BHBA (β-hydroxybutyric acid), carnitine, choline, citrate, creatine, creatinine, dimethylamine, dimethylglycine, dimethyl sulfone, ethanol, formate, glucose, glutamate, glutamine, glycerol, glycine, hippurate, histidine, isobutyrate, isoleucine, isopropanol, lactate, leucine, lysine, malonate, mannose, methanol, methionine, ornithine, oxoglutarate, phenylalanine, proline, pyruvate, sarcosine, serine, threonine, tyrosine, urea, and valine.

Moreover, because of the different sample preparation protocol used (no ultrafiltration), in the 300 MHz spectra of serum samples it was also possible to recognise the presence of low-density/very-low-density lipoproteins (VLDL/LDL), N-acetyl glycoproteins, O-acetyl glycoproteins, lipid chains, phosphotidylcholine, and unsaturated fatty acids (**Figure 3.6**).

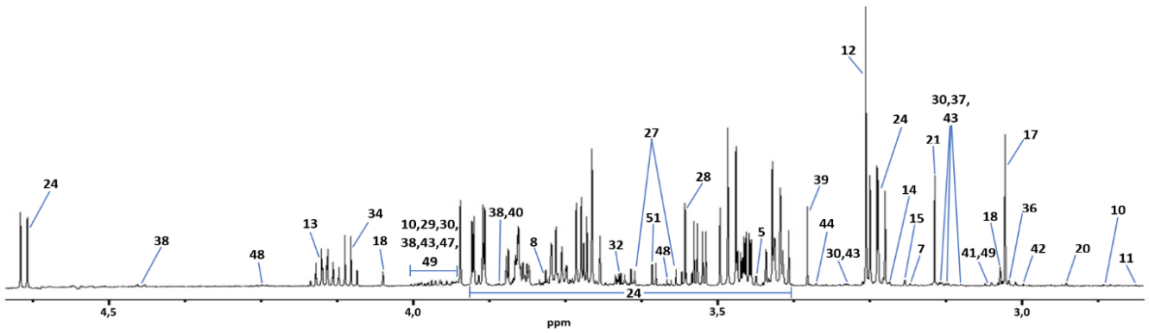
The PCA analysis of the 300 MHz ^{1}H NMR spectra did not highlight any general trend in blood serum metabolic profiles neither according to the treatment (**Figure 3.7 A**) nor according to the experimental period (**Figure 3.7 B**). However, it was possible to notice that samples in t0 tended to separate from those collected in t1, t2, and t3 (**Figure 3.7 A, B**) and cows ID 437 and 988 clustered together in all the experimental periods (**Figure 3.7 B**).

Figure 3.5. Typical 700 MHz ^{1}H NMR spectrum obtained from blood serum samples: **A)** 0.80 - 2.80 ppm region, **B)** 2.80 - 4.67 ppm region, and **C)** 5.10 - 8.50 ppm region. A convenient vertical zoom was chosen for each spectral region. Identification numbers: (1) 1-methylhistidine, (2) 2-hydroxybutyrate, (3) L-α-aminobutyrate, (4) acetate, (5) acetoacetate, (6) acetone, (7) acetylcarnitine, (8) alanine, (9) arginine, (10) asparagine, (11) aspartate, (12) betaine, (13) BHBA (β-hydroxybutyric acid), (14) carnitine, (15) choline, (16) citrate, (17) creatine, (18) creatinine, (19) dimethylamine, (20) dimethylglycine, (21) dimethyl sulfone, (22) ethanol, (23) formate, (24) glucose, (25) glutamate, (25) glutamine, (27) glycerol, (28) glycine, (29) hippurate, (30) histidine, (31) isobutyrate, (32) isoleucine, (33) isopropanol, (34) lactate, (35) leucine, (36) lysine, (37) malonate, (38) mannose, (39) methanol, (40) methionine, (41) ornithine, (42) oxoglutarate, (43) phenylalanine, (44) proline, (45) pyruvate, (46) sarcosine, (47) serine, (48) threonine, (49) tyrosine, (50) urea, and (51) valine.

A)



B)



C)

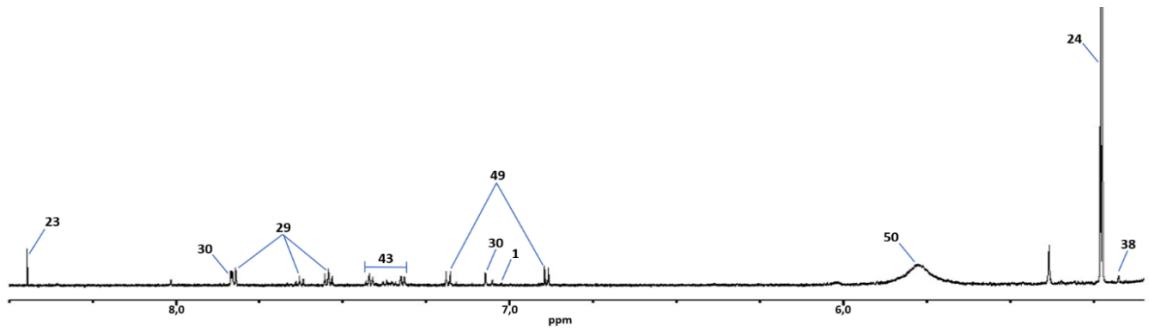


Figure 3.6. Typical 300 MHz ^{1}H NMR spectrum (–0.50 to 6.50 ppm) obtained from blood serum samples. Abbreviations: LCs = lipid chains, LPs = low-density/very-low-density lipoproteins, N-AcGPs = N-acetyl glycoproteins, O-AcGPs = O-acetyl glycoproteins, PTC = phosphotidylcholine, and UFAs = unsaturated fatty acids.

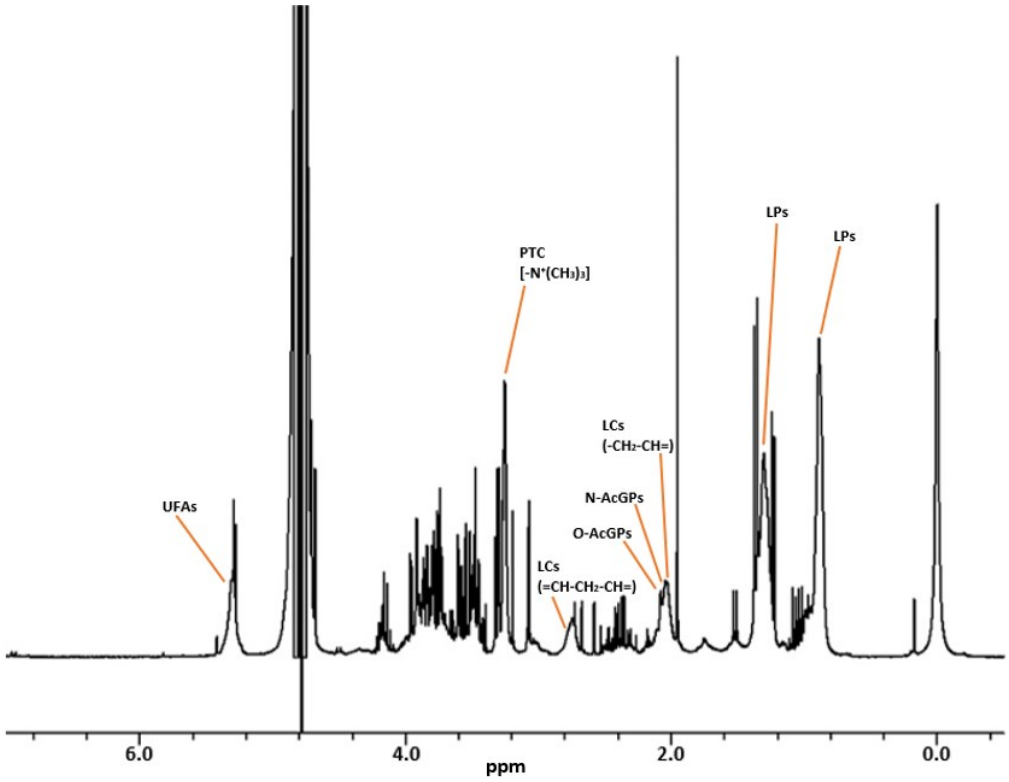
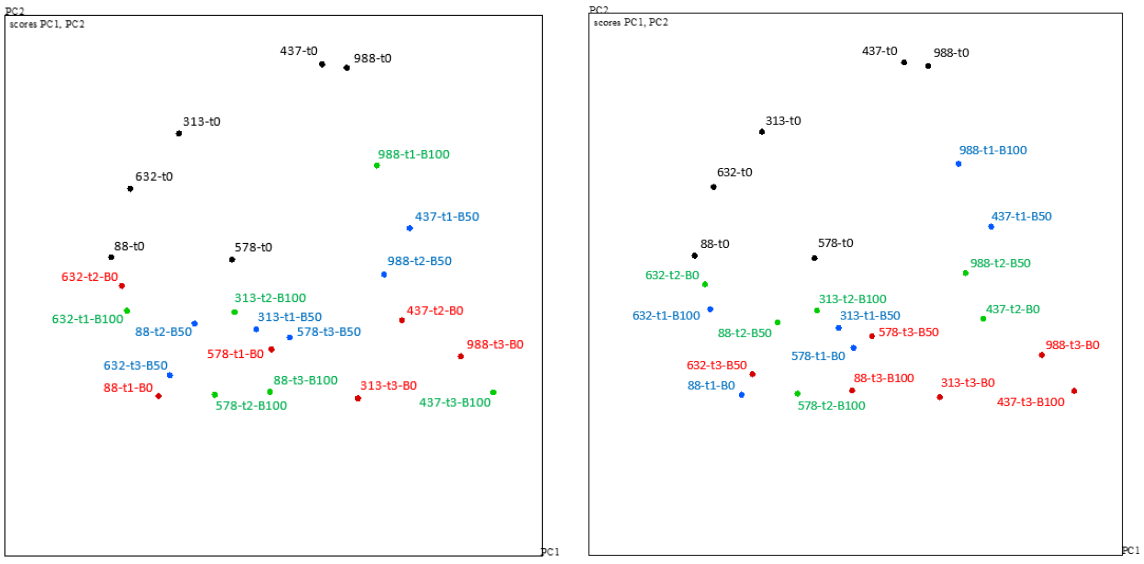


Figure 3.7. PCA analysis of the 300 MHz ^{1}H-NMR spectra of the rumen fluid samples: scores plot of the first two Principal Components of the variance (PC1 vs PC2). Each point represents a blood serum sample, coloured by **A)** bentonite treatment (black = t0, red = B0, blue = B50, and green = B100) or **B)** experimental period (black = t0, blue = t1, green = t2, and red = t3).

A) **B)**



3.3.6. Blood serum mineral content

Mineral concentrations measured by ICP-OES in blood serum samples from the measurement phase of each treatment period are shown in **Table 3.8**. Table does not report minerals below the instrument detection limit (As, Cd, Co, Mn, Mo, and Pb).

A significant increase in Sr (*P*=0.046) and Zn (*P*=0.005) concentrations was observed in serum as bentonite inclusion in the diet increased, while a significant quadratic period effect on Mg (*P*=0.026) concentration and a significant linear decrease in Ni (*P*=0.029) and Sr (*P*=0.012) content over time were reported.

Table 3.8. Least squares means (LS Means) and associated standard errors (SE) for blood serum mineral content of six cows receiving three bentonite treatments (B0 = 0 g/cow/d, B50 = 50 g/cow/d, and B100 = 100 g/cow/d) during three treatment periods (t1, t2, and t3).

	Treatment, LS Means			Period, LS Means				Treatment effect, *P*-value			Period effect, *P*-value		
									Contrast			Contrast	
Minerals (mg/L)	B0	B50	B100	t1	t2	t3	SE	ANOVA	Linear	Quadratic	ANOVA	Linear	Quadratic
Macrominerals													
Ca	94.27	99.24	105.49	90.43	103.05	105,53	5.41	0.416	0.213	0.927	0.215	0.115	0.484
K	118.31	118.67	121.81	118.49	115.64	124.65	5.45	0.868	0.658	0.831	0.505	0.426	0.411
Mg	20.12	20.21	21.32	20.27	22.67	18.72	0.77	0.490	0.317	0.590	0.048	0.199	0.026
Na	2851.79	2950.53	3081.91	2806.16	3070.17	3007.89	104.01	0.350	0.176	0.896	0.246	0.203	0.257
P	126.31	139.12	137.68	134.53	134.79	133.79	8.93	0.507	0.353	0.473	0.995	0.945	0.951
Microminerals and heavy metals													
Al	0.088	0.085	0.095	0.085	0.086	0.097	0.008	0.601	0.503	0.500	0.475	0.294	0.588
Ba	0.036	0.034	0.026	0.028	0.030	0.039	0.018	0.825	0.673	0.812	0.786	0.571	0.807
Cr	0.004	0.007	0.003	0.008	0.004	0.003	0.003	0.551	0.739	0.344	0.325	0.182	0.596
Cu	0.758	0.733	0.729	0.717	0.722	0.781	0.062	0.842	0.607	0.829	0.447	0.273	0.574
Fe	1.403	1.494	1.409	1.485	1.378	1.443	0.111	0.730	0.967	0.456	0.720	0.747	0.484
Li	0.089	0.094	0.092	0.092	0.093	0.090	0.007	0.851	0.766	0.652	0.949	0.830	0.829
Ni	0.005	0.006	0.002	0.009	0.003	0.001	0.002	0.351	0.391	0.270	0.058	0.029	0.309
Se	0.069	0.064	0.062	0.066	0.066	0.063	0.004	0.426	0.226	0.722	0.768	0.581	0.673
Sr	0.051	0.054	0.061	0.063	0.055	0.048	0.003	0.098	0.046	0.522	0.030	0.012	0.906
Zn	0.753	0.918	1.162	0.884	0.967	0.981	0.054	0.012	0.005	0.553	0.415	0.237	0.625

3.3.7. Milk quality and leukocyte subpopulations

Table 3.9 shows quality parameters and the relative percentages of leukocyte subpopulations (obtained by flow cytometry) in milk samples from the measurement phase of each treatment period. Regarding milk characteristics, bentonite treatment only significantly reduced lactose % (*P*=0.035). However, it was noted a significant linear enhancement of the total bacterial count (*P*=0.006) over time (t1<t2<t3). Also, a tendency to linear reduction in rennet coagulation time (RCT, min) (*P*=0.054) and a significant linear increase in curd firmness (a_{30}, mm) (*P*=0.019) and index of milk aptitude to coagulate (IAC) (*P*=0.033) were found from t1 to t3. No effect of treatment or period was observed on milk leukocyte subpopulations (%).

Table 3.9. Least squares means (LS Means) and associated standard errors (SE) for milk quality parameters and milk leukocyte subpopulations (WBCs, %) of six cows receiving three bentonite treatments (B0 = 0 g/cow/d, B50 = 50 g/cow/d, and B100 = 100 g/cow/d) during three treatment periods (t1, t2, and t3). BHB = β-hydroxybutyrate, Cls = chlorides, SCS = somatic cell score, TBC = total bacterial count, IAC = index of milk aptitude to coagulate, a_{30} = curd firmness, k_{20} = curd-firming time, RCT = rennet coagulation time, PMNs = neutrophils, MACs = macrophages, THLs = T-helper lymphocytes, CTLs = cytotoxic T lymphocytes, and BLs = B lymphocytes.

