UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

Cycle XXXV

S.S.D. AGR/18-19

Effects of genetic and anti-nutritional substances on performance, metabolism, feed efficiency and milk quality of lactating dairy cows

Coordinator:

Ch.mo Prof. Paolo Ajmone Marsan

Candidate:

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Matriculation n: 4915102

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A te,
Unica per sempre
H.

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Foreword of the thesis

All over the world, one of the objectives of dairy cattle breeding is to produce high quantity and high-quality milk at low cost and at the same time ensuring animal welfare and guaranteeing a low environmental impact. Reaching these goals, farmers can produce milk that is economically, environmentally, and socially sustainable. Different aspects of milk production interact with each other and influence the production process, from the production of feed in the field to the sale of milk on the external market. Among these factors, certainly the genetics of cows plays an important role. Different dairy breeds are bred around the world to produce milk, each with specific characteristics and dairy farmers choose the dairy breed considering various circumstances: destination of the milk market, farming environment, feed quality, presence/absence of pasture and others. Moreover, for many decades, farmers selected their cows to improve different aspects: milk production and quality, functional morphology, and health performance. This has led to the National Breeding Association to run different breeding programs to select specific traits for each breed's goals. The different genetic selection plans led to different adaptations ability and changed the breed's requirements for milk production. Another very important factors that can influence the efficiency of dairy cow breeding, there is the presence and control of anti-nutritional substances, among which, most importantly, mycotoxins stand out. This thesis aims to investigate the effect of different genetic dairy breeds and anti-nutritional substances (i.e., mycotoxins) on dairy performance, cow metabolism, feed efficiency and production quality. The thesis is divided into two parts. In the first part (chapters 1st and 2nd) the effect of breed on performance, metabolism and nutrition of cows is investigated. During this survey, two of the most bred dairy breeds in the world were compared: Holstein and Brown Swiss cows and their ability to adapt during the transition period which is one of the most studied and important periods in the life of the cow. In the second part (chapters 3rd and 4th) the effect of the presence of mycotoxins on the quantitative and qualitative production of milk, on the metabolism and on the reproductive aspect was investigated. In particular, the effect of mycotoxins of the genus fusarium (DON and ZEN) was studied.

1 Genetic effect

1.1Effect of dairy breed on farm performance

In dairy farms we can find different dairy breed. Typically, they manage milk purpose breeds (such as Holstein, Brown Swiss, Jersey, and others) or dual-purpose breeds (e.g., Simmenthal). Each breed is selected for its specific traits (Back et al., 2006; Bland et al., 2015; De Marchi et al., 2008; Gustavsson et al., 2014). The different genetic selection and breed goals depend on the breeding environment (Piccand et al., 2013; White et al., 2002). Milk purpose breeds are selected for milk production with different traits. Generally, Holstein is selected especially for high milk production, Jersey particularly for fat production and Brow Swiss for cheese yield. The most widespread dairy breed in Europe and North America is Holstein (Cunningham, 1983). In country across Alps Brown Swiss is managed especially for its milk quality (Cunningham, 1983; Mattiello et al., 2011; Zanon et al., 2020). In literature many studies compared different dairy breeds each other for specific traits explored: milk quality, reproductive performance, feed efficiency, rumination, fat and protein nutrition, immunity, metabolism, longevity, and resistance to environmental challenges. The choice to breed one breed or another bring to different milk performance as farm output and leads the farmer to manage their own animals in a different way. However, most of the studies in the dairy literature take into consideration one (principally Holstein) or eventually two (in addition Jersey) breeds. Besides, same nutritional model considers only the requirements of Holstein and Jersey (NASEM, 2021; NRC, 2001). Moreover, most of the papers on nutritional trials and immune-metabolic studies focus on Holstein nutrition and physiology. Several times, breed comparison studies pointed out differences' traits or different adoption on environmental or physiological challenges (Back et al., 2006; Barth et al., 2011; Gibson et al., 2016; Malacarne et al., 2006; Piccand et al., 2013; Turner et al., 2006). These differences are usually explained at the genetics level. Few times these paper (Braun et al., 2015b; Urdl et al., 2015) lead

information to breeders to better manage their cows taking into consideration their breed characteristic. We decided to better understand specific nutritional and immune-metabolic traits of the Brown Swiss breed, in comparison with the most widespread dairy breed in Italy, being Holstein breed. To the best of our knowledge, few studies in literature had investigated the nutritional and immune metabolic traits of the Brown Swiss breed. As we can see after, most of the Brown Swiss literature is focused on milk quality and cheese yield. For this reason, our studies focused on other aspect (e.g., nutritional, immune-physiological, adaptation to transition period, and so on) of the Brown Swiss breed.

1.2 Brown Swiss breed

The Brown Swiss breed is the second dairy breed in Italy as counted by the Italian national breeder's association (AIA) and widespread especially across Alps (Mattiello et al., 2011; Zanon et al., 2020). This diffusion is linked to the Alps regions (Italy, Switzerland, Austria, France, and German) because the place origin of the original breed (nowadays named as Original Brown or OB) in Switzerland. From this place, the Brown Swiss became widespread in different country. The principal country where Brown Swiss is breed is Switzerland with 520,000 heads, followed by Italy with 437,100, Germany with 453,000 and Austria. The breed is also popular in France, Spain, UK, Netherland, Portugal, Slovenia, Bulgaria, and Romania. In general, we can assume that here are over 6 million of Brown Swiss all over the world, especially in Europe, Canada, and USA. From a dual-purpose breed, it became to be milk selected at the end of the 800 centuries in Switzerland. However, an important genetic improvement occurred in the European Brown Swiss population around 1970s when European breeders began to use artificial insemination (AI) bulls from American Brown Swiss that was strongly selected for milk production. At the beginning of 1990s the national Italian Brown Swiss breeders association (ANARB) started to its own national genetic improvement selection. The breeding goals were the milk production, milk quality with high cheese yield, functional morphology, and longevity. Thanks to

European Brown Swiss Federation data, Brown Swiss is the second dairy breed for production after Holstein. However, its best feature is milk quality with a fat content about 4.00% and protein content about 3.50-3.80%. This was one of the most investigate breed traits in literature when Brown Swiss was considered, as described in the next paragraph. The second-best feature is the longevity: in 2018 in German dairy cows' population the mean age of leaving herd was higher in Brown Swiss population than Holstein and Simmental. Consequently, Brown Swiss is one of the most widespread dairy breeds also in Italy as the second Italian dairy breed with the 20% of the total dairy population (AIA). In 2022, the average milk production is about 7,764 kg of milk each lactation with 4.08% fat and 3.60% protein. For these reason Brown Swiss is well breed specially to produce cheese in different region and this is their breeding goals.