	Treatment, LS Means			Period, LS Means				Treatment effect, *P*-value			Period effect, *P*-value		
									Contrast			Contrast	
Item	B0	B50	B100	t1	t2	t3	SE	ANOVA	Linear	Quadratic	ANOVA	Linear	Quadratic
Milk quality													
Protein (%)	3.38	3.44	3.52	3.40	3.38	3.56	0.13	0.729	0.458	0.918	0.557	0.394	0.539
Casein (%)	2.64	2.65	2.74	2.64	2.60	2.80	0.11	0.768	0.523	0.798	0.428	0.335	0.393
Lactose (%)	4.74	4.46	4.55	4.58	4.49	4.68	0.12	0.035	0.054	0.033	0.122	0.244	0.077
Fat (%)	3.67	3.41	3.85	3.48	3.36	4.08	0.45	0.766	0.774	0.529	0.492	0.366	0.460
BHB (mmol/L)	0.05	0.06	0.05	0.06	0.07	0.04	0.01	0.560	0.901	0.313	0.182	0.136	0.239
Urea (mg/dL)	21.51	18.25	20.27	21.08	22.45	16.48	1.97	0.514	0.661	0.309	0.169	0.153	0.180
Cls (mg/dL)	153.67	181.83	171.17	173.17	174.00	159.50	12.68	0.093	0.137	0.076	0.328	0.220	0.401
pH	6.62	6.56	6.57	6.62	6.56	6.56	0.02	0.109	0.093	0.143	0.100	0.056	0.287
SCS*	1.67	2.77	1.09	1.07	2.16	2.29	0.79	0.285	0.566	0.147	0.408	0.254	0.566
LogTBC**	3.89	3.79	3.79	3.51	3.62	4.34	0.12	0.785	0.579	0.718	0.011	0.006	0.079
IAC	98.67	92.83	98.33	91.50	95.83	102.50	3.62	0.275	0.928	0.130	0.078	0.033	0.715
a_{30} (mm)	21.16	17.06	21.25	12.93	19.51	27.03	3.89	0.500	0.982	0.268	0.047	0.019	0.891
k_{20} (min)	8.48	9.71	8.03	9.84	9.13	7.26	1.03	0.444	0.734	0.243	0.211	0.104	0.612
RCT (min)	28.89	34.73	29.49	34.10	32.02	26.98	2.85	0.164	0.834	0.073	0.117	0.054	0.553
WBCs (%)													
PMNs	22.87	24.60	16.70	21.38	16.10	26.69	4.70	0.490	0.385	0.427	0.346	0.448	0.219
MACs	10.35	11.13	8.78	8.20	12.44	9.62	3.59	0.877	0.751	0.715	0.671	0.772	0.425
THLs	12.81	13.60	11.04	9.62	14.41	13.42	3.54	0.695	0.579	0.546	0.330	0.266	0.319
CTLs	8.77	7.58	6.36	6.76	8.19	7.77	1.89	0.509	0.274	0.992	0.758	0.623	0.607
BLs	1.59	2.64	1.90	2.40	2.99	0.74	0.67	0.378	0.690	0.212	0.067	0.086	0.080

* SCS = $\log_2$ (SCC/100,000) + 3
** Log TBC = Log CFU/mL; CFU = colony forming units

3.3.8. Milk whey oxidative stress biomarkers

Oxidative stress biomarkers evaluated in milk whey samples from the measurement phase of each treatment period are showed in **Table 3.10**. Both bentonite administration and treatment period did not produce any significant effect.

3.3.9. Milk whey proteome and peptidome

A total of 155 proteins and 1088 peptides were identified and quantified in milk whey.

The above peptides were found to be naturally occurring fragments of 17 cow milk proteins (**Table 3.11**).

Table 3.10. Least squares means (LS Means) and associated standard errors (SE) for oxidative stress parameters in milk whey of six cows receiving three bentonite treatments (B0 = 0 g/cow/d, B50 = 50 g/cow/d, and B100 = 100 g/cow/d) during three treatment periods (t1, t2, and t3). TP = total proteins, AOPP = advanced oxidation protein products, MDA = malondialdehyde, and CO = carbonyl groups.

	Treatment, LS Means			Period, LS Means				Treatment effect, *P*-value			Period effect, *P*-value		
									Contrast			Contrast	
Item	B0	B50	B100	t1	t2	t3	SE	ANOVA	Linear	Quadratic	ANOVA	Linear	Quadratic
TP (mg/mL)	15.75	16.93	17.17	15.07	17.52	17.27	1.42	0.612	0.379	0.729	0.284	0.201	0.340
AOPP (nmol/mL)	339.22	310.32	321.34	307.59	330.16	333.12	25.36	0.374	0.384	0.276	0.399	0.235	0.569
Thiols (nmol/mL)	312.56	278.81	274.48	203.84	359.30	302.79	91.28	0.924	0.732	0.878	0.404	0.395	0.304
MDA (nmol/mL)	0.24	0.75	0.27	0.49	0.18	0.58	0.16	0.144	0.903	0.063	0.286	0.717	0.142
CO (nmol/mL)	1.23	1.29	1.38	0.99	1.36	1.55	0.17	0.778	0.507	0.937	0.112	0.050	0.633

Table 3.11. List of the 17 cow milk proteins uniquely found by Proteome Discoverer 2.2 software, based on the 1088 peptide fragments identified in milk whey.

Protein[a]	Protein group	Organism	Sum PEP Score[b]	Sequence Coverage (%)[c]	Peptides[d]	PSMs[e]	Unique peptides[f]
P02662	Alfa-S1-casein	Bovine	2147,049	77	292	3624	67
A0A3Q1NG86	Alfa-S1-casein	Bovine	1361.511	76	211	2386	1
P02663	Alfa-S1-casein	Bovine	1303.276	93	185	2539	185
A0A3Q1MJE5	Alfa-S1-casein	Bovine	1204.499	76	185	2184	9
P02666	Alfa-S1-casein	Bovine	1188.064	93	193	2595	25
A0A452DHW7	Alfa-S1-casein	Bovine	1043.751	77	169	2402	1
P80195	Alfa-S1-casein	Bovine	403.361	58	63	545	63
P18892	Alfa-S1-casein	Bovine	233.557	39	36	241	36
P80025	Alfa-S1-casein	Bovine	113.442	11	30	140	30
P02668	Alfa-S1-casein	Bovine	58.762	47	15	101	1
A0A140T8A9	Alfa-S1-casein	Bovine	57.342	53	16	95	2
P31096	Alfa-S1-casein	Bovine	55.233	36	11	121	11
P08037	Alfa-S1-casein	Bovine	42.239	11	8	58	8
A0A023VWT2	Alfa-S1-casein	Bovine	34.974	53	9	41	9
Q9TUM6	Alfa-S1-casein	Bovine	34.238	24	11	36	11
E7E1P8	Alfa-S1-casein	Bovine	23.308	33	7	9	2
A0A3Q1LYE8	Alfa-S1-casein	Bovine	19.785	18	5	12	5

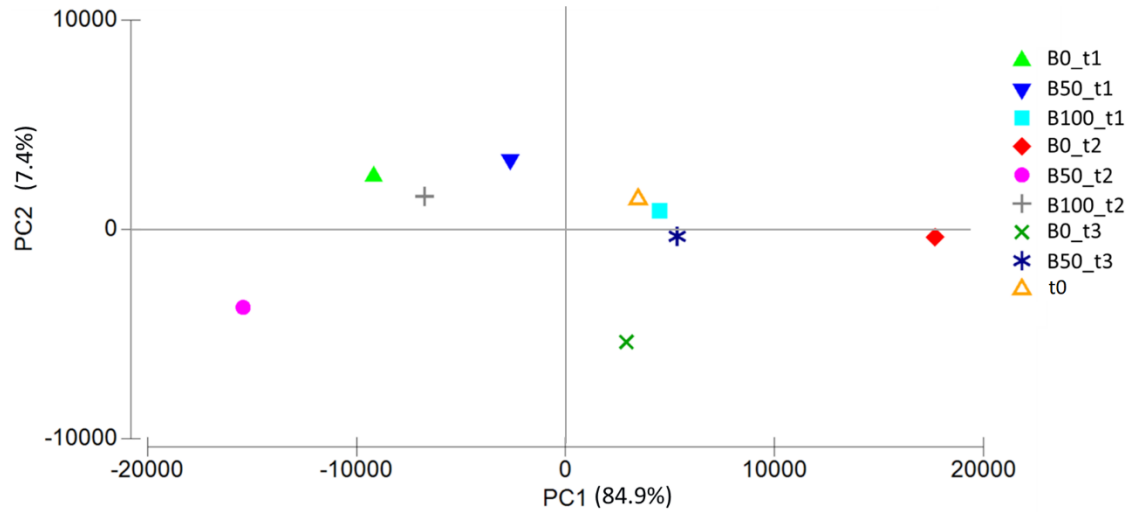
[a] Identification number in Uniprot database
[b] Sum Posterior Error Probability Score: error probability in attributing peptide fragments to each protein
[c] Percentage of the protein sequence covered with the peptide fragments attributed to each protein
[d] Number of peptide fragments attributed to each protein
[e] Peptide Spectral Matches: number of MS spectra that identified the peptide fragments attributed to each protein
[f] Number of peptide fragments uniquely attributed to each protein

Clustering protein profiles (both entire list and "consensus" list) and peptide profiles according to different factors (cow, treatment, and experimental period), no statistically significant differences (P>0.05) were observed in their abundance.

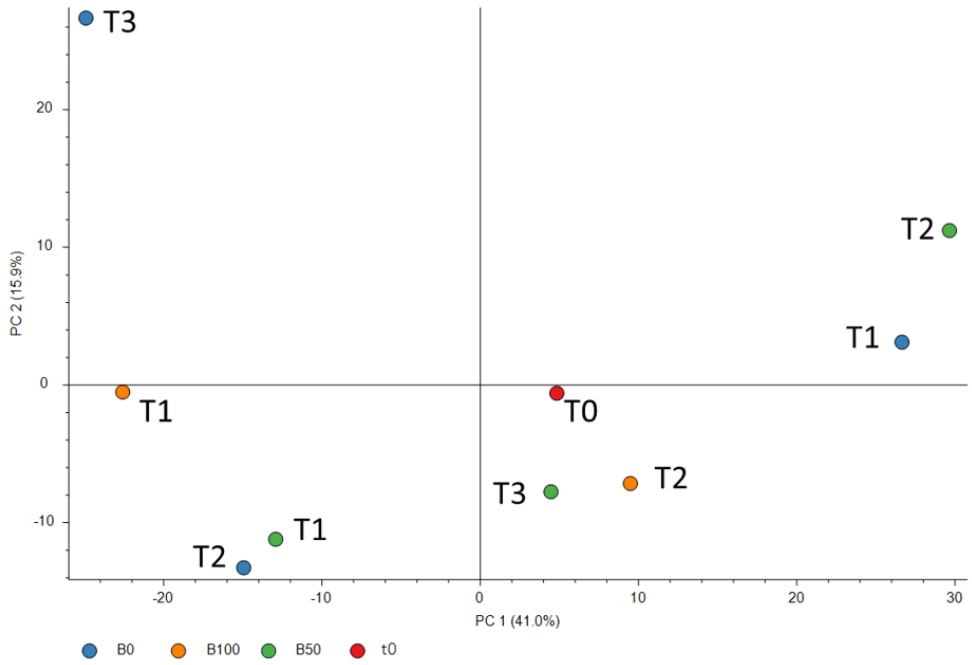
The Principal Component Analysis (PCA) of the abundance of both proteins and peptides, grouped by Latin square cells (Treatment x Period), did not reveal any clear separation, as shown by the scores plots in **Figure 3.8**.

Figure 3.8. Scores plots of the PCA analysis performed on the abundance of milk whey proteins (**A**) and peptides (**B**) identified, grouped by Latin square cells (Treatment x Period).

A)



B)



Although without statistically significant differences, it was observed that peptides attributable to alpha-S2-casein (P02663) were less expressed in t0 samples in comparison with B0, B50, and B100 samples. Butyrophilin subfamily 1 member A1 (P18892), Perilipin (Q9TUM6), and fragment 190-198 of Alpha-S2-casein (P02663), on the other hand, were more highly expressed in B0, B50, and B100 then in t0 samples. Moreover, the peptides belonging to the Glycosylation-dependent cell adhesion molecule protein (P80195) were more highly expressed in B50 and B100 samples than in B0 samples. Lastly, the B50 vs B100 comparison showed a higher expression of Alpha-S2-casein

(P02663) in B100 and a lower expression of peptides attributable to Kappa-casein fragment (E7E1P8) in B50 samples.

3.3.10. Milk whey metabolome

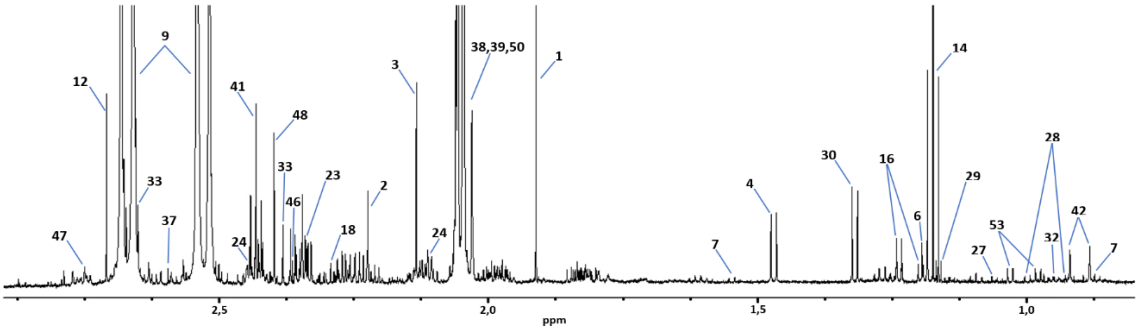
The pH of the NMR samples prepared with milk whey ranged from 6.90 to 6.95.

A total of 53 metabolites were identified in the ^{1}H NMR metabolic profile of milk whey samples **(Figure 3.9)**: acetate, acetone, acetyl carnitine, alanine, betaine, BHBA (β-hydroxybutyric acid), butyrate, choline, citrate, creatine, creatinine, dimethylamine, dimethyl sulfone, ethanol, formate, fucose, fumarate, GABA (γ-aminobutyric acid), galactose, galactose 1-P, glucose, glucose 1-P, glutamate, glutamine, glycerophosphocholine, hippurate, isobutyrate, isoleucine, isopropanol, lactate, lactose, leucine, malate, malonate, maltose, mannose, methylamine, N-acetyl-carbohydrates, N-acetyl-glucosamine, orotate, oxoglutarate, pantothenate, phenylalanine, phosphocholine, phosphocreatine, pyruvate, sarcosine, succinate, tyrosine, UDP-N-acetyl-glucosamine, urea, uridine, and valine.

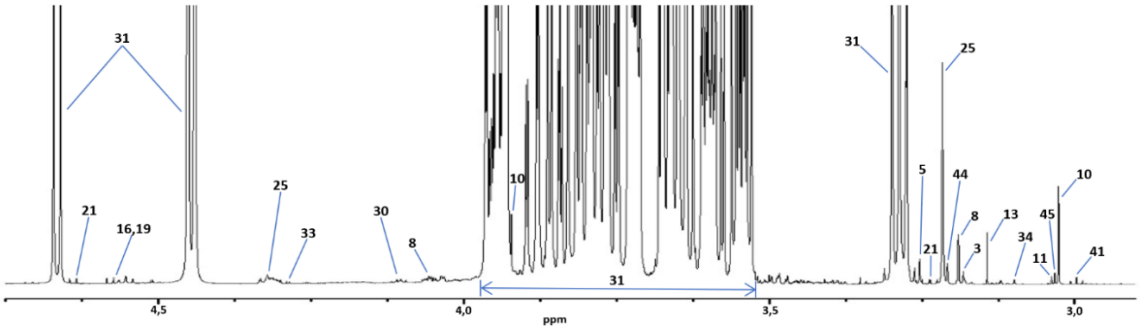
The PCA analysis of the 300 MHz ^{1}H NMR spectra revealed a slight tendency of the metabolic profiles of milk whey samples to cluster according to the bentonite treatment, with the first four PC explaining the 81% of the total variance (PC1 = 31.19%, PC2 = 22.63%, PC3 = 18.69%, PC4 = 8.51%), whereas no separation or clustering was observed considering the treatment periods. In particular, in the scores plot of the first two Principal Components of the variance (**Figure 3.10 A**), samples from untreated animals (t0 and B0) and the group of samples from treated animals (B50 and B100) tend to form two separate groups, albeit very close to each other. However, this trend is further weakened by the metabolic profiles belonging to cow ID 988: the sample B0 in t3 falls in the group of treated animals, while the sample B100 in t1 falls in the group of untreated animals, and sample B50 in t2 lies separate from the others. The associated loadings plot, in **Figure 3.10 B**, shows the spectral intervals mainly responsible for the clustering of the whey milk metabolic profiles described above. These spectral intervals were found to include the ^{1}H NMR peaks of acetyl-carnitine, betaine, BHBA, choline, citrate, creatine, creatinine, dimethylamine, dimethyl sulfone, ethanol, galactose, glucose, glycerophosphocholine, lactate, lactose, methylamine, N-acetyl carbohydrates, N-acetylnglucosamine, phosphocholine, phosphocreatine, and UDP-N-acetyl glucosamine.

Figure 3.9. Typical 700 MHz ^{1}H NMR spectrum obtained from milk whey samples: **A)** 0.80 - 2.90 ppm region, **B)** 2.90 - 4.75 ppm region, and **C)** 5.10 - 8.60 ppm region. A convenient vertical zoom was chosen for each spectral region. Identification numbers: (1) acetate, (2) acetone, (3) acetyl carnitine, (4) alanine, (5) betaine, (6) BHBA (β-hydroxybutyric acid), (7) butyrate, (8) choline, (9) citrate, (10) creatine, (11) creatinine, (12) dimethylamine, (13) dimethyl sulfone, (14) ethanol, (15) formate, (16) fucose, (17) fumarate, (18) GABA (γ-aminobutyric acid), (19) galactose, (20) galactose 1-P, (21) glucose, (22) glucose 1-P, (23) glutamate, (24) glutamine, (25) glycerophosphocholine, (26) hippurate, (27) isobutyrate, (28) isoleucine, (29) isopropanol, (30) lactate, (31) lactose, (32) leucine, (33) malate, (34) malonate, (35) maltose, (36) mannose, (37) methylamine, (38) N-acetyl-carbohydrates, (39) N-acetyl-glucosamine, (40) orotate, (41) oxoglutarate, (42) pantothenate, (43) phenylalanine, (44) phosphocholine, (45) phosphocreatine, (46) pyruvate, (47) sarcosine, (48) succinate, (49) tyrosine, (50) UDP-N-acetyl-glucosamine, (51) urea, (52) uridine, and (53) valine.

A)



B)



C)

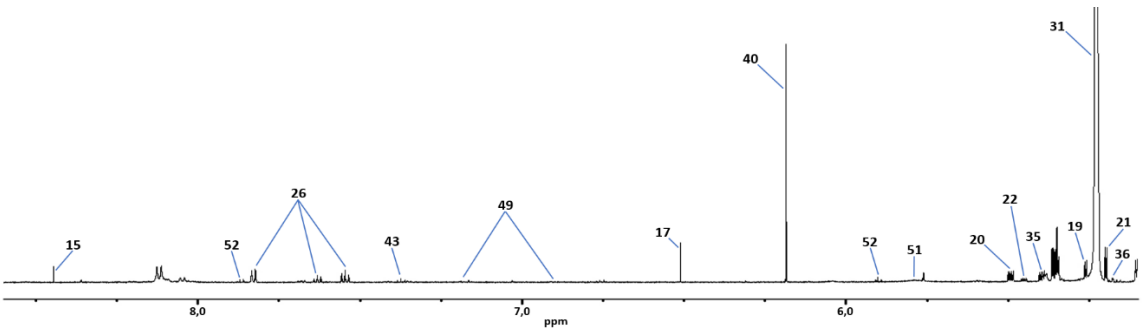
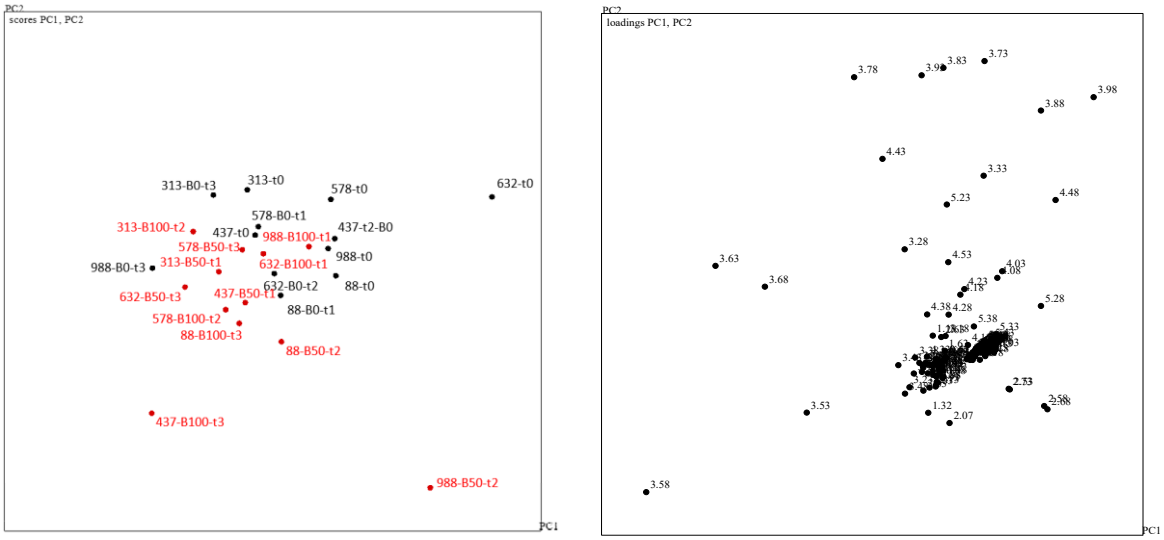


Figure 3.10. PCA analysis of the 300 MHz ^{1}H-NMR spectra of the milk whey samples: **A)** scores plot and **B)** corresponding loadings plot of the first two Principal Components of the variance (PC1 vs PC2). Each point in the score plot represents a milk whey sample, coloured by bentonite treatment [black = no bentonite treatment (t0 and B0) and red = bentonite treatments (B50 and B100)]. Each point in the loadings plot represent a ^{1}H-NMR spectral region, with the numbers indicating the ppm value of the region (centre of a spectral region 0.05 ppm wide).

A) **B)**



3.3.11. Milk whey mineral content

The mineral concentrations measured by ICP-OES in milk whey samples from the measurement phase of each treatment period are shown in **Table 3.12.** Table does not report minerals detected at concentrations below the instrument detection limit (Co, Cd, Mn, Mo, and Pb). In milk whey, no significant differences were highlighted between treatment groups for all minerals considered, while a significant quadratic period effect was found for As (P=0.038) and Li (P=0.040) content and a significant linear increase of Se concentration (P=0.038) was observed over time.

Table 3.12. Least squares means (LS Means) and associated standard errors (SE) for milk whey mineral content of six cows receiving three bentonite treatments (B0 = 0 g/cow/d, B50 = 50 g/cow/d, and B100 = 100 g/cow/d) during three treatment periods (t1, t2, and t3).

	Treatment, LS Means			Period, LS Means				Treatment effect, *P*-value			Period effect, *P*-value		
									Contrast			Contrast	
Minerals (mg/L)	B0	B50	B100	t1	t2	t3	SE	ANOVA	Linear	Quadratic	ANOVA	Linear	Quadratic
Macrominerals													
Ca	553.64	557.35	561.26	575.49	536.92	559.84	43.66	0.988	0.882	0.998	0.743	0.745	0.511
K	917.64	858.80	909.48	919.96	910.37	855.59	43.80	0.322	0.838	0.156	0.269	0.146	0.530
Mg	90.42	90.52	94.07	93.16	92.03	89.82	4.43	0.576	0.401	0.619	0.673	0.408	0.884
Na	542.57	559.96	565.64	527.93	587.77	552.47	75.47	0.887	0.652	0.888	0.512	0.604	0.322
P	485.06	474.76	497.13	484.45	490.39	428.10	26.53	0.588	0.612	0.417	0.931	0.914	0.734
Microminerals and heavy metals													
Al	0.166	0.178	0.188	0.188	0.169	0.176	0.012	0.410	0.208	0.918	0.507	0.451	0.391
As	0.059	0.062	0.062	0.061	0.057	0.064	0.002	0.359	0.202	0.513	0.064	0.194	0.038
Ba	0.028	0.044	0.024	0.033	0.037	0.026	0.008	0.131	0.674	0.061	0.421	0.354	0.360
Cr	0.009	0.008	0.008	0,008	0.009	0.008	0.001	0.151	0.077	0.351	0.404	0.778	0.212
Cu	0.018	0.017	0.017	0.016	0.017	0.020	0.003	0.934	0.787	0.791	0.549	0.325	0.770
Fe	0.060	0.035	0.054	0.040	0.050	0.059	0.012	0.364	0.711	0.186	0.527	0.286	0.989
Li	0.005	0.005	0.004	0.004	0.006	0.004	0.001	0.820	0.563	0.920	0.090	0.595	0.040
Ni	0.004	0.004	0.003	0.004	0.002	0.004	0.001	0.311	0.178	0.659	0.325	0.989	0.158
Se	0.111	0.109	0.108	0.104	0.107	0.117	0.004	0.806	0.537	0.945	0.081	0.038	0.467
Sr	0.110	0.124	0.125	0.137	0.110	0.112	0.013	0.550	0.341	0.601	0.186	0.122	0.297
Zn	1.077	1.166	1.047	1.055	0.971	1.265	0.260	0.889	0.918	0.664	0.562	0.448	0.472

3.4.Discussion and conclusions

The aim of this study was to evaluate possible side effects of bentonite administration on the physiology and production of lactating Holstein cows and on milk characteristics. The approach applied in this study (3 x 3 Latin square crossover design) allowed to reduce the number of animals for experimental purposes, in compliance with the EU Directives on the protection of animals used for scientific purpose (European Parliament and Council, 2010). Moreover, given the large number of parameters to be considered for purpose of the experiment, different analytical methodologies, including Omics, were employed. The animals selected for the trial were cows in mid-late lactation in order to avoid the considerable physiological changes associated with the onset of lactation and to reduce the likelihood of mastitis occurrence (Sordillo and Raphael, 2013). Lastly, given the absence of aflatoxins and the negligible levels of fumonisins in the diet fed to the animals, it can be assumed that the observed variations in the biomarkers considered can be attributed primarily to the administration of bentonite and physiological factors of individual subjects.

In the experimental conditions applied, bentonite treatments did not affect cows' general physiological parameters. Rather, the modifications observed in these variables were caused by the period effect. In particular, the reduction of milk yield and the increase in BCS (associated with a

higher BW at the end of the trial) are in accordance with the normal lactation cycle, considering that the six cows were in middle-late lactation (DIM: 192.5 ± 22.7 at the beginning of t1 and 266.5 ± 22.7 at the end of t3; mean ± SD) (Strucken et al., 2015; Stanton et al., 1992; Souissi and Bouraoui, 2020; Mishra et al., 2016; VandeHaar and Pierre, 2006). The significant reduction of RT observed over time seems to be in contrast to the findings from Krpalkova et al. (2022), who recorded the longest rumination times at the end of lactation analyzing data from 2,777 cows on nine different commercial dairy farms located in different countries (Ireland, Germany, and Australia). However, the late lactation phase considered in that study ranged from 201 to 305 days in milk and during this long phase different sub-trends can be observed. In particular, due to a decline started at the end of the mid lactation phase, RT reached the minimum levels between 200 and 250 d and then rapidly increased till a maximum around 300 d of lactation. The lactation period in the six cows used in the present experiment approximately coincides with the end of the mid lactation and the first half of the late lactation in Krpálková et al. (2022). Therefore, the RT trends over the course of lactation observed in the two studies are compatible.

The metabolic profile found in rumen fluid samples is similar to those reported in other studies (Ametaj et al., 2010; Eom et al., 2020; Malheiros et al., 2021; Saleem et al., 2013; Zhao et al., 2014). However, the pronounced presence of glycerol observed in some samples was found to derive from a contamination from a batch of syringe filters used for sample pre-treatment. For this reason, the spectral ranges corresponding to glycerol peaks in the rumen fluid spectra were excluded performing the PCA analysis. A glycerol contamination was also reported by Bica et al. (2020).

The results of the PCA analysis revealed a clearer separation of rumen fluid metabolic profiles by period effect (t0, t1, and t2+t3 clusters in PC1 vs PC2 scores plot) than treatment effect (t0, B0, and B50+B100 in PC2 vs PC4 scores plot). The observed trends could be related to RT decline over time, as rumination is essential for the reduction of feed particle size improving rumen fermentations (Beauchemin K.A., 2018; Sjaastad et al., 2010) on one hand, and possible clay-dependent changes in rumen microbiota (Neubauer et al., 2019) on the other. Indeed, the study of the microbial populations included in this project revealed some significant differences in rumen fluid samples between the control (B0) and treated animals (B50 and B100) (B. Cardazzo, M. Cardin, E. Novelli; personal communication). In particular, differences in the relative abundance of phylum of Proteobacteria were reported due to the increase in the family of Succinivibrionaceae (B0: 17.41 ± 1.85%; B50: 20.05 ± 4.69%; B100: 22.20 ± 4.68%; mean ± SD) following the administration of bentonite. Moreover, a reduction in the relative abundance of the family of Ruminococcaceae (B0: 6.24 ± 1.84%; B50: 4.74 ± 1.79%; B100: 4.69 ± 0.90%; mean ±standard deviation) was recorded in treated cows. Lastly, treatment-induced changes to the relative abundance of low-abundant microbial populations were also reported, including a reduction in the order of Gastranaerophilales and the family of Clostridiaceae, while the family of Pseudomonadaceae increased in B100. No

differences were found in the eukaryotic communities of rumen fluid between treatment groups. Further investigations are needed to better clarify the association between bentonite-induced changes in microbial populations and metabolome in rumen fluid. Moreover, given the results obtained from the metabolomics study, it would be interesting to assess whether and how ruminal microbial communities varied as a function of the experimental period. Indeed, although rumen and gut microbiota are considered quite stable in adult ruminants and only limited day-to-day variations have been reported (Skarlupka et al., 2019; Huang et al., 2020), longer period studies have found significant changes in ruminal bacterial composition throughout the lactation cycle in dairy cows (Bainbridge et al., 2016; Jewell et al., 2015).