1.3 Holstein breed

One of the technical and economic goals of each dairy breeds to maximize their profits is the milk production. Holstein is the most widespread in USA and Europe (Cunningham, 1983) due to its high milk production, with lower butterfat and protein based on percentage in the milk if compared to other dairy breeds (like Jersey and Brown Swiss). It is a specified milk purpose selected breed. The Italian national Holstein breeder's association (ANAFIBJ) count approximately 1 million of registered Holstein cows in Italy. The origin of the Holstein breed come from the Friesians cows in Netherlands. The first Dutch breeders started to select their local cows to improve the amount of milk produced. Originally, the breed was known as Holstein-Friesians and nowadays more simply as Holstein. The first Holstein cows were imported in US in the half of the 800' century and American breeders started a strong and efficient genetic selection focused on the milk production. This selection though the years resulted in the dairy cow that everyone in the world nowadays known as Holstein. Mean lactation records of Italian registered Holstein in 2022 showed an average production of 10,396 kg of milk with 3.88% fat and 3.34% protein. In comparison with Brow Swiss, they

produce more milk but with lower fat and protein content. Thanks to its diffusion, Holstein is usually considered the model cow for the milk production, especially for high milk yield. Most of the nutritional model (NASEM_2021; NRC, 2001) have calculated the cow requirements referring to Holstein milking, dry cows, heifer, and calves. To the best of our knowledge, in the dairy literature the predominant breed in many trials and studies concerning nutrition, welfare, genetic, physiology, immunity, management and other aspects, is Holstein. Few times dairy studies take into consideration non-Holstein dairy breeds, expect for specific purpose.

1.4 Literature on the comparison between Brown Swiss and Holstein

As the second dairy breed in many countries, Brown Swiss was many times compared with the most widespread dairy breed in the world, being Holstein. To the best of our knowledge, the majority part of the dairy literature considers Holstein in their studies and papers for many and different aspects and purposes in the dairy sector: milk production, milk quality, reproduction, nutrition, welfare, genetic selection, management, and others. Thanks to its world diffusion and high dairy performance Holstein is quite contemplated as the dairy model cow for many breeders and technicians, but also for dairy studies. Most of the cows involved in studies are Holstein cows. However, few times the cows involved in some papers are cows of different breeds, like Brown Swiss, Jersey, Simmental, and other local dairy breeds. In these cases, this king of studies that didn't' involved Holsteins, are very few compared with Holstein based studies. They are mainly performed in country where Holstein is quite widespread as other breeds, like in many Swiss (Braun et al., 2013, 2015a, 2015b; Kessler et al., 2020; Piccand et al., 2013) or German studies (Gruber et al., 2014). Furthermore in Italy, due the Brown Swiss diffusion, many studies had investigated same specific traits of the breed, especially for milk quality, cheese yield and coagulation properties (Bittante, 2022; Bittante et al., 2021; Cecchinato et al., 2015; De Marchi et al., 2007, 2008a, 2008b; Franceschi, Malacarne, Faccia, et al., 2020; Franceschi, Malacarne, Formaggioni, et al., 2020; Franzoi et al., 2020; Gottardo et al., 2017; Malacarne et al.,

2006; Martinez-Castillero et al., 2021; Zanon et al., 2020). Same of these were performed in regions where there are multi-breeds dairy farms (Gottardo et al., 2017; Mattiello et al., 2011; Zanon et al., 2020), such as the Italian Alpen regions. The principle aims of this kind of studies was to find the most important difference in milk performance between Brown Swiss and Holstein in the same farm or among different dairy farms. A lot of paper in literature pointed out the differences in milk yield and quality between the two breeds reflecting the different genetic selection goals. Several studies confirmed the higher milk yield of Holstein breed but at the same Brown Swiss showed more fat, protein, and casein milk content (Bittante et al., 2021; Carroll et al., 2006; Cecchinato et al., 2015; De Marchi et al., 2007, 2008a; Dechow et al., 2007; Franceschi, Malacarne, Formaggioni, et al., 2020; Gottardo et al., 2017). These milk production trends confirmed the different genetic selection. The major aim of the Brown Swiss breeders is the final cheese production. Several papers confirmed that Brown Swiss produced milk with higher cheese yield in comparison with Holstein (Bittante et al., 2021; Cecchinato et al., 2015; De Marchi et al., 2008a; Franceschi, Malacarne, Faccia, et al., 2020; Franceschi, Malacarne, Formaggioni, et al., 2020) and better coagulation properties (De Marchi et al., 2007, 2008a; Malacarne et al., 2006). About the milk cell somatic count some of the studies pointed out an equal content between Brown Swiss and Holstein (Dechow et al., 2007). However, others showed a less content in Brown Swiss milk (Benedet et al., 2020; Gibson et al., 2016). In addition, Gottardo et al., (2017) found a different milk fat acids profile between two breeds: Brown Swiss milk was higher in C14:0 and lower in C18:1 than Holstein. Another milk parameter well discussed in dairy literature between Brown Swiss and Holstein is the milk urea (i.e., MUN) content. As we know, MUN is strongly related with the blood urea (Baker et al., 1995; Broderick & Clayton, 1997; Burgos et al., 2007). These are parameters are useful for farmers, nutritionist, veterinarian, and researchers because are linked with the protein metabolism, rumen fermentation pathway (Bastin et al., 2009; Hof et al., 1997; Nousiainen et al., 2004) and energy-protein balance (Broderick & Clayton, 1997; Wattiaux & Karg, 2004). Most papers found higher urea in Brown Swiss milk than Holstein (Benedet et al., 2020; Bittante, 2022; Carroll et al., 2006; Doska et al., 2012; Kessler et al., 2020; Wattiaux et al., 2005). As well described above, we can find different studies on milk differences

between two breeds in literature and this is the most debated and discussed argument. In recent years, some authors started to better understand the difference adaptation to environmental challenges between different breeds, including Brown Swiss and Holstein. Studies investigated the different adaptation to heat stress especially during the summer season in different world areas, according with many authors (Correa-Calderon et al., 2004; El-Tarabany & El-Tarabany, 2015; Lacetera et al., 2006; Maggiolino et al., 2020). Brown Swiss seems to be more tolerant to heat stress than Holstein in different environments. Correa-Calderon et al., (2004) noted that Brown Swiss cows had a lower respiration rate and rectal temperature than Holstein in the same environmental conditions and Lacetera et al., (2006) found that immune functions under heat stress decrease more rapidly in Holsten than in Brown Swiss. Also with Italian data, Brown Swiss reveal a lower reduction in productive performance under heat stress (Maggiolino et al., 2020). For this reason, Maggiolino et al. (2020) proposed and higher THI breakpoints, 2/3 point over than Holstein cows, when the heat stress effects occur. In literature, few studies had investigated specific traits of the feeding behavior, feed efficiency and nutrition of Brown Swiss in comparison with Holstein. In dated American studies, Brown Swiss cows were less efficient in feed efficiency than Holstein (Dickinson et al., 1969). About dry matter intake (DMI), Gruber et al. (2014) pointed out that Brown Swiss cows eat less dry matter than Holstein when feed with the same diets in lactation, both as total daily dry matter intake for each cow both as dry matter intake referred to body weight. However, at dry period Brown Swiss cow had similar DMI than Holstein. Trend in DMI showed by Gruber et al., (2014) is in accordance with Carroll et al., (2006). Regarding feeding behavior, rumination time is an important parameter for the animal health status (Kaufman et al., 2018; Paudyal et al., 2018; Soriani et al., 2012, 2013). In 1970s, Welch et al. (1970) founded differences in rumination time between different breeds, but Brown Swiss wasn't included. Few studies compared the rumination time between two breeds. Some authors found that BS spent less time to ruminate during the day when both in the same farm conditions (Grodkowski et al., 2023) and in various farms (Braun et al., 2015b). However, these studies were performed on pasture-based system and without DMI individual detection. Braun et al., (2015b) evaluate the rumination time with a nose base sensor (MSR Electronics, Seuzach, Switzerland) in