The PCA analysis of rumen fluid NMR spectra also revealed that samples collected in t0 differed sharply from all the others, including B0, even if animals were not receiving bentonite in both t0 and B0. This could be explained by the fact that, at the time of sampling in t0, animals had not yet fully acclimated to the new environment and diet. Moreover, it was also found that samples from cows 313 and 988 did not meet the general trends discussed above. It is possible to hypothesize a "delayed" bentonite effect on cow 313 due to individual response to the treatments and a carryover effect due to insufficient washout interval on cows 313 and 988, i.e., the two subjects receiving B0 diet during the last experimental period (t3). The latter hypothesis deserves attention because it could imply that long-term bentonite administration in dairy farms may significantly alter the ruminal metabolome and, consequently, animal and mammary metabolism. However, possible interferences due to individual physiological conditions can not be ruled out, as the two "anomalous" animals were those with the most advanced lactation (DIM of cow 988: 210 d at the beginning of t1 and 284 d at the end of t3; DIM of cow 313: 205 d at the beginning of t1 and 279 d at the end of the t3) and cow 313 was the one with most advanced pregnancy (129 d at the beginning of t1 and 203 d at the end of t3), while cow 988 was the only nonpregnant subject among the experimental animals.

The mean concentrations of the minerals detected in rumen fluid samples exhibited a good agreement with values from Foroutan et al. (2020) and Saleem et al. (2013), with some exceptions, i.e., Al, about one order of magnitude higher in Saleem et al. (2013), and Se and As, not reported in the literature consulted. Despite the high cation exchange capacity (CEC) of bentonite (Adamis et al., 2005), B50 and B100 treatments did not cause any significant variation in the mineral content in rumen fluid, except for Zn, the concentration of which was affected by a significant quadratic treatment effect. To the best of our knowledge, there are no studies evaluating possible bentonite-induced changes in the concentration of minerals in the ruminal fluid of cows. Ivan et al. (1992) reported reduced ruminal solubilities of Cu, Mg, and Zn in rams treated with 0.50 % as fed of bentonite. However, comparing the results of the two experiments, consideration should be given to the different animal species used, the different duration and dosages of treatments, the

bentonite concentrations reached in rumen, possible differences in composition and chemical-physical properties of the bentonite administered, and the differences in the basal diet fed. In fact, mineral content, surface charge, particle size, and adsorption and ion exchange capacity of clays are extremely variable and are also affected by environmental factors (e.g., pH and composition of the medium) (Damato et al., 2022; Magnoli et al., 2013; Otunola and Ololade, 2020), which may vary in the gastrointestinal tract depending on the animal species and the diet. In the present study, variations of few microelements (Cu, Li, Ni, and Zn) were observed in rumen fluid over the time. Such changes could depend on slight variations in basal diet composition over the trial.

Regarding blood serum samples, the metabolites identified by ^{1}H NMR spectroscopy are similar to those reported in other studies (Xu et al., 2016; López Radcenco et al., 2021; Chen et al., 2013; Sun et al., 2014; Tiziani et al., 2008; Eom et al., 2020). The results of the PCA analysis suggested that the administration of bentonite does not affect serum metabolome of dairy cows. In contrast, serum metabolome seemed to be markedly affected by the status and stage of pregnancy. In fact, the metabolic profiles of the two animals that were nonpregnant at the beginning of the experiment, i.e., ID 437 and 988, clearly differed from those of all other cows and clustered together throughout the course of the experiment, despite the development of pregnancy in cow 437 at t1. Once again, the metabolic profiles in t0 tended to cluster separately from the samples collected in the other periods and treatments. Therefore, the incomplete acclimatization of cows to the new farming conditions at the time of sampling could also have affected serum metabolome.

Rindsig and Schultz (1970) reported mineral imbalances in lactating Holstein dairy cows due to the addition of 5% and 10% of sodium bentonite to a high-grain fat-depressing diet. In particular, authors reported that the clay reduced the availability and/or the retention of Ca, Mg, and P. In the present experiment, the amounts of bentonite administered were much lower and only few and slight variations were observed in serum mineral content. Among macrominerals, only Mg showed significant fluctuations over time, with the lowest concentration recorded in t3 (**Table 3.8**). As the maintenance of physiological Mg levels in cattle primarily depends on the absorption from rumen (Martens and Stumpff, 2019), it is interesting to note that a nonsignificant tendency to a linear decrease in Mg concentration (P = 0.074) was present in ruminal fluid samples over time (**Table 3.6**). Importantly, despite the decrease in t3, Mg serum concentration was within the normal range of 1.85-3.17 mg/100 mL (Halse K., 1970). Regarding microelements, Ni showed a significant linear decrease over time similar to that reported in rumen fluid. Lastly, the significant linear increase in serum concentration of Zn due to bentonite treatments could be related to the Zn fluctuations observed in rumen fluid samples. In any case, the concentration measured for the elements detected in serum samples exhibited a good agreement with literature (Foroutan et al., 2020). Unfortunately, we could not find any reference value for serum Cr, Li and Ni in the dairy cow.

No significant variations of oxidative stress biomarkers were found in blood plasma due to bentonite administration. There was only a period effect on carbonyl groups, probably related to the proceeding of pregnancy (Castillo et al., 2005; Celi and Gabai, 2015).

The qualitative assessment of milk samples as well as the flow cytometry and the oxidative stress evaluation did not highlight any relevant changes induced by bentonite administration. This means that the macronutrient content, the milk coagulation properties (MCP), and the indicators of udder health (e.g., SCC, leukocyte subpopulation profile, and oxidative stress parameters) were not affected by the bentonite doses administered in the present experiment. On the other hand, some modifications due to the period effect were noticed. In particular, the improvement of MCP recorded over time is compatible with the progress of pregnancy and lactation in the experimental cows. Indeed, Penasa et al. (2016), using a huge number of records provided by the Breeders Association of Veneto region (Padova, Italy), found a decrease in RCT and an increase in a_{30} from the first to the last period of pregnancy in Italian Holstein cattle, with a stronger pregnancy effect on a_{30} than on RCT. According to the study conducted by Ostersen et al. (1997) on 39 Danish Holstein first lactation cows, the RCT was shortest and a_{30} was best in early and late lactation than in mid lactation. In the present study, a significant increase in milk total bacterial count (TBC) was also reported due to period effect. In case of mastitis, cows' udder can shed large numbers of pathogenic microorganisms (Skeie et al. 2019). In fact, as mentioned in "Materials and methods", three mastitis episodes were reported in cow ID 437 throughout the experiment. While the first two episodes occurred after the sampling day in t0, the initial signs of the third mastitis episode arose on the day of sampling in t3: the SCC value and the relative percentage of neutrophils were increased (data not shown), as typical during mastitis (Alhussien and Dang, 2018; Li et al., 2014; Rivas et al., 2001). In particular, the SCC value in the milk sample collected from cow ID 437 in t3 was very high, about 20 to 2000 times higher than all the other SCC values recorded in the trial (data not shown), and was therefore excluded from the statistical analysis as outlier. However, other milk parameters, such as milk pH and oxidative stress biomarkers, which were expected to increase (Luangwilai et al., 2020; Celi and Gabai, 2015; Guzzo et al., 2015; Gabai et al., 2014), and lactose, which usually decrease in case of udder infection/inflammation (Luangwilai et al., 2020), were not affected. Checking the TBC values recorded in cow 437, the one in t3 was higher than those in the previous experimental period, but a similar trend was noted in three other cows (ID 313, 88, and 578) having normal SCC. Therefore, the mastitis episode can not be considered as the only cause of the TBC enhancement in t3. Likely, it depended also on environmental factors and the progression of lactation in the experimental subjects. Indeed, besides from within the mammary gland and teat canal, microbial contamination of milk can originate from multiple other sources, such as the skin surface of udder and teats, the environment in which cows are housed and milked (grass, soil, bedding materials, animal faeces, feed, water, air etc.), milking and storage equipments,

and people (Bramley et al., 1984; Quigley et al. 2013; Deddefo et al., 2023). Moreover, the study conducted by Paludetti et al. (2019) found higher TBC in bulk tank milk samples from 150 farms in late-lactation than in those from 67 farms in mid-lactation, providing indications that the microbiological quality of milk may also be influenced by the stage of lactation.

Regarding the ^{1}H NMR spectroscopy, the compounds identified in the metabolic profiles of milk whey samples are in good agreement with literature (Boiani et al., 2019; Foroutan et al., 2019; Conde-Báez et al., 2017; Rysova et al., 2021; Klein et al., 2010; Sundekilde et al., 2013; Yanibada et al., 2018). Although a tendency for separation between treated and non-treated animals resulted from the PCA analysis, it was not so pronounced to confirm or rule out a significant effect of bentonite administration on milk metabolome. Regarding the anomalous "behavior" of cow ID 988, it should be considered that it was the only nonpregnant subject for the entire duration of the trial. In fact, milk metabolites derive from different cell types, metabolisms, and compound transport pathways and mechanisms operated by the mammary gland, which can change in particular conditions, such as pregnancy (Sundekilde et al., 2013; McManaman and Neville, 2003). De Nicola et al. (2020) found significant differences in the metabolic profiles of milk samples from pregnant and nonpregnant buffaloes. Moreover, while not deviating from the general clustering (treated vs untreated cows), it can be noted in the scores plot that the metabolic profile of cow 437 in t3 is arranged quite far from the other treated animals. Probably such "behavior" can be attributed to the early stage of pregnancy and/or to the third mastitis episode in this subject. In fact, mastitis is also responsible for metabolite variations in milk (Hu et al., 2021). In this regard, among the metabolites identified as responsible for the slight separation between milk metabolic profiles, some compounds (e.g., BHBA, lactate, and N-acetylnglucosamine) have been identified as potential biomarkers of mastitis in bovine milk (Luangwilai et al., 2020).

Comparing the findings of the metabonomic analysis of milk whey with those of the other biofluids, it is possible to hypothesise that the (slight) bentonite-induced changes in ruminal fluid metabolome were compensated by the animals' metabolism and, therefore, slighter differences were found in milk whey samples. Any differences between treatments in blood serum samples were maybe masked by stronger effects of other factors, primarily the stage of pregnancy. Moreover, the effect of the acclimatization was clear on rumen fluid and blood serum metabolome, but appeared to not affect the metabolic profiles of milk whey.

Considering the two biofluids (slightly) affected by the treatment, dimethylamine, ethanol, lactate, and methylamine were identified as metabolites responsible for the variance in both rumen fluid and milk whey samples. All of them are products of the microflora metabolism according to the information reported in the online Livestock Metabolome Database (LMDB). Moreover, butyrate and β-hydroxybutyrate (BHBA) were also reported as responsible for the variance in rumen fluid and milk whey samples, respectively. Indeed, butyrate represents one of the main volatile fatty

acids (VFAs) produced by rumen fermentations and, during its transport through the ruminal epithelium into the blood system, it is converted in BHBA which, among other functions, serves as a source for lipid synthesis by the mammary tissue (Sjaastad et al., 2010).

The proteomic and peptidomic analyses did not reveal any significant qualitative or quantitative differences between milk samples according to both bentonite treatment and experimental period. However, some nonsignificant differences were noted, mostly between t0 and all the treatment groups, including B0 (in accordance with the observations previously made for the metabolic profiles of ruminal fluid and blood serum), and secondarily between treatment groups.

The slight/nonsignificant changes in the metabolic and proteomic/peptidomic profiles of milk samples were supported by the results of the study of the microbial populations (B. Cardazzo, M. Cardin, E. Novelli; personal communication). Indeed, some mild effects of bentonite treatments were noted on microbial populations, as the redundancy analysis (RDA) revealed some differences between B0 and treated cows, while no statistically significant variations were highlighted by means of the permutational multivariate analysis of variance (PERMANOVA). Likely, it depended on the presence of variations in low-abundant microbial populations. In particular, it was noted a decrease in the relative abundance of the genera *Lysinibacillus* (B0: 0.14 ± 0.14%; B50: 0 ± 0%; B100: 0.04 ± 0.10%; mean ±standard deviation) and *Coprobacter* (B0: 0.15 ± 0.19%; B50: 0.04 ± 0.08%; B100: 0.04 ± 0.10%; mean ±standard deviation) due to bentonite administration. Moreover, variations (without a clear trend) between treatment groups were observed in the relative abundance of Lachnospiraceae_AC2044 group (B0: 0.17 ± 0.26%; B50: 0.61 ± 0.47%; B100: 0.09 ± 0.15%; mean ±standard deviation).

Finally, the mineral concentrations measured in milk whey samples are quite similar (mostly slightly lower) to those reported by Foroutan et al. (2019) and no clay effect was observed on both macro and microminerals as previously reported by Maki et al. (2016; 2017). Only slight fluctuations of few microminerals were found due to the period effect.

In conclusion, on the basis of the results obtained, dietary administration of bentonite to dairy cows (within the doses prescribed by the manufacturer in compliance with the EU regulation (European Commission, 2013) does not appear to negatively affect animal physiology and milk characteristics. Therefore, it can be considered a safe practice for both animals' and consumers' health. However, since some findings suggested the possibility of carryover effects, further investigations would be needed to study the effects of longer-term bentonite administration.

3.5. References

Adamis, Z., Williams, R.B., and International Programme on Chemical Safety, 2005. Bentonite, kaolin and selected clay minerals. World Health Organization. Available online: https://apps.who.int/iris/handle/10665/43102

Alhussien, M.N. and Dang, A.K. 2018. Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: An overview. *Veterinary World*, 11(5), 562-577. doi: 10.14202/vetworld.2018.562-577

Ametaj, B.N., Zebeli, Q., Saleem, F., Psychogios, N., Lewis, M.J., Dunn, S.M., Xia, J., and Wishart, D.S. 2010. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics*, 6, 583–594. doi: 10.1007/s11306-010-0227-6

Bailoni, L., Carraro, L., Cardin, M., Cardazzo, B. 2021. Active Rumen Bacterial and Protozoal Communities Revealed by RNA-Based Amplicon Sequencing on Dairy Cows Fed Different Diets at Three Physiological Stages. *Microorganisms*, 9(4), 754. doi: 10.3390/microorganisms9040754

Bainbridge, M.L., Cersosimo, L.M., Wright, A.-D.G., and Kraft, J. 2016. Rumen bacterial communities shift across a lactation in Holstein, Jersey and Holstein × Jersey dairy cows and correlate to rumen function, bacterial fatty acid composition and production parameters. *FEMS Microbiol Ecol*. 92, fiw059. doi: 10.1093/femsec/fiw059

Beauchemin, K.A. 2018. Invited review: Current perspectives on eating and rumination activity in dairy cows. *J. Dairy Sci*., 101(6), 4762–4784. doi: 10.3168/jds.2017-13706

Bica, R., Palarea-Albaladejo, J., Kew, W., Uhrin, D., Pacheco, D., Macrae, A., Dewhurst, R.J., 2020. Nuclear Magnetic Resonance to Detect Rumen Metabolites Associated with Enteric Methane Emissions from Beef Cattle. *Sci Rep.* 10, 5578. doi: 10.1038/s41598-020-62485-y

Bittante, G., Amalfitano, N., Bergamaschi, M., Patel, N., Haddi, M.L., Benabid, H., Pazzola, M., Vacca, G.M., Tagliapietra, F., Schiavon, S. 2022. Composition and aptitude for cheese-making of milk from cows, buffaloes, goats, sheep, dromedary camels, and donkeys. *J Dairy Sci*. 105(3), 2132-2152. doi: 10.3168/jds.2021-20961

Boiani, M., Sundekilde, U., Bateman, L.M., McCarthy, D.G., Maguire, A.R., Gulati., A., Guinee, T.P., Fenelon, M., Hennessy, D., FitzGerald, R.J., Kelly, P.M. 2019. Integration of high and low field H NMR to analyse the effects of bovine dietary regime on milk metabolomics and protein-bound moisture characterisation of the resulting mozzarella cheeses during ripening. *International Dairy Journal*, 91, 155-164. doi: 10.1016/j.idairyj.2018.08.016

Bordignon, M., Da Dalt, L., Marinelli, L., and Gabai, G. 2014. Advanced oxidation protein products are generated by bovine neutrophils and inhibit free radical production in vitro. *Vet J*. 199(1), 162–168. doi: 10.1016/j.tvjl.2013.10.028

Bramley, A.J., McKinnon, C.H., Staker, R.T., Simpkin, D.L., 1984. The effect of udder infection on the bacterial flora of the bulk milk of ten dairy herds. *J Appl Bacteriol*, 57(2), 317-323. doi: 10.1111/j.1365-2672.1984.tb01396.x

Brown, M., and Wittwer, C. 2000. Flow Cytometry: Principles and Clinical Applications in Hematology. *Clinical Chemistry*, 46(8), 1221–1229. doi: 10.1093/clinchem/46.8.1221

Castillo, C., Hernandez, A., Bravo, M., Lopez-Alonso, M., Pereira, V., and Benedito, J.L. 2005. Oxidative status during late pregnancy and early lactation in dairy cows. *Vet J*. 169(2), 286–292. doi: 10.1016/j.tvjl.2004.02.001

Celi, P., and Gabai, G. 2015. Oxidant/antioxidant balance in animal nutrition and health: the role of protein oxidation. *Front. Vet. Sci*., 2, 48. doi: 10.3389/fvets.2015.00048

Chen, Y., Wu, J., Tu, L., Xiong, X., Hu, X., Huang, J., Xu, Z., Zhang, X., Hu, C., Hu, X., Guo, A., Wang, Y., Chen, H. 2013. ^{1}H-NMR Spectroscopy Revealed *Mycobacterium tuberculosis* Caused Abnormal Serum Metabolic Profile of Cattle. *PLoS ONE*, 8(9), e74507. doi: 10.1371/journal.pone.0074507

Conde-Báez, L., Castro-Rosas, J., Villagómez-Ibarra, J.R., Páez-Lerma, J.B., Gómez-Aldapa, C. 2017. Evaluation of Waste of the Cheese Industry for the Production of Aroma of Roses (Phenylethyl Alcohol). *Waste Biomass Valor*, 8, 1343–1350. doi: 10.1007/s12649-016-9654-6

Cosentino, C., Paolino, R., Rubino, M., Freschi, P. 2022. Effect of the Addition of Donkey Milk on the Acceptability of Caciotta Cow Cheese. *Animals*, 12, 1444. doi: 10.3390/ani12111444

Damato, A., Vianello, F., Novelli, E., Balzan, S., Gianesella, M., Giaretta, E., and Gabai, G. 2022. Comprehensive Review on the Interactions of Clay Minerals With Animal Physiology and Production. *Front. Vet. Sci*. 9, 889612. doi: 10.3389/fvets.2022.889612

Deddefo, A., Mamo, G., Asfaw, M., Amenu, K., 2023. Factors affecting the microbiological quality and contamination of farm bulk milk by *Staphylococcus aureus* in dairy farms in Asella, Ethiopia. *BMC Microbiol*, 23(1), 65. doi: 10.1186/s12866-022-02746-0

De Nicola, D., Vinale, F., Salzano, A., d'Errico, G., Vassetti, A., D'Onofrio, N., Balestrieri, M.L., and Neglia, G. 2020. Milk Metabolomics Reveals Potential Biomarkers for Early Prediction of Pregnancy in Buffaloes Having Undergone Artificial Insemination. *Animals (Basel)*, 10(5), 758. doi: 10.3390/ani10050758

Edmonson, A.J., Lean, I.J., Weaver, L.D., Farver, T., Webster, G. 1989. A Body Condition Scoring Chart for Holstein Dairy Cows. *Journal of Dairy Science*, 72, 1, 68-78. doi: 10.3168/jds.S0022-0302(89)79081-0

Elliott, C.T., Connolly, L., Kolawole, O., 2020. Potential adverse effects on animal health and performance caused by the addition of mineral adsorbents to feeds to reduce mycotoxin exposure. *Mycotoxin Res*. 36, 115–126. https://doi.org/10.1007/s12550-019-00375-7.

Eom, J.S., Lee, S.J., Kim, H.S., Choi, YY, Kim, SH, Lee, Y.G., and Lee, S.S. 2020. Metabolomics Comparison of Hanwoo (*Bos taurus coreanae*) Biofluids Using Proton Nuclear Magnetic Resonance Spectroscopy. *Metabolites*. 10(8), 333. doi: 10.3390/metabo10080333

European Commission, 2006. Commission Recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. *Official Journal of the European Union*, L229, 7-9. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:229:0007:0009:EN:PDF

European Commission, 2013. Commission Implementing Regulation (EU) No 1060/2013 of 29 October 2013 concerning the authorisation of bentonite as a feed additive for all animal species. *Official Journal of the European Union*. L289, 33-37. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:289:0033:0037:EN:PDF

European Parliament and Council, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union*. L276, 33-79. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF

Foroutan, A., Guo, A.C., Vazquez-Fresno, R., Lipfert, M., Zhang, L., Zheng, J., Badran, H., Budinski, Z., Mandal, R., Ametaj, B.N., and Wishart, D.S. 2019. Chemical Composition of Commercial Cow's Milk. *J. Agric. Food Chem*. 67(17), 4897-4914. doi: 10.1021/acs.jafc.9b00204

Foroutan, A., Fitzsimmons, C., Mandal, R., Piri-Moghadam, H., Zheng, J., Guo, A., Li, C., Guan, L.L., Wishart, D.S., 2020. The Bovine Metabolome. Metabolites, 10(6), 233. doi: 10.3390/metabo10060233

Gabai, G., Testoni, S., Piccinini, R., Marinelli, L., Howard, C., Stradaioli, G. 2004. Oxidative stress in primiparous cows in relation to dietary starch and the progress of lactation. *Animal Science*, 79(1), 99-108. doi:10.1017/S1357729800054576

Gabai, G., Magro, M., Baratella, D., Miotto, G., Da Dalt, L., Vianello, F. (2014). Oxidized whey proteins as potential biomarkers of oxidative stress: preliminary studies of milk exposure to the myeloperoxidase product HOCl. *Proceedings of the First Dairy Care Conference*. 2015 Aug 22–23, Copenhagen, Denmark: DairyCare, p. 42.

Guzzo, N., Bailoni, L., Mantovani, R., Dalt, L. Da, & Gabai, G., 2015. Oxidized protein biomarkers in the blood and milk of cows supplemented with flaxseed during the dry period. *Proceedings of the Third DairyCare Conference, 3rd and 4th March 2015, Cordoba, Spain: p.60*.

Halse, K. 1970. Individual variation in blood magnesium and susceptibility to hypomagnesaemia in cows. *Acta Vet. Scand*. 11(3), 394-414. doi: 10.1186/BF03547967

Hu, M.L., 1994. Measurement of protein thiol and glutathione in plasma. *Methods Enzymol*. 233, 380-385. doi: 10.1016/s0076-6879(94)33044-1

Hu, H., Fang, Z., Mu, T., Wang, Z., Ma, Y., and Ma, Y. 2021. Application of Metabolomics in Diagnosis of Cow Mastitis: A Review. *Front. Vet. Sci*., 8, 747519. doi: 10.3389/fvets.2021.747519

Huang, S., Ji, S., Yan, H., Hao, Y., Zhang, J., Wang, Y., Cao, Z., Li, S. 2020. The day-to-day stability of the ruminal and fecal microbiota in lactating dairy cows. *Microbiologyopen*., 9, 5, e990. doi: 10.1002/mbo3.990

Ivan, M., Dayrell, M. de S., and Hidiroglou, M. 1992. Effects of Bentonite and Monensin on selected Elements in the Stomach and Liver of Fauna-Free and Faunated Sheep. *J Dairy Sci*. 75(1), 201–208. doi: 10.3168/jds.S0022-0302(92)77754-6

Jewell, K.A., McCormick, C.A., Odt, C.L., Weimer, P.J., and Suen, G. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. *Appl Environ Microbiol*. 81, 4, 4697–4710. doi: 10.1128/AEM.00720-15

Klein, M.S., Almstetter, M.F., Schlamberger, G., Nürnberger, N., Dettmer, K., Oefner, P.J., Meyer, H.H.D., Wiedemann, S., Gronwald, W. 2010. Nuclear magnetic resonance and mass spectrometry-based milk metabolomics in dairy cows during early and late lactation. *J. Dairy Sci*., 93(4), 1539–1550. doi: 10.3168/jds.2009-2563

Krpálková, L., O'Mahony, N., Carvalho, A., Campbell, S., and Walsh, J. 2022. Association of rumination with milk yield of early, mid and late lactation dairy cows. *Czech J. Anim. Sci*., 67(3), 87–101. doi: 10.17221/94/2021-CJAS

Levine, R.L, Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S. and Stadtman, E.R. 1990. Determination of Carbonyl Content in Oxidatively Modified Proteins. *Methods in Ezymology*, 186, 464-478. doi: 10.1016/0076-6879(90)86141-h

Li, N., Richoux, R., Boutinaud, M., Martin P., Gagnaire, V. 2014. Role of somatic cells on dairy processes and products: a review. *Dairy Sci Technol*. 94(6), 517–538. doi: 10.1007/s13594-014-0176-3

Li, N., Richoux, R., Perruchot, M.-H., Boutinaud, M., Mayol, J.-F., Gagnaire, V., 2015. Flow Cytometry Approach to Quantify the Viability of Milk Somatic Cell Counts after Various Physico-Chemical Treatments. *PLOS One*, 10(12), e0146071. doi: 10.1371/journal.pone.0146071

López Radcenco, A., Adrien, M.d.L., Ruprechter, G., de Torres, E., Meikle, A., Moyna, G. 2021. Monitoring the Transition Period in Dairy Cows through ^{1}H NMR-Based Untargeted Metabolomics. *Dairy*, 2(3), 356–366. doi: 10.3390/dairy2030028

Luangwilai, M., Duangmal, K., Chantaprasarn, N., Settachaimongkon, S. 2020. Comparative metabolite profiling of raw milk from subclinical and clinical mastitis cows using ^{1}H-NMR combined with chemometric analysis. *International Journal of Food Science & Technology*, 56(1), 493-503. doi: 10.1111/ijfs.14665

Magnoli, A.P., Alonso, V.A., Cavaglieri, L.R., Dalcero, A.M., Chiacchiera, S.M., 2013. Effect of monogastric and ruminant gastrointestinal conditions on in vitro aflatoxin B1 adsorption ability by a montmorillonite. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 30(4):743–749. doi: 10.1080/19440049.2013.784398

Maki, C.R., Thomas, A.D., Elmore, S.E., Romoser, A.A., Harvey, R.B., Ramirez-Ramirez, H.A. and Phillips, T.D. 2016. Effects of calcium montmorillonite clay and aflatoxin exposure on dry matter intake, milk production, and milk composition. *J Dairy Sci*., 99(2), 1039–1046. doi: 10.3168/jds.2015-10242

Maki, C.R., Haney, S., Wang, M., Ward, S.H., Rude, B.J., Harvey, R.B., Phillips, T.D. 2017. Calcium montmorillonite clay for the reduction of aflatoxin residues in milk and dairy products. *Dairy and Vet Sci J*. 2(3), 555587. doi: 10.19080/JDVS.2017.02.555587

Malheiros, J.M., Barbosa Correia, B.S., Ceribeli, C., Rodrigues Cardoso, D., Colnago, L.A., Bogusz Junior, S., Reecy, J.M., Gerson Barreto Mourão, G., Lehmann Coutinho, L., Pascale Palhares, J.C., Berndt, A., and Correia de Almeida Regitano, L. 2021. Comparative untargeted metabolome analysis of ruminal fluid and feces of Nelore steers (*Bos indicus*). *Sci Rep*, 11, 12752. doi: 10.1038/s41598-021-92179-y

Martens, H., and Stumpff, F. 2019. Assessment of magnesium intake according to requirement in dairy cows. *J Anim Physiol Anim Nutr*., 103, 1023–1029. doi: 10.1111/jpn.13106

McKinnon, K.M. 2018. Flow Cytometry: An Overview. *Curr Protoc Immunol*., 120, 5.1.1–5.1.11. doi: 10.1002/cpim.40

McManaman, J.L., and Neville, M.C. 2003. Mammary physiology and milk secretion. *Adv Drug Deliv Rev*. 55(5), 629–641. doi: 10.1016/s0169-409x(03)00033-4

Mishra, S., KumarI, K., and Dubey, A. 2016. Body Condition Scoring of Dairy Cattle: A Review. *RRJVS*, 2(1), 58-65. Available online: https://www.rroij.com/open-access/body-condition-scoring-of-dairy-cattle-a-review-.pdf

Neubauer, V., Humer, E., Mann, E., Kröger, I., Reisinger, N., Wagner, M., Zebeli, Q., Petri, R.M., 2019. Effects of clay mineral supplementation on particle-associated and epimural microbiota, and gene expression in the rumen of cows fed high-concentrate diet. *Anaerobe* 59, 38–48. doi: 10.1016/j.anaerobe.2019.05.003

Nur Atikah, I., Alimon, A.R., Yaakub, H., Abdullah, N., Jahromi, M.F., Ivan, M., Samsudin, A.A., 2018. Profiling of rumen fermentation, microbial population and digestibility in goats fed with dietary oils containing different fatty acids. *BMC Vet Res*., 14, 1, 344. doi: 10.1186/s12917-018-1672-0

Ostersen, S., Foldager, J., and Hermansen, J.E. 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. *J Dairy Res*, 64(2), 207-219. doi: 10.1017/s0022029996002099

Otunola, B.O., Ololade, O.O., 2020. A review on the application of clay minerals as heavy metal adsorbents for remediation purposes. *Environ Technol Innov*. 18, 100692. doi: 10.1016/j.eti.2020.100692

Paludetti, L.F., Kelly, A.L., O'Brien, B., Jordan, K., Gleeson, D., 2019. Microbiological quality of milk from farms to milk powder manufacture: an industrial case study. *J Dairy Res*, 86(2), 242-247. doi: 10.1017/S0022029919000347

Penasa, M., De Marchi, M., and Cassandro, M. 2016. Short communication: Effects of pregnancy on milk yield, composition traits, and coagulation properties of Holstein cows. *J. Dairy Sci*., 99(6), 4864–4869. doi: 10.3168/jds.2015-10168

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D., 2013. The complex microbiota of raw milk. FEMS Microbiol Rev, 37(5), 664-698. doi: 10.1111/1574-6976.12030

Rappsilber, J., Mann, M., Ishihama, Y. 2007. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature Protocols*, 2(8), 1896-1906. doi: 10.1038/nprot.2007.261

Reznick, A.Z., and Packer, L. 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods in Enzymology,* 233, 357-363. doi: 10.1016/S0076-6879(94)33041-7

Rindsig, R.B., Schultz, L.H., 1970. Effect of Bentonite on Nitrogen and Mineral Balances and Ration Digestibility of High-Grain Rations Fed to Lactating Dairy Cows. *J Dairy Sci*. 53(7), 888–892. doi: 10.3168/jds.S0022-0302(70)86313-5

Rivas, A.L., Quimby, F.W., Blue, J., and Coksaygan, O. 2001. Longitudinal evaluation of bovine mammary gland health status by somatic cell counting, flow cytometry, and cytology. *J Vet Diagn Invest*, 13(5), 399–407. doi: 10.1177/104063870101300506

Roncada, P., Piras, C., Soggiu, A., Turk, R., Urbani, A., Bonizzi, L. 2012. Farm animal milk proteomics. *J Proteomics*, 75(14), 4259 – 4274. doi: 10.1016/j.jprot.2012.05.028

Rysova, L., Legarova, V., Pacakova, Z., Hanus, O., Nemeckova, I., Klimesova, M., Havlik, J. 2021. Detection of bovine milk adulteration in caprine milk with N-acetyl carbohydrate biomarkers by using ^{1}H nuclear magnetic resonance spectroscopy. *J Dairy Sci*. 104(9), 9583–9595. doi: 10.3168/jds.2020-20077

Saleem, F., Ametaj, B.N., Bouatra, S., Mandal, R., Zebeli, Q., Dunn, S.M., and Wishart, D.S. 2012. A metabolomics approach to uncover the effects of grain diets on rumen health in dairy cows. *J Dairy Sci*. 95, 11, 6606-6623. doi: 10.3168/jds.2012-5403

Saleem, F., Bouatra, S., Guo, A.C., Psychogios, N., Mandal, R., Dunn, S.M., Ametaj, B.N., and Wishart, D.S. 2013. The Bovine Ruminal Fluid Metabolome. *Metabolomics*, 9, 360–378. doi: 10.1007/s11306-012-0458-9

Sjaastad, O.V., Sand, O., Hove, K., 2010. *Physiology of Domestic Animals*, Editor: Scandinavian Veterinary Press, Oslo.