different multi breeds farms trying to detect the “standard” rumination time for each breed. They found that probably Brown Swiss had less daily rumination time as specific breed trait. To the best of our knowledge, few studies in literature had discussed the different response to nutritional change in diets between Brown Swiss and Holstein. Probably no studies accurately investigated the nutrient utilization differences between two breed and the rumen environment. Carroll et al. (2006) found no breed differences when dietary fat increased in the diets on milk yield performance between Brown Swiss and Holstein. In addition, our literature research found few papers involving the immune metabolic profile and energy metabolism between the two breeds, especially in transition period and early lactation stage. Benedet et al., (2020) noted equal blood BHOB among Brown Swiss and Holstein in multi breeds farms. However Brown Swiss showed blood urea content and less NEFA. About immunology functions, Gibson et al., (2016) compared the macrophage function of Brown Swiss and Holstein and they found many differences. Brown Swiss macrophage produced more ROM when stimulated and they can better destroy live bacteria with higher uptake of bio-particle than Holstein. Moreover, Holstein macrophage produced more IL-1β as marker of the inflammasome activation than Brown Swiss. Author concluded that two breed had different killing bacteria capacity and inflammation response. This data is quit in agreement with results obtained by Lacetera et al., (2006) that worked on peripheral blood mononuclear cell: two breeds had different response under heat stress conditions. In conclusion, we found different studies of comparison of various traits between Brown Swiss and Holstein. Principally they are focused on milk quality and cheese yield attitude. These traits are well described by literature. On the other hands, few studied pointed out the differences in other aspect of the dairy cow breeding: nutrition, energy metabolism, feeding behavior, DMI capacity, immune functions, rumen environment and adaptation to environmental challenges. In addition, many studies have been performed in extensive farming system based principally on pasture and not in intensive farming system with confined cows, like the majority part of dairy cow’s breed in Italy, especially in northern Italy. Moreover, many of this trial are performed in commercial dairy farm where some information and data are missing or impossible to detects like

individual DMI or daily BW. These data could only be recorded with specific technologies that are mainly present in dairy research center.

2 Anti nutritional substances effect

2.1 Mycotoxins

Among anti-nutritional substance can be ingested by dairy cows there are mycotoxin. Mycotoxins are a heterogeneous group of secondary metabolites produced by many filamentous fungi belonging the genera *Fusarium*, *Aspergillus* and *Penicillium* that can cause toxic responses when ingested by humans and other vertebrates (Fink-Gremmels, 2008; Zinedine et al., 2007). Moreover, other fungi genera can include mycotoxigenic fungi like *Alternaria*, *Chaetomium*, *Cladosporium*, *Claviceps*, *diplodia*, *Myrothecium*, *Monacus*, *Phoma*, *Phomopis*, *Pithomyces*, *Trichoderma* and *Stachybotrys* (Bryden, 2012; Nielsen et al., 2006). Mycotoxins are generally stable in animal feeds, and they can originate from forage cultivation (Cheli et al., 2013; Gallo et al., 2015; Kabak et al., 2006). A worldwide survey of occurrence of mycotoxins found that 81% of the 7049 livestock feed samples collected in America, Europe and Asia contained at least one mycotoxin (Rodrigues & Naehrer, 2012). Many studies in literature had described the toxicological effects of mycotoxins in farm animals (Riley & Pestka, 2005; Fink-Gremmels & Malekinejad, 2007; Pestka, 2007; Voss et al., 2007; Antonissen et al., 2014). The most studied mycotoxins are regulated mycotoxins: aflatoxins (AFs), citrinin, trichothecenes such as dexynivalenol (DON), patulin, ochratoxin A (OTA), fumonisins (FBs) and zerealenone (ZEN). Fungi can grow in forages, in particular silage and hay (Drejer Storm et al., 2014; Keller et al., 2013; Skládanka et al., 2011; Storm et al., 2008). Forages can contaminate diets especially with mycotoxin from *Aspergillus*, *Fusarium* and *Alternaria* fungi.

2.2 Fusarium mycotoxins: DON, ZEN, and FBs

Most of the investigated and discussed *Fusarium* mycotoxin in literature for dairy cows are DON, ZEN, and FBs (Gallo et al., 2022). They are more debated in different studies than another minor fusarium mycotoxin like T-2/HT-2 and beavericin.

The DON, also known as vomitoxin, is a type B trichothecene with a carbonyl group at C-8 position that can disrupt ekaryotic protein biosynthesis (Tittlemier et al., 2019). It is primally produced by *F. graminearum* (*Gibberellazeae*), *F culmorum*, *F. nivale*, *F. poae*, *F. roseum* and *F. tricintum*. DON had two less toxic forms: 3-acetyl-deoxynivalenol (3-Ac-DON) and 15-acetyl-deoxynivalenol (15-Ac-DON). Trichothecenes impacted the health status of animals in many ways, including reduced productivity, weight loss, diarrhea, drop in dry matter intake (Foroud et al., 2019; Wegulo, 2012a). Mean, the 7/10% of the DON ingested is adsorbed in ruminants (Payros et al., 2016) In the Rumen, the DON is degraded in its derivate, the de-epoxy-DON (DOM-1) (King et al., 1984) We can primally detect DON and its metabolites in feces, but a little part is also present in urines. (Larsen et al., 2004). After absorption, DON and its metabolites are conjugation with glucuronic acid that increases the water solubility and then they are excreted by urines and feces (by bile; Gamage et al., 2006).

ZEN is a mycotoxin mainly produced by *F-graminearum, F-roseum, F.culmorum* and *F.crook-wellense*. It is a resorcyclic acid lactone structure, like estradiol, particularly the sex-hormone 17 β-estradiol (E2). Due to is structure and conformation, ZEN is capable to bind estrogenic receptors (Riley & Pestka, 2005). The effects and adsorption of ZEN range between species (Catteuw et al., 2019). In ruminant the rumen microbiota degrades ZEN in two metabolites: α-zearalenol (α-ZEL) and β -ZEL (Knutsen et al., 2017). The effect of each ZEN metabolite is different. α-ZEL is 60 times more potent than ZEN while the potency β -ZEL is one fifth of that ZEN. The proportion between α and β ZEL changes in different *in vivo* and *in vitro* studies. *In vitro* studies α-ZEL seems to be more relevant than β ZEL (Debevere et al., 2020). However *in vivo* studies found more β ZEL than α-ZEL (Knutsen et al., 2017). Many studies on ZEN concentration ant its metabolites in urine, feces and bile found more β ZEL, followed by ZEN and then α-ZEL (Dänicke et al., 2014;

Mirocha et al., 1981). Females are more sensitive to ZEN than males (Kuiper-Goodman et al., 1987). In the urine excretion, most of ZEN and its metabolites were glucuronide conjugate (Mirocha et al., 1981)