Skarlupka, J.H., Kamenetsky, M.E., Jewell, K.A., Suen, G. 2019. The ruminal bacterial community in lactating dairy cows has limited variation on a day-to-day basis. *J Animal Sci Biotechnol*, 10, 66. doi: 10.1186/s40104-019-0375-0

Skeie, S.B., Håland, M., Thorsen, I.M., Narvhus, J., Porcellato, D., 2019. Bulk tank raw milk microbiota differs within and between farms: A moving goalpost challenging quality control. *J Dairy Sci*, 102(3), 1959-1971. doi: 10.3168/jds.2017-14083

Sordillo, L.M., Raphael, W. 2013. Significance of metabolic stress, lipid mobilization, and inflammation on transition cow disorders. *Vet Clin North Am Food Anim Pract*., 29(2), 267–278. doi: 10.1016/j.cvfa.2013.03.002

Souissi, W., and Bouraoui, R., 2020. Relationship between Body Condition Score, Milk Yield, Reproduction, and Biochemical Parameters in Dairy Cows. In: M'Hamdi, N. (ed.), *Lactation in Farm Animals - Biology, Physiological Basis, Nutritional Requirements, and Modelization*. IntechOpen. doi: 10.5772/intechopen.85343.

Stanton, T.L., Jones, L.R., Everett, R.W., and Kachman, S.D. 1992. Estimating Milk, Fat, and Protein Lactation Curves with a Test Day Model. *Journal of Dairy Science*, 75(6), 1691-1700. doi: 10.3168/jds.S0022-0302(92)77926-0

Strucken, E.M., Laurenson, Y.C.S.M., and Brockmann, G.A. 2015. Go with the flow-biology and genetics of the lactation cycle. *Front Genet*. 6, 118. doi: 10.3389/fgene.2015.00118

Sun, L.W., Zhang, H.Y., Wu, L., Shu, S., Xia, C., Xu, C., and. Zheng, J. S. 2014. ^{1}H-Nuclear magnetic resonance-based plasma metabolic profiling of dairy cows with clinical and subclinical ketosis. *J Dairy Sci*. 97(3), 1552–1562. doi:10.3168/jds.2013-6757

Sundekilde, U.K., Larsen, L.B., and Bertram, H.C. 2013. NMR-Based Milk Metabolomics. *Metabolites*, 3(2), 204-222. doi: 10.3390/metabo3020204

Talukder, S., Kerrisk, K.L., Gabai, G., Fukutomi, A., and Celi, P. 2015. Changes in milk oxidative stress biomarkers in lactating dairy cows with ovulatory and an-ovulatory oestrous cycles. *Animal Reproduction Science*, 158, 86–95. doi: 10.1016/j.anireprosci.2015.05.004

Taylor, B.C., Keefe, R.G., Dellinger, J.D., Nakamura, Y., Cullor, J.S. and Stott, J.L. 1997. T Cell Populations and Cytokine Expression in Milk Derived from Normal and Bacteria-Infected Bovine Mammary Glands. *Cell Immunol*., 182(1), 68–76. doi: 10.1006/cimm.1997.1215

Tiziani, S., Emwas, A.-H., Lodi, A., Ludwig, C., Bunce, C.M., Viant, M.R., and Günther, U.L. 2008. Optimized metabolite extraction from blood serum for ^{1}H nuclear magnetic resonance spectroscopy. *Anal Biochem*, 377(1), 16–23. doi: 10.1016/j.ab.2008.01.037

VandeHaar, M.J. and St-Pierre, N. 2006. Major Advances in Nutrition: Relevance to the Sustainability of the Dairy Industry. *Journal of Dairy Science*, 89(4), 1280-1291. doi: 10.3168/jds.S0022-0302(06)72196-8

Vinagre, C., Madeira, D., Narciso, L., Cabral, H.N. and Diniz, M. 2012. Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. *Ecological Indicators*, 23, 274–279. doi: 10.1016/j.ecolind.2012.04.009

Wasowicz, W., Nève, J. and Peretz, A. 1993. Optimized steps in fluorimetric determination of thiobarbituric acidreactive substances in serum: importance of extraction pH and influence of sample preservation and storage. *Clinical Chemistry.* 39(12), 2522-2526.

Wilkerson, M. J. 2012. Principles and Applications of Flow Cytometry and Cell Sorting in Companion Animal Medicine. *Vet Clin North Am Small Anim Proct*, 42(1), 53–71. doi: 10.1016/j.cvsm.2011.09.012

Wilkerson, M. J. and Springer, N. L., 2020. Cluster of Differentiation (CD) Antigens. In: Brooks, M.B., Harr, K.E., Seelig, D.M., Wardrop, K.J., Weiss, D.J. (eds.), *Schalm's Veterinary Hematology*. John Wiley & Sons, Inc. pp. 41-47.

Witko-Sarsat, V., Friedlander, M., Capeillère-Blandin, C., Nguyen-Khoa, T., Nguyen, A.T., Zingraff, J., Jungers, P. and Descamps-Latscha, B. 1996. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney International*, 49(5), 1304—1313. doi: 10.1038/ki.1996.186

Witko-Sarsat, V., Friedlander, M., Nguyen Khoa, T., Capeillère-Blandin, C., Nguyen, A.T., Canteloup, S., Dayer, J.M, Jungers, P., Drüeke, T. and Descamps-Latscha, B. 1998. Advanced Oxidation Protein Products as Novel Mediators of Inflammation and Monocyte Activation in Chronic Renal Failure. *J Immunol*, 161(5), 2524–2532.

Xu, C., Sun, L.-W., Xia, C., Zhang, H.-Y, Zheng, J.-S. and Wang., J.-S. 2016. ^{1}H-Nuclear Magnetic Resonance-Based Plasma Metabolic Profiling of Dairy Cows with Fatty Liver. *Asian-Australas J Anim Sci*. 29(2), 219-229. doi: 10.5713/ajas.15.0439

Yang, J., Zhao, X., Lu, X., Lin, X., Xu, G., 2015. A data preprocessing strategy for metabolomics to reduce the mask effect in data analysis. *Front Mol Biosci*. 2, 4. doi: 10.3389/fmolb.2015.00004

Yanibada, B., Boudra, H., Debrauwer, L., Martin, C., Morgavi, D.P., Canlet, C. 2018. Evaluation of sample preparation methods for NMR-based metabolomics of cow milk. *Heliyon*, 4(10), e00856. doi: 10.1016/j.heliyon.2018.e00856

Yoshida, Y., Itoh, N., Hayakawa, M., Piga, R., Cynshi, O., Jishage, K.-I., Niki, E. 2005. Lipid peroxidation induced by carbon tetrachloride and its inhibition by antioxidant as evaluated by an oxidative stress marker, HODE. *Toxicology and Applied Pharmacology*, 208(1), 87 – 97. doi: 10.1016/j.taap.2005.01.015

Zhao, S., Zhao, J., Bu, D., Sun, P., Wang, J. and Dong, Z. 2014. Metabolomics analysis reveals large effect of roughage types on rumen microbial metabolic profile in dairy cows. *Letters in Applied Microbiology*, 59(1), 79—85. doi: 10.1111/lam.12247

4. General discussion and conclusions

The experimental part of this dissertation aimed to fill the gaps in knowledge of possible side effects of bentonite administration on *i)* ruminal fermentations, rumen fluid composition and characteristics, *ii)* the physiology and production of dairy cows, and *iii)* milk characteristics. Indeed, despite some indications of possible undesirable effects of clay minerals have been reported in different animal species and cell cultures, most of the studies performed in farm animals have focused on a limited number of parameters to evaluate the effectiveness of clays as sequestrants of feed contaminants (especially mycotoxins) and usually did not include a control with the clay alone (i.e., without contaminants) (Damato et al., 2022; Döll and Dänicke, 2004; Elliott et al., 2020). Therefore, in Section 2 of the present dissertation, a commercial bentonite was added to a total mixed ration (TMR) with no or negligible mycotoxin contamination and different experimental approaches (*in vitro* and *in vivo*) and numerous analytical methods were applied in order to investigate the putative effects of the clay mineral on different physiological and production parameters of lactating dairy cows.

While no bentonite-induced change in gas production resulted from study 1, a slight decrease in both cumulative GP (mL/g DM) and GP rate (mL/g DM) due to bentonite treatment (significant at the highest dose used = 50 mg in 1 g of diet) was observed in study 2. In both the two *in vitro* studies and *in vivo* experiment, bentonite administration produced some slight modifications to the metabolome and mineral content (mainly microelements) of the rumen fluid, as summarized in **Table 4.1**. Some similarities could be observed among the three experiments. For instance, the concentrations of butyrate and propionate varied due to bentonite supplementation in study 1 and they were also reported among the metabolites responsible for the clusterization (Principal Component Analysis - PCA) of the rumen fluid NMR spectra in study 3. Another metabolite responsible for the clusterization in study 3 was trimethylamine, which was found to decrease in B1000 treatment in study 2. Bentonite treatments caused a significant increase in Al and Sr concentrations in study 1 and 2 and affected Zn ruminal content in study 2 and 3.

The differences observed in the results of the three experiments could be determined by numerous factors, including: *i)* the different experimental approach (*in vitro* vs *in vivo*), *ii)* the different experimental design (e.g., the number of biological replicates), *iii)* the influence of the animal(s) used, the time of rumen fluid collection, and the acclimatization process on rumen fluid physicochemical characteristics and microbiological and metabolic composition, *iv)* differences in sample preparation protocols and analytical methods applied.

Table 4.1. Main results obtained from the metabolomic and mineral content analyses of rumen fluid samples in the three studies presented in Chapters 1, 2, and 3 of Section 2 of this dissertation. Abbreviations: ICP-OES = Inductively Coupled Plasma-Optical Emission Spectroscopy; ICP-MS = Inductively Coupled Plasma- Mass Spectrometry; LC/DFI-MS/MS = Liquid Chromatography/Direct Flow Injection-Tandem Mass Spectrometry; PC = principal component; PCA = principal component analysis.

Study	Experimental approach	Experimental design	Analytical methods	Main results
1	*In vitro* ruminal fermentations	• One biological replicate (one cow as rumen fluid donor) • Five technical replicates for each treatment • Five bentonite treatments: 0, 2.5, 5, 10 and 50 mg in 1 g of diet (B0, B50, B100, B200, and B1000, respectively) • 24 h incubation at 39 °C	• Qualitative/ Semiquantitative metabolomics by 300 MHz ^{1}H NMR spectroscopy and PCA analysis • Qualitative and quantitative mineral content analysis by ICP-OES and regression analysis • Analyses performed on pooled technical replicates	• Clear differences between the metabolic profile of B0 sample and those of B50, B100, B200, and B1000 samples mainly related to bentonite-induced decrease in butyrate and increase in propionate • Significant decrease of Ba, Ca, and Mn and significant increase of Al, Cr, Mo, and Sr due to treatments
2	*In vitro* ruminal fermentations	• Three biological replicates (three different cows as rumen fluid donor) • Five technical replicates for each treatment • Three bentonite treatments: 0, 5, and 50 mg in 1 g of diet (B0, B100, and B1000, respectively) • 24 h incubation at 39 °C	• Qualitative and quantitative metabolomic study by LC/DFI-MS/MS and 700 MHz ^{1}H NMR spectroscopy • Trace element content analysis by ICP-MS • Statistical analysis of metabolites and trace elements concentrations by means of a mixed model considering treatment as fixed effect and biological replicate as random effect	• Significant treatment effect on 7 metabolites: decrease of LysoPC C14:0 in B100 and B1000, increase of methylmalonic acid and spermidine in B1000, decrease of 4-hydroxybutyrate, 4-hydroxyphenylacetate, and trimethylamine in B1000, and decrease of fumaric acid in B100 • Significant treatment effect on 4 trace elements: increase of Al in B1000, increase of Sr in B100 and B1000, decrease of Zn in B1000, and increase of Co as bentonite dose increases
3	*In vivo*	• Six multiparous lactating Holstein cows • 24-d acclimatization (t0) • 3 × 3 Latin square crossover design with 19-d treatment periods (t1, t2, and t3) interspersed with 9-d washout periods • Three bentonite treatments: 0, 50, and 100 g/animal/d (B0, B50, and B100, respectively)	• Qualitative/ Semiquantitative metabolomics by 300 MHz ^{1}H NMR spectroscopy and PCA analysis • Qualitative and quantitative mineral content analysis by ICP-OES and statistical analysis of the concentrations measured by means of a mixed model considering period and treatment as fixed effects and cow and cell (treatment × period) as random effects	• Separation of the rumen fluid metabolic profiles in clusters due to: - period effect: clusters t0, t1, and t2+t3 in PC1 vs PC2 scores plot (with the exception of two samples from cow 313) - treatment effect and acclimatization process: clusters t0, B0, and B50+B100 in PC2 vs PC4 scores plot (with the exception of four samples, three from cow 313 and one from cow 988) • Metabolites potentially responsible for the clusterization of the rumen fluid metabolic profiles: acetate, alanine, butyrate, dimethylamine, ethanol, GABA, isopropanol, lactate, lysine, methylamine, trimethylamine, propionate, and valerate. • Significant period effect on 5 minerals: linear decrease in Cu, Ni, and Zn concentrations over time and quadratic effect on Li content • Significant quadratic effect of bentonite treatment on Zn concentration