The FBs are secondary metabolites of *F.verticillioides*, *F. proliferatum*, *F. anthophilum* and *F. nygamai*. They are a C-20 or C-19 long chain ammino-polyol backbone with two methyl groups on the backbone and two propane-1,2,3-tricaboxylic acid (TCA) and the side chains are esterified to hydroxy groups at positions C14 and C15. In feed commodities we principally found fumonisins B1 (FB1), fumonisins B2 (FB2), fumonisins B3 (FB3) and fumonisins B4 (FB4) (Knutsen, Alexander, et al., 2018). The backbone of group fumonisins B is like the sphinganine (Sa) and sphingosine (So). So, FBs are toxic because it interacts into the disruption of the sphinolipid metabolism (Knutsen, Barregård, et al., 2018) Ruminants are less sensitive than other animals (Kemboi et al., 2020), because these molecules are poorly adsorbed. The adsorbed fractions are rapidly distributed in the liver and excreted via feces. However, FBs, when adsorbed at the same time, are rapidly eliminated by the organism (Knutsen, Alexander, et al., 2018). To confirm this rapid flow, in plasma from cow that received FB1 orally, FB1 plasma concentration were no detectable (Prelusky et al., 1995). FB could be detected in tissues after hydrolyzation as HFB1 which is less toxic (K. A. Voss et al., 2007). FB and its metabolites are mainly excreted via feces and lesser via urine in cattle (Rice & Ross, 1994).

2.3 Effect on productive performance

Most of the trials with fusarium mycotoxin contamination have analyzed together the effects on cattle of many *Fusarium* mycotoxin (DON, ZEN and FB). The majority part of studies concerning the interaction of mycotoxins with other toxins on living organism are mainly focused on monogastric (Grenier et al., 211). Many times, in cow's feed contaminated by fusarium mycotoxin we can find a co-existence of ZEN, DON and FBs contamination (Pinton & Oswald, 2014). For this reason, most of the literature related to fusarium mycotoxin effects on cattle took into consideration the simultaneous contamination effects of ZEN, DON, and FBs. However, in same trials one of the 3

previous cited mycotoxins could be more present than others, so the effects investigated related to this major contamination. In literature, many studies investigated the effects of fusarium mycotoxin on performances of beef and dairy cattle, and, in addition, to other ruminants. Many trials pointed out modifications of the diet digestibility and rumen environment with lower DM and NDF digestibility in dairy cows (Gallo et al., 2020a), protein digestibility (Gallo et al., 2020a) and altered rumen Ph in beef cattle (Duringer et al., 2020) and lower gas production in vitro (Gallo et al., 2021) by DON and FB contamination at different levels. Milk yield seems to be affected by DON and FB contamination with a production decrease (Gallo et al., 2020b; Seeling et al., 2006) and decrease in fat content (Gallo et al., 2020b; Seeling et al., 2006). Some studies, analyzing the performance effects of fusarium mycotoxin, took also into consideration the variation on immune metabolic cow status. Theu found an increase in liver enzymes (Hartinger et al., 2022) and transaminases (Gallo et al., 2020a) and decrease of total leukocyte count (140). Many studies confirm that DON could cause an oxidative stress status both in vitro (da Silva et al., 2014) and in vivo trails (Osselaere et al., 2013; Zhao et al., 2022). Moreover, ZEN, combined to FBs, seems to alter the rumen environment (Hartinger et al., 2022).

2.4 Effects on reproductive performance

Considering fusarium mycotoxin, ZEN is probably the most investigated mycotoxin on reproductive effects in literature. This is due to the ZEN capacity to bind estrogenic receptors as an agonist and cause clinical/reproductive effects. In addition, ZEN metabolites, α-ZEL and β-ZEL interfere with hormonal-related effects in steroid metabolism. The hydroxysteroid dehydrogenases enzymes (HSD) plays an important role in the estrogen and testosterone hormones synthesis in many steroidogenic tissues (Olsen, 1989). ZEN is a competitive substrate of 3α-HSD and 3 β-HSD and can cause an accumulation of active components. This can cause a variation of cholesterol conversion in testosterone and progesterone modifying the sexual behavior and ovarian follicular development (Olsen, 1989). Many *in vivo* studies have investigated the effects of ZEN on cattle reproduction. ZEN feed contamination seems to decrease the oocyte quality and

cause abortion in dairy heifer (Silva et al., 2021). In bulls, ZEN can decrease the testicular weight (Dänicke et al., 2002) and in cows increases the ovarian antral follicle population (Fushimi et al., 2015). DON is not an estrogenic toxin, but it can cause ribotoxic stress in cells, including reproductive cells. In literature, many *in vitro* studies confirm this effect as described below. However, in *in vivo* trials, DON affects the reproduction system with a decrease in cow follicular size (Guerrero-Netro et al., 2021). More studies were performed in literature in vitro trials to better understand the reproductive effect of fusarium mycotoxin contamination in comparison with in vivo trials. In vitro trials are more common because reproductive cells are complex structure and more suitable for in vitro studies to evaluate their development over the time (Santos et al., 2013). Many trials were performed in granulosa cells to evaluate the toxin's effects. Frequently, reproductive cells were collected from small ovarian follicles (1-7mm) (Li et al., 2020; Pizzo et al., 2016; Yang et al., 2019). Some authors find that DON can decrease the progesterone secretion in Theca cells (Guerrero-Netro et al., 2021). In granulosa cells DON seems to decrease both estradiol and progesterone secretion (Guerrero-Netro et al., 2015; Pizzo et al., 2015, 2016). Moreover, in the same cells, ZEN metabolites increase the estradiol secretion (Minervini & Aquila, 2008; Pizzo et al., 2016) and decrease progesterone (Pizzo et al., 2016). In addition, they increase the oxidative stress (Li et al., 2020; Yang et al., 2019). In cumulus-oocyte complexes ZEN stalls the oocyte maturation (Minervini & Aquila, 2008; Takagi et al., 2008).

2.5 Mitigate the mycotoxin contamination effects in diets: Preventing field contamination and detoxification.

Good agriculture practices and good manufactures practices can help farmer, breeders and technicians to minimize the mycotoxin contamination in ruminant diets at the field level. Wegulo (Wegulo, 2012b) reported many practices to mitigate the field contamination: cultivate crop resistance plants, sowing at correct time, using fungicides, avoiding damage to kernel, culture rotation, ploughing the crop residues and others. Some authors evaluated

the effective technical and economic convenience of some of the previous practices described to mitigate fusarium mycotoxin contamination (Zorn et al., 2017). Excess of precipitation or irrigation at flowering stage increases the *Fusarium* spp. infection risks. Good storage practices can help to mitigate the contamination. When the field and storage practices are insufficient to prevent mycotoxin contamination, the last chance to mitigate the negative effects is the detoxification. It can be carried out by breeders via dietary supplementation with mycotoxin decontaminants. Many of these products are usually called mycotoxin mitigating products (MMP) or mycotoxin deactivation products (MDP). Unfortunately, fusarium mycotoxins are resistance to processing before dietary uses, for DON (Mishra et al., 2014), ZEN (Ryu et al., 2003), and FBs (Bryła et al., 2017), except to high temperature nearly to 200 C°. Some grains processing methods can reduce the contamination, like extrusion cooking (Cazzaniga et al., 2001; Ryu et al., 1999; K. Voss et al., 2017; Wu et al., 2017), hulling and milling (Cheli, Pinotti, et al., 2013; Lee et al., 1987), ozonation (Grenier et al., 2014; McKenzie et al., 1997) and hydrothermal treatment (Scott, 1998). At dietary levels detoxification can be performed or via adsorption (adsorbents) or biotransformation before gut absorption (enzymes-based additives). Most of the adsorbents contains organoclays that can bind mycotoxin during the digestive process reducing the toxin availability. However, the binding capacity isn't so specific, and they are not so efficient for fusarium mycotoxins like DON, ZEN, and FBs (Colović et al., 2019). On the other hand, biotransformation is highly specific and irreversible. Mycotoxins are transformed into less toxic or non-toxic molecules by enzymes or microorganisms. A sufficient moisture (>20%) during the storage must be performed to ensure the enzymes stability. Besides these, mitigating products also usually contains antioxidants to minimize the negative impact of mycotoxin.

Tables and figures are insert though the text of each chapter. Only in the chapter number 2 tables of results and figures are reported at the end due to the large number of parameters evaluated in over two years of trial.

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Chapter 1

Metabolic and inflammatory responses reveal different adaptation to the transition period challenges in Holstein, Brown Swiss, and Simmental dairy cows.

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Abstract

Twelve Holstein (HOL), 9 Brown Swiss (BRO), and 9 Simmental (SIM) dairy cows raised in the same barn and managed identically were enrolled to characterize the responses to the transition period. From –21 to 28 days from calving (DFC), body condition score (BCS) and milk yield were measured. Blood samples were collected to assess plasma biomarkers of metabolism, minerals, liver function, inflammation, and oxidative status. Compared with SIM, HOL and BRO had higher milk yield during the first week of lactation. HOL had the highest milk yield from the second to the fourth week and the lowest BCS at 28 DFC. SIM had the highest BCS and the highest plasma creatinine and P, reflecting a greater attitude to gain muscle mass. Compared with SIM, BRO and HOL had lower plasma fructosamine, indicating reduced glucose availability driven by lactose synthesis. SIM had the mildest acute phase response at the onset of lactation, as suggested by the lowest ceruloplasmin concentration. HOL had the highest cholesterol and paraoxonase concentrations, and the greatest interleukin-1β production by leukocytes following ex-vivo stimulation, suggesting that they had the quickest resolution of the acute phase response due to the fastest immune cell activation. BRO had the highest concentration of reactive oxygen metabolites, ceruloplasmin, bilirubin, glutamate-oxaloacetate transaminase, γ-glutamyl transferase, and β-hydroxybutyrate, and the lowest concentration of paraoxonase, reflecting the strongest activation of leukocytes and the most severe acute phase response. Together, these results highlight different metabolic and inflammatory conditions around calving in the three explored breeds.

Key words: *acute phase response, liver function, metabolism, milk yield, peripartum*

1. Introduction

The physiological adaptation of dairy cows to the transition period challenges has become a widely debated topic over the last decades (Grummer 1995; Drackley 1999; LeBlanc 2010). In this context, the pivotal role played by the immune system in affecting the success of such adaptation has been recently recognized (Bertoni and Trevisi 2013; Mezzetti et al. 2020; Cattaneo et al. 2021). Acute phase response has been documented as a physiological condition in dairy cows during the transition period (Van Knegsel et al. 2014; Premi et al. 2021), although the magnitude and duration of this process varies markedly among individuals, deeply affecting performances at the onset of lactation. During the acute phase, liver synthesizes positive acute phase proteins (APP; i.e. haptoglobin and ceruloplasmin) (Ceciliani et al. 2012), decreasing the production of other proteins referred to as negative APP (i.e. albumin, lipoproteins, retinol binding protein and paraoxonase) (Bionaz et al. 2007; Bertoni et al. 2008).

Most of the research exploring this topic focuses on Holstein Friesian as a model for the modern high-yielding cow. Despite that, dairy breeders all over the world manage various breeds selected to maximize different production traits (Back et al. 2006; De Marchi et al. 2008; Gustavsson et al. 2014; Bland et al. 2015) or adapted to face different environmental conditions (White et al. 2002; Piccand et al. 2013; Curone et al. 2018). Besides Holstein (HOL), Brown Swiss (BRO) and Simmental (SIM) are among the most widespread cosmopolitan dairy breeds, raised particularly for their milk composition traits and muscle gain, respectively. Literature documents large variability among these breeds in their adaptive response to environmental stressors (Mylostyvyi et al. 2021), reproductive performances (Piccand et al. 2013), feed efficiency (Dickinson et al. 1969), metabolism and milk composition (Benedet et al. 2020; Franzoi et al. 2020). Conversely, little is known about the immune system of the different breeds (Curone et al. 2019), and existing results were mostly obtained through ex-vivo approaches. According to Lacetera et al. (2006), BRO peripheral blood mononuclear cells are less tolerant to chronic heat exposure than those from HOL, suggesting a different decline in immune functions between these two breeds when exposed to high temperatures. Compared with HOL, BRO cows have greater resistance to bacterial infections, and their macrophages produce more reactive oxygen

species (ROS) and less interleukin (IL)-1β, after a challenge stimulation with bacterial lipopolysaccharides (LPS) (Gibson et al. 2016).
To the best of our knowledge, studies comparing the adaptive metabolic responses of different dairy breeds at calving are lacking. We hypothesized that dairy breeds could have different haematochemical profile during the transition period, likely driven by the different breeding objectives and milk yield among them. Thus, this observational study aimed at exploring the metabolic and inflammatory adaptations during the transition period in HOL, BRO, and SIM dairy cows managed identically.

2. Material and Methods

The study was carried out in a commercial dairy farm located in the Parmigiano Reggiano production area (Terenzo, Parma, Italy), from November 2017 to May 2018, in accordance with Italian laws on animal experimentation (DL n. 116, 27/01/1992) and ethics (authorization of the Italian Ministry of Health N°851/2018).

2.1 Experimental design and animal management

The herd included HOL, BRO, and SIM dairy cows raised together, under the same management practices. Cows were dried off 60 days before the expected calving and housed in a bedded-pack pen until calving. Thereafter, cows were moved to a postpartum bedded-pack pen for two weeks and finally moved to a lactation freestall pen, with cubicles bedded with straw. Lactating cows were milked twice daily at 4.00 am and pm.
All the cows were fed the same TMR diet formulated to satisfy the average herd requirements, according to the "Parmigiano Reggiano" production regulation, and distributed twice daily for lactating cows (at 6.00 am and pm) and twice weekly for dry cows. A 3–5% refusal was guaranteed to ensure that cows had ad libitum access to feed. Representative samples of TMR were collected monthly. Samples were ground and characterized by a Foss NIR systems 5000 spectrophotometer (Hillerød, Denmark) equipped with a monochromator and transport module, scanning over the wavelength range 1100-2500 nm every 2 nm. The calibrations used to obtain the forage characterizations

were produced by a commercial forage testing laboratory (CRPA Lab, Reggio Emilia, Italy), and samples were analysed for crude protein, NDF, ether extract, starch, and sugar. Analysis results were used to calculate the nutritional value of the feed, in accordance with NRC (2001) guidelines. Diet composition is reported in Table 1.