In any case, the modifications observed in the three studies can be related to the capacity of clay minerals in interacting with micro- and macronutrients and microorganisms (Cuadros J., 2017; Elliott et al., 2020; Wallace and Newbold, 1991; Williams L.B., 2019). In particular, in the *in vivo* experiment, the modifications in rumen metabolome were accompanied by the finding of a decrease in daily rumination time over the experimental periods and slight bentonite-induced changes in ruminal fluid bacterial communities (increase in Succinivibrionaceae and Pseudomonadaceae, reduction in Ruminococcaceae and Clostridiaceae and Gastranaerophilales; relative abundances). The potential of clay minerals to interact with rumen microbes deserves attention, as the exact mechanisms are not completely understood and the phenomenon and its consequences have been poorly studied, especially *in vivo*. It is important to bear in mind that some clays possess antibacterial activity through physical and chemical mechanisms of action (e.g. physical damage to the cell membrane thanks to the mineralogical textures of clays, such as fibrous needles; binding and encasement of bacteria within clay particles, preventing uptake of essential nutrients, efflux of waste products, and respiration; adsorption of cations competing with bacterial uptake of essential elements; release of cytotoxic metals; increased cell membrane permeability through lipid peroxidation and oxidative stress; altered pH and oxidation state of the microenvironment) (Williams L.B., 2019). Such mechanisms have low specificity and could involve both pathogens and commensal bacteria once the clay is administered as feed additive. Moreover, based on the influence of pH on the surface charge and the resulting binding capacity of clay minerals, it cannot be ruled out that microorganisms bound in the upper tract of the digestive system may be translocated and released in a lower tract. These considerations might suggest the risk of onset of gastrointestinal dysmicrobism and altered host metabolism. However, only some natural clays have manifested antibacterial activities, linked to their mineralogical texture, composition, and small particle size (< 200 nm) (Williams, 2019). In the present dissertation, the clay administered to the experimental cows was composed of selected bentonites with montmorillonite content about 85% (Technical Data Sheet of GLOBALFEED® T1, Laviosa SpA: https://www.laviosa.com/wp-content/uploads/2017/01/LAVIOSA_AnimalFeed_GLOBALFEED-T1_TDS_ENG-1.pdf). Hu et al. (2002) did not observe bacteriostatic or bactericidal effect of montmorillonite *in vitro* on both *Escherichia coli* and *Staphylococcus aureus*. Antibacterial activities are reported for modified bentonites/montmorillonites (Hu et al., 2005; Santos et al., 2011; Holešová et al., 2013; Martsouka et al., 2021). In addition, considering the *in vivo* study conducted by Neubauer et al. (2019), the administration of a bentonite and plant extract-based product exerted a positive effect on rumen microbiota composition in non-lactating Holstein cows fed high-concentrate diet, as the treatment increased the relative abundance of some commensal bacteria and reduced the relative abundance of potentially harmful microrganisms. In particular, authors reported that clay supplementation increased the relative abundance of genus *Butyrivibrio* and

decreased the genera *Lactobacillus*, *Fusobacterium*, and *Treponema* in the particle-associated microbiota and decreased *Succiniclasticum* genus and increased *Campylobacter* genus in the epimural microbiota.

Despite the bentonite-induced changes in rumen fluid samples discussed above, study 3 of the present dissertation showed a good ability of cows in preserving their homeostasis, since, negligible or no effects of bentonite were found in the metabolome and mineral content of blood serum and milk whey. Considering also the other parameters assessed *in vivo* (i.e., general indicators of animal health and production, oxidative stress biomarkers, milk quality and leukocyte subpopulations, milk proteome and peptidome), most of the observed modifications are more likely attributable to factors other than bentonite administration (such as animals' acclimatization, period effect, lactation phase, pregnancy status, and occurrence of mastitis), at least at the doses employed in this experiment, that meet the EU regulation of the use of bentonite against dietary mycotoxins in ruminants (European Commission, 2013).

The effects of the acclimatization process, the inter-individual and intra-individual variability on the results reported in the present dissertation deserve consideration, as they can produce experimental bias. The cows used for the three trials were purchased from a commercial dairy farm and relocated at the "L. Toniolo" experimental farm (Legnaro, Padua, Italy). They found a new farm environment, received the standard diet for lactating cows used at the new farm, and were housed and managed in accordance with the experimental needs (i.e., separation from the resident herd, individual boxes, milking by bucket system, close monitoring and handling by operators etc.). Such changes are recognized as stressors able to affect animal physiological and behavioral parameters (Conour et al., 2006; Obernier and Baldwin, 2006; Grandin and Shivley, 2015; Sevi et al., 2001; Pošćić et al., 2017; Mamuad et al., 2017). Moreover, the proceeding of lactation, onset of mastitis, pregnancy, status and stage can produce inter-individual variability and intra-individual changes over time in numerous parameters of cows and other dairy ruminants, including oxidative stress status (Castillo et al., 2005; Celi and Gabai, 2015), BCS and milk yield (Ostersen et al. 1997; Strucken et al., 2015; Stanton et al., 1992; Souissi and Bouraoui, 2020), various milk characteristics, and milk and blood metabolome (Penasa et al., 2016; Sundekilde et al., 2013; McManaman and Neville, 2003; de Nicola et al., 2020; Hu et al., 2021; Guo and Tao, 2018). Bainbridge et al. (2016) and Jewell et al. (2015) found significant changes in the rumen microbiota of lactating dairy cows over the lactation cycle. Moreover, several authors reported variations in ruminal microbiome within the animal groups studied, although all the subjects were of the same breed and farm of origin, fed the same diet, and kept under the same experimental conditions, with possible or proven changes in microbial metabolic activity and rumen metabolome (Clemmons et al., 2022; Jami and Mizrahi, 2012; Mizrahi and Jami, 2018; Sasson et al., 2017; Shabat et al., 2016; Xue et al., 2020). Some of these studies found a relationship between rumen microbiome composition/metabolome and

genetic, physiological, and productive differences among animals, including host metabolism and serum metabolome, milk yield, milk protein yield and lactose content, feed efficiency, and methane emission (Clemmons et al., 2022; Mizrahi and Jami, 2018; Sasson et al., 2017; Shabat et al., 2016; Xue et al., 2020).

In the *in vivo* trial (study 1) of the present dissertation, the inter-individual variability and intra-individual changes depending on sampling time were expected due to the experimental design. In fact, the mixed model applied for the statistical analysis of data (except the results of the omics analyses) was designed in order to discriminate changes induced by bentonite treatment from those due to the period and cow effect. Regarding the results of the PCA analysis on the NMR spectra, the period effect contributed (together with bentonite treatment) to the clusterization of the rumen metabolic profiles, while it appears not to have affected the separation of blood serum and milk whey samples. Moreover, the PCA analysis of rumen fluid NMR spectra revealed the separation of samples collected in t0 from those collected during t1, t2, and t3, which were grouped according to the treatment (cluster B0 for controls and cluster B50+B100 for treated animals). A similar but less clear separation was obtained from the PCA analysis of the metabolomic profiles of blood serum samples, as t0 tended to separate from all the other samples. In milk, the acclimatization process does not seem to have affected the metabolome (t0 samples tended to cluster with B0 samples and separate from the metabolic profiles of treated animals), but it was responsible for some (nonsignificant) differences in the whey proteomic/petidomic profiles between t0 samples and those collected from cows in B0, B50, and B100 treatments. Lastly, in all the biofluids considered, a few metabolic profiles did not meet the general clusterization/separation highlighted by the PCA analysis of the NMR spectra. In most cases, the presence of such "outliers" could be explained by the source of inter-individual and intra-individual variability discussed above. However, two "outliers" observed in the metabolic study of rumen fluid samples deserve attention. In fact, the samples collected from the two control subjects (B0) in the last experimental period (t3) clustered together with treated animals, suggesting a possible carryover effect of bentonite due to insufficient washout interval.

It is possible that the individual variability and uncomplete animal acclimatization might have affected the experimental outcomes of the first *in vitro* experiment, as the rumen fluid used in study 1 was taken from one cow (one biological replicate) at the end of t0, but not those of the *in vitro* study 2, performed using the rumen fluid collected from three different cows (three biological replicates) after concluding the *in vivo* trial.

Another important aspect that deserves attention is that the three experiments conducted evaluated the effects of bentonite administration over a short time interval. Indeed, the *in vitro* fermentations were performed in 24 h and, even if the *in vivo* trial lasted about three months, it included 19-d treatment periods and 9-d washout periods. Nevertheless, the results obtained

showed some slight metabolic and mineral changes attributable to bentonite treatments. In addition, as mentioned above, indications of possible carryover effects of clay on the metabolome of rumen fluid emerged from the *in vivo* study. Therefore, it cannot be ruled out that prolonged and/or continuous treatments with bentonite may have a greater impact.

It can be concluded that short-term administration of bentonite within the maximum levels set by the European regulations did not produce overt undesirable effects on dairy cows' physiology, health, and production as well as on milk quality. In particular, the nutritional and hygienic characteristics of milk and its cheesemaking properties were not compromised, suggesting that the use of bentonite in farms has no negative implications for consumers and dairy industry. However, as our results showed some slight metabolic and mineral alterations induced by the treatments, further experiments would be needed to investigate possible effects of long-term continuative administration of bentonite in dairy cows.

4.1. References

Bainbridge, M.L., Cersosimo, L.M., Wright, A.-D.G., and Kraft, J. 2016. Rumen bacterial communities shift across a lactation in Holstein, Jersey and Holstein × Jersey dairy cows and correlate to rumen function, bacterial fatty acid composition and production parameters. *FEMS Microbiol Ecol*. 92, fiw059. doi: 10.1093/femsec/fiw059

Castillo, C., Hernandez, A., Bravo, M., Lopez-Alonso, M., Pereira, V., and Benedito, J.L. 2005. Oxidative status during late pregnancy and early lactation in dairy cows. *Vet J*. 169(2), 286–292. doi: 10.1016/j.tvjl.2004.02.001

Celi, P., and Gabai, G. 2015. Oxidant/antioxidant balance in animal nutrition and health: the role of protein oxidation. *Front. Vet. Sci*., 2, 48. doi: 10.3389/fvets.2015.00048

Clemmons, B.A., Mulon, P.-Y., Anderson, D.E., Ault-Seay, T.B., Henniger, M.T., Schneider, L.G., Staton, M., Voy, B.H., Donohoe, D.R., Campagna, S.R., McLean, K.J., and Myer, P.R. 2022. Ruminal Bacterial Communities and Metabolome Variation in Beef Heifers Divergent in Feed Efficiency. *Ruminants*, 2, 282–296. doi: 10.3390/ruminants2020019

Conour, L.A., Murray, K.A., Brown, M.J. 2006. Preparation of Animals for Research—Issues to Consider for Rodents and Rabbits. *ILAR Journal*, 47(4), 283–293. doi: 10.1093/ilar.47.4.283

Cuadros, J., 2017. Clay minerals interaction with microorganisms: A review. *Clay Miner*. 52(2), 235–261. doi: 10.1180/claymin.2017.052.2.05

Damato, A., Vianello, F., Novelli, E., Balzan, S., Gianesella, M., Giaretta, E., and Gabai, G. 2022. Comprehensive Review on the Interactions of Clay Minerals With Animal Physiology and Production. *Front Vet Sci*. 9, 889612. doi: 10.3389/fvets.2022.889612

De Nicola, D., Vinale, F., Salzano, A., d'Errico, G., Vassetti, A., D'Onofrio, N., Balestrieri, M.L., and Neglia, G. 2020. Milk Metabolomics Reveals Potential Biomarkers for Early Prediction of Pregnancy in Buffaloes Having Undergone Artificial Insemination. *Animals (Basel)*, 10(5), 758. doi: 10.3390/ani10050758

Döll, S., Dänicke, S. 2004. *In vivo* Detoxification of *Fusarium toxins*. *Arch Anim Nutr*. 58(6), 419–441. doi: 10.1080/00039420400020066

Elliott, C.T., Connolly, L., Kolawole, O., 2020. Potential adverse effects on animal health and performance caused by the addition of mineral adsorbents to feeds to reduce mycotoxin exposure. *Mycotoxin Res*. 36, 115–126. doi: 10.1007/s12550-019-00375-7

European Commission, 2013. Commission Implementing Regulation (EU) No 1060/2013 of 29 October 2013 concerning the authorisation of bentonite as a feed additive for all animal species. *Official Journal of the European Union*. L289, 33-37. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:289:0033:0037:EN:PDF

Grandin, T., and Shivley, C. 2015. How Farm Animals React and Perceive Stressful Situations Such As Handling, Restraint, and Transport. *Animals (Basel)*, 5(4), 1233-51. doi: 10.3390/ani5040409

Guo, Y.S., and Tao, J.Z. 2018. Metabolomics and pathway analyses to characterize metabolic alterations in pregnant dairy cows on D 17 and D 45 after AI. *Sci Rep*, 8(1), 5973. doi: 10.1038/s41598-018-23983-2

Holešová, S., Samlíková, M., Pazdziora, E., Valášková, M. 2013. Antibacterial activity of organomontmorillonites and organovermiculites prepared using chlorhexidine diacetate. *Appl Clay Sci*, 83–84, 17–23. doi: 10.1016/j.clay.2013.07.013

Hu, X.-R., Lu, G.-L., Chen, L.-S., Gu, J.-M., Zhang, Y., 2002. [Study on the mechanism of the interaction between montmorillonite and bacterium]. *Yao Xue Xue Bao*, 37, 9, 718—720. https://pubmed.ncbi.nlm.nih.gov/12567898/

Hu, C.H., Xu, Z.R., Xia, M.S., 2005. Antibacterial effect of Cu^{2+}-exchanged montmorillonite on *Aeromonas hydrophila* and discussion on its mechanism. *Vet Microbiol*. 109, 1-2, 83–88. doi: 10.1016/j.vetmic.2005.04.021

Hu, H., Fang, Z., Mu, T., Wang, Z., Ma, Y., and Ma, Y. 2021. Application of Metabolomics in Diagnosis of Cow Mastitis: A Review. *Front. Vet. Sci*., 8, 747519. doi: 10.3389/fvets.2021.747519

Jami, E., and Mizrahi, I. 2012. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One*, 7(3), e33306. doi: 10.1371/journal.pone.0033306

Jewell, K.A., McCormick, C.A., Odt, C.L., Weimer, P.J., and Suen, G. 2015. Ruminal bacterial community composition in dairy cows is dynamic over the course of two lactations and correlates with feed efficiency. *Appl Environ Microbiol*. 81, 4, 4697–4710. doi: 10.1128/AEM.00720-15

Mamuad, L.L., Kim, S.H., Lee, S.S., Dae, C. 2017. 869 Adaptation period during changing diet of dairy cows changes rumen fermentation characteristics and microbial qualities and communities. *Journal of Animal Science*, 95(4), 372. doi: 10.2527/asasann.2017.869

Martsouka, F., Papagiannopoulos, K., Hatziantoniou, S., Barlog, M., Lagiopoulos, G., Tatoulis, T., Tekerlekopoulou, A.G., Lampropoulou, P., Papoulis, D. 2021. The Antimicrobial Properties of Modified Pharmaceutical Bentonite with Zinc and Copper. *Pharmaceutics*, 13, 8, 1190. doi: 10.3390/ pharmaceutics13081190