A group of 30 parous dairy cows was enrolled in the experiment after the dry off as follows: 12 HOL (parity: 2.7 ± 0.8, BCS: 3.25 ± 0.30, average milk production previous lactation: 11,000 ± 585 kg [mean ± SD]); 9 BRO (parity: 3.0 ± 0.5, BCS: 3.25 ± 0.31, average milk production previous lactation: 9,484 ± 354 kg); and 9 SIM (parity: 3.5 ± 2.1, BCS: 4.0 ± 0.38 average milk production previous lactation: 9,858 ± 405 kg). Subjects in each group were chosen to represent the proportion of each breed within the herd, according also to production relative to herd and breed average. Periodical checks were performed between −21 and 28 days from calving (DFC), as described in the following sections.

2.2 Body Condition Score and Milk Yield

The BCS was determined by the same operator using a 1 to 5 scale (Edmonson et al. 1989) at −21 ± 2, 3, 7, and 28 DFC. The ΔBCS was calculated as the difference between BCS values measured at 3 and 28 DFC. The milk yield was automatically measured in the milking parlour using the Afikim system (SAE Afikim, Kibbutz Afikim, Israel) at each milking between 1 and 30 DFC. Data about milk yield were expressed as average weekly values.

2.3 Health Status

Health status was monitored daily from −21 to 30 DFC. Hoof health was assessed daily by the breeder. Mastitis was diagnosed by visual evaluation of abnormal milk from each quarter. Retained placenta was recorded when foetal membranes were not expelled within 24 h after calving. Endometritis and metritis were diagnosed by a veterinary practitioner through rectal ultrasonography according to Sheldon et al. [24]. Milk fever, displacement of the abomasum, and ketosis were diagnosed by veterinarian examination.

Table 1. Diets offered to the cows during the study

Item[1]	Lactating cows	Dry cows
Diet, % DM		
Grass hay	25.1	50.0
Alfalfa hay	25.0	–
Straw	–	20.7
Corn grain ground dry	19.1	–
Corn grain flaked	5.12	3.51
Barley flaked	4.16	–
Soybean meal	–	4.97
Soybean flaked	3.50	–
Beet molasses	2.73	2.94
Flaxseed	1.81	–
Flaxseed meal	3.54	3.64
Beet sugar pulp dry	1.39	–
Soy hulls	1.41	10.8
Supplement[2]	1.88	3.0
Chemical Composition		
NE_L, Mcal kg of DM^{-1}	1.59	1.30
Crude protein, % DM	15.1	12.3
Starch + sugar, % DM	27.8	17.9
Ether extract, % DM	3.28	2.50
NDF, % DM	34.8	49.6
MP, % CP	10.7	8.50
RUP, % DM	5.50	3.80

[1] DM is dry matter; NE_L is net energy for lactation; NDF is neutral detergent fiber; CP is crude protein; MP is metabolizable protein and RUP is rumen undegradable protein (both estimated using NRC 2001)

[2] Lactating cows' supplement contained 15,5% Ca, 2,8% P, 9% Na, 7,5% Mg, 400,000 IU/kg of vitamin A, 100,000 IU/kg of vitamin D, 2.000 mg/kg of vitamin E, 50 mg/kg of vitamin K, 110 mg/kg of vitamin B1, 1.2 mg/kg of vitamin B12, 4.200 mg/kg of vitamin PP, 5.200 mg/kg of choline, 3.350 mg/kg of Mn, 7.500 mg/kg of Zn, 2.400 mg/kg of Cu, 400 mg/kg of I, 30 mg/kg Se.

Dry cows' supplement contained 3,5% Ca, 4,0% P, 1,5% Na, 5,0% Mg, 200,000 IU/kg of vitamin A, 50,000 IU/kg of vitamin D, 2.800 mg/kg of vitamin E, 100 mg/kg of vitamin K, 350 mg/kg of vitamin B1, 3 mg/kg of vitamin B12, 3.500 mg/kg of vitamin PP, 4.500 mg/kg of choline, 1.000 mg/kg of Mn, 2.800 mg/kg of Zn, 150 mg/kg of Cu, 100 mg/kg of I, 12 mg/kg Se.

2.4 Blood sample collection and analysis

At −21 ± 2, 3, 7, and 28 DFC, before the morning feeding, blood was collected from the jugular vein into 10-mL evacuated heparinized tubes (BD Vacutainer, BD Diagnostics, Franklin Lakes, New Jersey, United States). After collection, blood was processed and analysed as described by Calamari et al. (2016). Briefly, blood was centrifuged (3500×g, 15 min at 4°C), and the packed cell volume (PCV) was directly measured after centrifugation through a capillary column (ALC Centrifugette 4203). A clinical autoanalyzer (ILAB-650, Werfen – Instrumentation Laboratory, Bedford, MA, United States) was used to measure the concentration of glucose, nonesterified fatty acids (NEFA), β-hydroxybutyrate (BHB), urea, creatinine, Ca, P, Mg, Na, K, Cl, Zn, haptoglobin, ceruloplasmin, total protein, albumin, globulin, cholesterol, total bilirubin, aspartate aminotransferase (AST-GOT), γ-glutamyl transferase (GGT), and alkaline phosphatase according to Calamari et al. (2016). Furthermore, the same instrument was used to determine reactive oxygen metabolites (ROMt) and ferric ion reducing antioxidant power (FRAP) according to Jacometo et al. [28], paraoxonase according to Bionaz et al. [29], thiol groups according to Minuti et al. [30], myeloperoxidase according to Bradley et al. [31], fructosamine according to Caré et al. [33]. Moreover, IL-1β and IL-6 according to Mezzetti et al. (2019). Further details on the analytical procedures adopted for blood analyses are reported in Table S1.

Whole blood stimulation assay. At 3 DFC, an additional sample was collected in heparinized tubes under aseptic conditions and processed according to Jahan et al. (2015), with some modifications. Briefly, a 5 µg/mL solution of Escherichia coli LPS (O111:B4, Sigma Aldrich Company Ltd., UK, Cat. No. L3012) and Dulbecco's modified Eagle medium (Sigma Aldrich Company Ltd., UK, Cat. No. D6046) was injected through the rubber cap of the tube using a 1-mL syringe. After injection, the tubes were gently inverted 10 times and placed in a water bath at 38°C for 3.5 h. After the whole blood stimulation assay (WBA), the plasma was collected by centrifugation at 3500×g for 16 min at 4 °C and stored at −80 °C for IL-1β and IL-6 measurements. After the WBA, variations in the plasma cytokines were expressed as the fold change relative to the baseline.

2.5 Statistical analysis

Data were analysed using SAS software, version 9.4 (SAS Inst. Inc., Cary, NC, USA) and are presented in graphs and tables as the least squares mean and pooled standard error for individual means of breeds over time. Data underwent ANOVA testing using a mixed models for repeated measures (Glimmix Procedure, SAS Inst. Inc.). The statistical models included the fixed effect of breed (Br), time (DFC for BCS and plasma analytes, weeks from calving for milk yield), their interaction (Br × Time), and the random effect of the individual cow. For WBA, the model included only Br as fixed effect. Parity was firstly included in the models as a covariate but was not retained because of the lack of significance for the great majority of the variables investigated. The analysis was carried out using two covariance structures: autoregressive order and spatial power, with their heterogeneous counterparts. The covariance structures were ranked according to their Akaike information criterion, with the one having the lowest criterion being chosen (Littell et al. 1998). Distribution of residuals was visually assessed. The pairwise comparison was done using the least significant difference test with the Tukey adjustment for multiple comparisons. Significance was declared $p \leq 0.05$, and differences for $p \leq 0.1$ were discussed in the context of tendencies.

3. Results

3.1 Body condition score, milk yield, and health status

The BCS was higher in SIM compared with the other breeds (3.56 vs 2.95 and 3.08 ± 0.10 for SIM, HOL, and BRO, respectively; $p < 0.05$ for both HOL and BRO), and a Br x Time interaction was also observed ($p = 0.04$, Figure 1a). Compared with the other breeds, SIM had the highest BCS at -21, 3 (3.61 vs 3.00 vs 2.99 ± 0.12 for SIM, HOL, and BRO, respectively), and 7 DFC ($p < 0.01$ for both HOL and BRO), and HOL had the lowest BCS at 28 DFC (3.22 vs 2.71 vs 3.02 ± 0.12 for SIM, HOL, and BRO, respectively; $p < 0.05$ and $p < 0.01$ for BRO and SIM, respectively). After calving, BCS remained unchanged in BRO, thus the ΔBCS was lower in BRO compared with the other breeds (0.00 vs. 0.29 and 0.39 ± 0.09 for BRO, HOL, and SIM, respectively; $p < 0.05$ for both HOL and SIM). Milk yield

tended to be higher in HOL than in the other breeds (37.7 vs 32.6 and 31.9 ± 1.85 L/d in HOL, BRO, and SIM, respectively; $p < 0.1$ for both BRO and SIM), and a Br x Time interaction was also observed ($p < 0.01$, Figure 1b). Compared with other breeds, SIM had the lowest milk yield during the first week of lactation ($p < 0.05$ for both HOL and BRO), whereas HOL had the highest milk yield during the second, third, and fourth weeks of lactation ($p < 0.05$ for both BRO and SIM). Disease incidence was similar among breeds (Table S2).

3.2 Metabolic Profile

Energy and protein metabolism biomarkers, kidney function indicators, and mineral metabolism biomarkers.

Among energy metabolism biomarkers, fructosamine concentration was higher in SIM than in HOL and tended to be higher in SIM than in BRO ($p < 0.05$ and $p = 0.1$, respectively; Table 2). Plasma BHB concentration was higher in BRO than in HOL ($p = 0.03$, Table 2). No effect was detected for PCV, glucose, and NEFA (Figure S1a-e). Among the protein metabolism and kidney function biomarkers, creatinine concentration was higher in SIM than in HOL and tended to be higher in SIM than in BRO ($p < 0.01$ and $p = 0.1$ for HOL and BRO, respectively; Table 2). No effect was detected for urea (Figure S1f). Among the mineral metabolism biomarkers, P concentration was highest in SIM ($p < 0.05$ and $p < 0.01$ for HOL and BRO, respectively; Table 2), and a Br x Time interaction was also observed ($p = 0.02$, Figure 2a). Compared with the other breeds, SIM had the highest P concentration at 3 ($p = 0.02$ and $p = 0.03$ for BRO and HOL, respectively) and 7 DFC ($p < 0.01$ for both BRO and HOL). The P concentration was higher in HOL than in BRO at 28 DFC ($p < 0.05$). No effect was detected for the other minerals (Figure S2).

Liver function and inflammation biomarkers.

Among the liver function biomarkers, total bilirubin concentration was higher in BRO than in HOL cows ($p = 0.04$; Table 2), and a Br x Time interaction was also observed ($p < 0.01$; Figure 2b). Compared with the other breeds, BRO had the highest bilirubin concentration at 3 DFC ($p < 0.01$). The AST-GOT and GGT concentration was the highest in BRO ($p = 0.01$ for AST-GOT and $p < 0.05$ for GGT for both HOL and SIM, Table 2), and a tendency

towards a Br x Time interaction was also observed for AST-GOT ($p = 0.09$; Figure 2c). Compared with the other breeds, BRO had the highest AST-GOT concentration at 3 ($p < 0.01$ for both HOL and SIM) and 7 DFC ($p < 0.05$ for both HOL and SIM). No effect was detected for the alkaline phosphatase concentration (Figure S3b). Among the inflammation biomarkers, total protein concentration was the lowest in BRO ($p < 0.05$ and $p < 0.01$ for HOL and SIM, respectively) and tended to be higher in SIM than in HOL ($p < 0.1$; Table 2). No effect was detected for myeloperoxidase, IL-1β, IL-6, and globulin (Figure S3e; S4a-b). Among the positive APP, ceruloplasmin concentration was the highest in BRO ($p < 0.01$ for both HOL and SIM) and was higher in HOL than in SIM ($p = 0.04$; Table 2). A Br x Time interaction was also observed ($p < 0.01$; Figure 2d). Compared with BRO, SIM had lower ceruloplasmin at -21 DFC ($p = 0.04$), and both SIM and HOL had lower ceruloplasmin at 3, 7, and 28 DFC ($p \leq 0.01$). Compared with SIM, HOL had higher ceruloplasmin at 3 DFC ($p = 0.01$). No difference was observed for haptoglobin concentration (Figure S3f). Among the negative APPs, cholesterol concentration was higher in HOL than in SIM and tended to be higher in HOL than in BRO ($p < 0.05$ and $p = 0.08$, respectively; Table 2). Plasma paraoxonase concentration was higher in HOL than in BRO ($p = 0.05$; Table 2), and a tendency towards a Br x Time interaction was also observed ($p = 0.06$; Figure 2e). At 7 DFC, paraoxonase concentration was higher in HOL than in BRO ($p < 0.01$) and tended to be higher in HOL than in SIM ($p = 0.09$) and in SIM than in BRO ($p = 0.07$). No effect was noted for albumin concentration (Figure S2d).

Redox Balance Biomarkers.

Among the antioxidant systems biomarkers, no effect was detected for thiol groups and FRAP concentrations (Figure S3e-f). Among the oxidant species biomarkers, ROMt concentration was the highest in BRO ($p = 0.04$ for both HOL and SIM; Table 2), and a Br x Time interaction was also observed ($p = 0.02$; Figure 2f). Compared with the other breeds, BRO had the highest ROMt concentration at 3 ($p < 0.01$ for both HOL and SIM) and 7 DFC ($p < 0.05$ and $p < 0.01$ for HOL and SIM, respectively).