McManaman, J.L., and Neville, M.C. 2003. Mammary physiology and milk secretion. *Adv Drug Deliv Rev*. 55(5), 629–641. doi: 10.1016/s0169-409x(03)00033-4

Mizrahi, I., and Jami, E. 2018. Review: The compositional variation of the rumen microbiome and its effect on host performance and methane emission. *Animal,* 12(S2), S220-S232. doi:10.1017/S1751731118001957

Neubauer, V., Humer, E., Mann, E., Kröger, I., Reisinger, N., Wagner, M., Zebeli, Q., Petri, R.M., 2019. Effects of clay mineral supplementation on particle-associated and epimural microbiota, and gene

expression in the rumen of cows fed high-concentrate diet. *Anaerobe*. 59, 38–48. doi: 10.1016/j.anaerobe.2019.05.003

Obernier, J.A., and Baldwin, R.L. 2006. Establishing an Appropriate Period of Acclimatization Following Transportation of Laboratory Animals. *ILAR Journal*, 47(4), 364–369. doi: 10.1093/ilar.47.4.364

Ostersen, S., Foldager, J. and Hermansen, J.E. 1997. Effects of stage of lactation, milk protein genotype and body condition at calving on protein composition and renneting properties of bovine milk. *Journal of Dairy Research*, 64(2), 207-219. doi: 10.1017/s0022029996002099

Penasa, M., De Marchi, M., and Cassandro, M. 2016. Short communication: Effects of pregnancy on milk yield, composition traits, and coagulation properties of Holstein cows. *J. Dairy Sci.*, 99(6), 4864–4869. doi: 10.3168/jds.2015-10168

Pošćić, N., Gabai, G., Stefanon, B., Da Dalt, L., and Sgorlon, S. 2017. Milk cortisol response to group relocation in lactating cows. *Journal of Dairy Research*, 84(1), 36-38. doi:10.1017/S0022029916000790

Santos, M.F., Oliveira, C.M., Tachinski, C.T., Fernandes, M.P., Pich, C.T., Angioletto, E., Riella, H.G., Fiori, M.A. 2011. Bactericidal properties of bentonite treated with Ag+ and acid. International Journal of Mineral Processing, 100, 1-2, 51-53. doi: 10.1016/j.minpro.2011.04.012

Sasson, G., Kruger Ben-Shabat, S., Seroussi, E., Doron-Faigenboim, A., Shterzer, N., Yaacoby, S., Berg Miller, M.E., White, B.A., Halperin, E., Mizrahi, I. 2017. Heritable bovine rumen bacteria are phylogenetically related and correlated with the cow's capacity to harvest energy from its feed. mBio 8:e00703-17. doi: 10.1128/mBio.00703-17

Sevi, A., Taibi, L., Albenzio, M., Muscio, A., Dell'Aquila, S., Napolitano, F. 2001. Behavioral, adrenal, immune, and productive responses of lactating ewes to regrouping and relocation. *J Anim Sci*, 79(6), 1457-65. doi: 10.2527/2001.7961457x

Shabat, S., Sasson, G., Doron-Faigenboim, A., Durman, T., Yacoby, S., Miller, M.E.B., White, B.A., Shterzer, N., and Mizrahi, I. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J*, 10, 2958–2972. doi: 10.1038/ismej.2016.62

Souissi, W., and Bouraoui, R., 2020. Relationship between Body Condition Score, Milk Yield, Reproduction, and Biochemical Parameters in Dairy Cows. In: M'Hamdi, N. (ed.), *Lactation in Farm Animals - Biology, Physiological Basis, Nutritional Requirements, and Modelization*. IntechOpen. doi: 10.5772/intechopen.85343

Stanton, T.L., Jones, L.R., Everett, R.W., and Kachman, S.D. 1992. Estimating Milk, Fat, and Protein Lactation Curves with a Test Day Model. *Journal of Dairy Science*, 75(6), 1691-1700. doi: 10.3168/jds.S0022-0302(92)77926-0

Strucken, E.M., Laurenson Y.C.S.M., and Brockmann, G.A. 2015. Go with the flow-biology and genetics of the lactation cycle. *Front. Genet*., 6, 118. doi: 10.3389/fgene.2015.00118

Sundekilde, U.K., Larsen, L.B., and Bertram, H.C. 2013. NMR-Based Milk Metabolomics. *Metabolites*, 3(2), 204-222. doi: 10.3390/metabo3020204

Wallace, R.J., and Newbold, J.1991. Effects of bentonite on fermentation in the rumen simulation technique (Rusitec) and on rumen ciliate protozoa. *The Journal of Agricultural Science*, 116(1), 163-168. doi:10.1017/S0021859600076279

Williams L.B. 2019. Natural antibacterial clays: historical uses and modern advances. *Clays Clay Miner*. 67, 7–24. doi: 10.1007/s42860-018-0002-8

Xue, M.-Y., Sun, H.-Z., Wu, X.-H., Liu, J.-X., and Guan, L.L. 2020. Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome*, 8, 64. doi: 10.1186/s40168-020-00819-8

APPENDIX 1

Operating procedure for 700 MHz ^{1}H NMR spectroscopy analysis of rumen fluid, blood serum, and milk whey samples

A subset of representative rumen fluid, blood serum, and milk whey samples were sent in dry ice to TMIC (The Metabolomics Innovation Centre) at the University of Alberta (Edmonton, Alberta, Canada) to be analyzed by 700 MHz ^{1}H NMR spectroscopy.

NMR sample preparation

The day of the analysis, all samples (stored at -80 °C) were thawed on ice, vorexed, and centrifuged at 14,000 rpm for 15 min at 4 °C (Eppendorf™ Centrifuge 5810 R). The sample preparation protocol used for the ^{1}H NMR spectroscopy of rumen fluid samples was the same described for the *in vitro* fermented fluid in Section 2, chapter "2. *In vitro* experiment 2" of the present dissertation. Milk whey and blood serum samples, on the other hand, were prepared following the operating procedures described by Foroutan et al. (2019, 2020) with slight modification. In brief, 3 kDa Amicon® 0.5 mL ultrafilter systems (Millipore®, Sigma-Aldrich) (one per sample) were washed 5 times with 500 mL HPLC Grade water (Fisher chemical) centrifuging at 10,000 rpm for 10 min at room temperature (Eppendorf™ Centrifuge 5810 R) in order to remove glycerol from the ultrafilter membrane. Water residues were eliminated by repeating centrifugation with empty ultrafilters at 4,000 rpm for 1 min. Then, 400 µL of blood serum and milk whey samples were transferred in the ultrafilters and centrifuged at 11,000 rpm for 15 min at 4 °C to eliminate proteins and lipoproteins, which compromise the quality of ^{1}H NMR spectra by generating intense and broad signals covering the small and thin peaks of metabolites. Then, 200 µL of the ultrafiltered biofluids were combined in a 1.5 mL Eppendorf tube with 50 µL of two different standard NMR buffer solutions: the buffer added to serum ultrafiltrates contained 250 mM potassium phosphate (pH 7.0), 5 mM DSS-d6 (3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt), 5.84 mM 2-chloropyrimidine-5-carboxylic acid, and D_2O 54% v/v in H_2O, while the buffer added to milk whey ultrafiltrates consisted of 750 mM potassium phosphate (pH 7.0), 5 mM DSS-d6 (3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt), 5.84 mM 2-chloropyrimidine-5-carboxylic acid, and D_2O 54% v/v in H_2O. The final 250 µl NMR samples contained 150 mM (rumen fluid and milk whey samples) or 50 mM (blood serum samples) potassium phosphate, 1 mM DSS-d6 as calibrate internal standard, and 10% D_2O. Blanks (50 µl of buffer solution plus 200 µl of 5 mM NaN_3 aqueous solution) were also prepared following the same protocols applied for sample preparation in order to exclude any contamination from the buffer solutions or the ultrafilters and any interference due to the presence of NaN_3 in the samples.

All samples and blanks were vortexed and centrifuged at 13,000 rpm for 10 min at 4 °C (Eppendorf™ Centrifuge 5810 R) and then transferred into 3 mm NMR tubes using glass Pasteur pipettes.

^{1}H NMR spectra acquisition and processing

All ^{1}H-NMR spectra were acquired on Bruker Avance III Ascend 700 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a 5 mm cryo-probe and an autosampler (SampleJet, Bruker Biospin) and controlled by means of IconNMR™ Automation Software (Bruker BioSpin). The acquisition and processing parameters used were 298 K temperature, NOESY-presaturation pulse sequence, 128 scans, 4 s acquisition time, 1 s recycle delay, 0.5 Hz line broadening, DSS singlet as chemical shift reference standard at 0.00 ppm.

^{1}H NMR spectra compound identification

Chenomx NMR Suite 8.2 software (Chenomx Inc., Edmonton, AB, Canada) was used for metabolite identification in rumen fluid and milk whey by comparing manually the spectral signals of each sample with the spectra of pure compounds included in Spectral Reference Libraries. For the blood serum samples, spectra were imported in MagMet web system (https://magmet.ca) able to automatically identify metabolites in 1D ^{1}H NMR spectra of serum.

References

Foroutan, A., Guo, A.C., Vazquez-Fresno, R., Lipfert, M., Zhang, L., Zheng, J., Badran, H., Budinski, Z., Mandal, R., Ametaj, B.N. and Wishart, D.S. 2019. Chemical Composition of Commercial Cow's Milk. *J Agric Food Chem*. 67(17), 4897-4914. doi: 10.1021/acs.jafc.9b00204

Foroutan, A., Fitzsimmons, C., Mandal, R., Piri-Moghadam, H., Zheng, J., Guo, A., Li, C., Guan, L.L., Wishart, D.S., 2020. The Bovine Metabolome. *Metabolites*, 10(6), 233. doi: 10.3390/metabo10060233

APPENDIX 2 – Other projects

1. Cryopreservation and oxidative stress in porcine oocytes

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Received: 12 October 2020
Received in revised form: 4 December 2020
Accepted: 29 December 2020
Available online: 2 January 2021

Citation:
Mateo-Otero Y, Yeste M, Damato A and Giaretta E (2021) Cryopreservation and oxidative stress in porcine oocytes. Research in Veterinary Science. Volume 135, Pages 20-26, ISSN 0034-5288, https://doi.org/10.1016/j.rvsc.2020.12.024.

Abstract

Several vitrification protocols have been established for porcine oocytes so as to facilitate gene banking of female germplasm. Although live piglets have been successfully produced from pig oocytes vitrified at the germinal vesicle (GV) stage, the competence of vitrified oocytes to develop into the blastocyst stage is greatly compromised following cryopreservation. The focus of this review is to elucidate the impact of cryopreservation on the redox balance of pig oocytes, making special reference to the relevance of non-enzymatic and enzymatic antioxidant defences. Besides, the regulation of gene expression in response to oxidative stress is also considered. Finally, we discuss the effects of supplementing maturation and vitrification media with the exogenous non-enzymatic antioxidants that have hitherto yielded the most relevant results.

Keywords: Oocyte, Cryopreservation, Oxidative stress, Antioxidants, Pigs

2. Evaluation of metabolomic profiling and oxidative stress markers of bull seminal plasma and their relationship with sperm motility before and after thawing

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Published: December 2022

Citation:
Giaretta E, Gabai G, Mislei B, Bucci D, Damato A, Vigolo V, Falomo M E and Zennaro L (2022) Evaluation of metabolomic profiling and oxidative stress markers of bull seminal plasma and their relationship with sperm motility before and after thawing. Animal Reproduction Science. Volume 247, 107107, ISSN 0378-4320, https://doi.org/10.1016/j.anireprosci.2022.107107.

Mechanisms underlying differentiation of good or poor freezability ejaculates in bull are still poorly understood. This study aims at investigating oxidative stress parameters and metabolomic profiling of seminal plasma (SP) in relation to bull semen motility before and after cryopreservation.
Semen samples were collected by artificial vagina from 20 bulls aged from 10 to 12 mo. An aliquot of semen was centrifuged to obtain SP (stored at -80°C), another was diluted 1:1 with a pre-warmed extender and cryopreserved. Motility assessment (CASA) was performed on fresh and thawed semen (0 and 3 h after thawing). Semen samples were divided into good freezers (GF) and bad freezers (BF) groups according to thawed semen motility, with cutoff values as follows: TM ≥ 60% and PM ≥ 30%. SP was used for oxidative stress markers analysis by spectroscopy techniques and metabolomic profile by nuclear magnetic resonance (NMR). The relationship between NMR spectra and semen quality was evaluated by the PCA/PLS-DA analysis. General linear models and mixed linear models were used to evaluate the relationship between seminal plasma oxidative stress parameters and semen quality. A clear separation between GF and BF ejaculates was observed on the first two principal components of the variance (PC1 vs PC2) of NMR spectra of SP. The increase of advanced oxidative protein products (AOPP) and thiols concentrations on SP were significantly related with higher straightness (STR), beat cross frequency (BCF) and linearity (LIN) and the decrease of amplitude of lateral head displacement (ALH) of fresh semen. Thiols and AOPP concentrations on SP were positively related to semen freezability. In addition, higher AOPP concentration was significantly related to higher TM, PM and LIN of thawed spermatozoa, whereas higher levels of thiols in SP concentration were positively related to the increment of sperm PM

after thawing. These findings suggested that SP composition can modulate bull semen freezabilty. Further analyses could elucidate which metabolites and oxidative stress parameters could be used as biomarkers of good semen quality after cryopreservation.

Acknowledgements

I would like to conclude by thanking the people who contributed their knowledge, expertise, practical or emotional support to my research and goals achieved during my Doctoral Course.

First of all, I express my deepest gratitude to my supervisor Prof. Gianfranco Gabai and my co-supervisor Prof. Lucio Zennaro for their invaluable help, patience, and guidance.

My gratitude extends to all those who collaborated in the research activities reported in the present dissertation. In particular, I would like to thank Prof. Enrico Novelli, who coordinated the project BENFELAT, and Profs. Giulio Cozzi, Antonio Berti and Dr. Alberto Simonetto for the management and care of the animals used in the experiments. For their indispensable contribution in sample collection, laboratory activities and/or data analysis, I also thank Profs. Giovanni Bittante, Barbara Cardazzo, Maria Elena Gelain, Matteo Gianesella, Elisa Giaretta, Paola Roncada, Franco Tagliapietra, Bruno Tilocca, Fabio Vianello, Drs. Nicolò Amalfitano, Federico Bonsembiante, Laura Da Dalt, Enrico Fiore, Paola Vanzani, my colleagues Marco Cardin and Selene Masaro, and the undergaduate students Giosuè Gentili, Daniele Lanza, Esmaeil Montazeri Najafabadi, and Hirak Krushnachandra Tandel.

Moreover, I am very grateful to Profs. Burim Ametaj and David Wishart for the opportunity they gave me to visit the University of Alberta (Edmonton, Alberta, Canada) in order to receive a training in Metabolomics and conduct part of my research at The Metabolomic Innovation Centre (TMIC). I also thank the team of TMIC who kindly dedicated their time to me to carry out the above activities. My experience abroad was so exciting and constructive also thanks to other wonderful people I met both on and off campus, such as Alyaa, Azat, Debby, Dorsa, Eric and his family, Fabiano, Jenna, Karla, Marco, Marina, Michela, Raheel, Roz, and Wei.

I would like to acknowledge my friends, in particular Gigi, Giorgio, Giovanni, Nunzio, Raffaella, and Jack, for their support in the hardest days.

Finally, I am very thankful to my family, as they supported me in every single moment of my PhD studies.