Table 2. Plasma concentrations from -21 to 28 days from calving of biomarkers of metabolism, minerals, liver function, inflammation, and oxidative status in Holstein (HOL), Brown Swiss (BRO), and Simmental (SIM) dairy cows managed identically

	Breed[2]				*p*-value[4]		
Item[1], unit	HOL	BRO	SIM	SEM[3]	Br	Time	Br x Time
PCV, L/L	0.343	0.336	0.360	0.010	0.22	<0.01	0.38
Metabolism							
Glucose, mmol/L	4.14	4.05	4.21	0.08	0.39	<0.01	0.16
Fructosamine, µmol/L	295^{b}	299^{ab}	311^{a}	4.30	0.03	<0.01	0.32
NEFA, mmol/L	0.45	0.51	0.53	0.06	0.60	<0.01	0.88
BHB, mmol/L	0.44^{b}	0.56^{a}	0.49^{ab}	0.03	0.04	<0.01	0.14
Urea, mmol/L	4.06	4.38	4.33	0.33	0.72	<0.01	0.18
Creatinine, µmol/L	95.5^{b}	99.4^{ab}	108.3^{a}	2.31	<0.01	<0.01	0.87
Minerals							
Ca, mmol/L	2.49	2.49	2.47	0.03	0.88	<0.01	0.68
P, mmol/L	1.73^{b}	1.61^{b}	1.92^{a}	0.07	0.01	<0.01	0.02
Mg, mmol/L	0.96	0.92	0.97	0.02	0.25	<0.01	0.18
Na, mmol/L	146	147	146	0.73	0.72	<0.01	0.68
K, mmol/L	4.31	4.21	4.26	0.07	0.56	0.34	0.85
Cl, mmol/L	106	107	106	0.71	0.36	<0.01	0.81
Zn, µmol/L	11.4	12.1	11.3	0.49	0.44	<0.01	0.22
Liver function							
Bilirubin, µmol/L	3.38^{b}	5.28^{a}	3.95^{ab}	0.56	0.04	<0.01	<0.01
AST-GOT, U/L	101.6^{a}	150.8^{b}	90.3^{a}	14.6	0.01	<0.01	0.09
GGT, U/L	22.0^{a}	37.2^{b}	19.6^{a}	5.12	0.04	0.39	0.70
Alkaline phosphatase, U/L	59.4	47.9	56.2	8.42	0.58	0.02	0.11
Inflammation							
Total protein, g/L	79.7^{a}	76.3^{b}	82.5^{a}	1.16	<0.01	<0.01	0.88
Albumin, g/L	35.0	34.7	36.2	0.61	0.20	0.01	0.38
Globulin, g/L	44.6	41.6	46.2	1.49	0.10	<0.01	0.91
Haptoglobin, g/L	0.40	0.44	0.39	0.05	0.76	<0.01	0.30
Ceruloplasmin, µmol/L	2.65^{a}	3.33^{b}	2.37^{c}	0.10	<0.01	<0.01	<0.01
IL-1β, pg/mL	309	1212	247	488	0.30	0.06	0.23
IL-6, pg/mL	592	1094	458	242	0.16	0.02	0.22
Myeloperoxidase, U/L	431	478	448	19.4	0.21	0.02	0.41
Cholesterol, mmol/L	3.91^{a}	3.40^{ab}	3.20^{b}	0.22	0.04	<0.01	0.86
Paraoxonase, U/mL	84.2^{a}	67.4^{b}	73.2^{ab}	5.20	0.05	<0.01	0.06
Oxidative status							
Thiol groups, µmol/L	313	318	334	13.9	0.51	0.31	0.72
FRAP, µmol/L	176	155	153	20.9	0.64	0.29	0.74
ROMt, mg H_2O_2/100 mL	20.7^{b}	23.9^{a}	20.1^{b}	1.06	0.03	<0.01	0.02

[1] PCV is packed cell volume, NEFA is nonesterified fatty acids, BHB is β-hydroxybutyrate, AST-GOT is glutamate-oxaloacetate transaminase, GGT is γ-glutamyl transferase, IL-1β is interleukin-1β, IL-6 is interleukin-6, FRAP is ferric ion reducing antioxidant power, ROMt is total reactive oxygen metabolites.

[2] Within a row, means without a common superscript differ for $p < 0.05$.

[2] Standard error = greatest standard error of the means among breeds

Table 3. Fold changes of cytokines following a whole blood stimulation assay from blood collected 3 days after calving in Holstein (HOL), Brown Swiss (BRO), and Simmental (SIM) dairy cows managed identically

	Breed[1]				
Item	HOL	BRO	SIM	SEM[2]	*p*-value
Interleukin-6	3.63	2.86	2.95	0.64	0.55
Interleukin-1β	45.3[a]	16.9[b]	15.2[b]	9.3	0.01

[1] Within a row, means without a common superscript differ for $p < 0.05$.
[2] SEM = greatest standard error of the means among breeds

Figure 1.

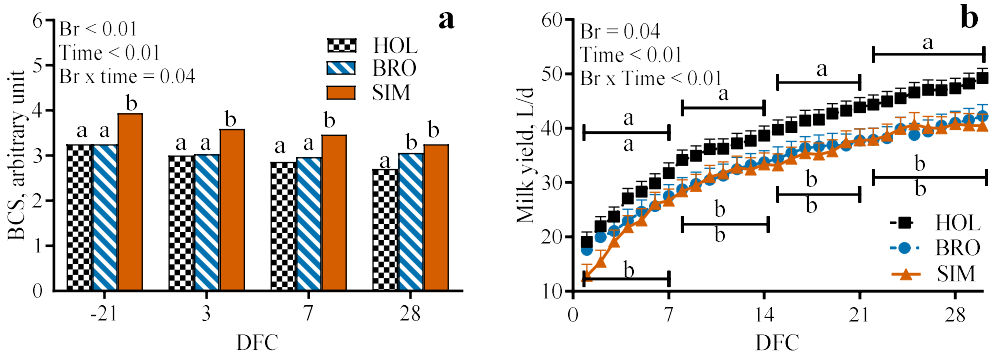

Figure 2.

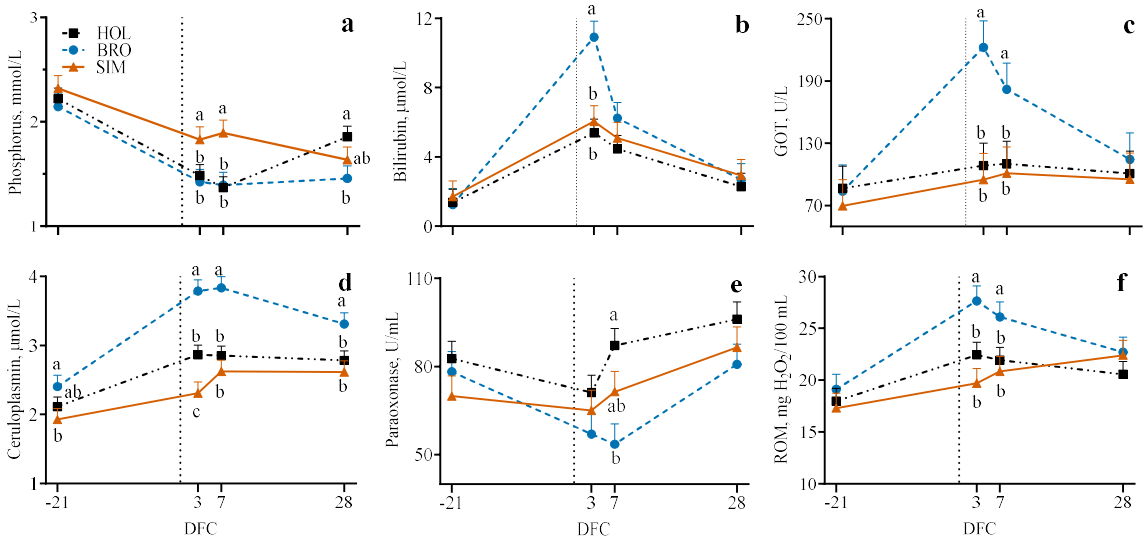

Figure captions

Figure 1. Values of body condition score (BCS; **a**) and milk yield (**b**) from -21 to 28 days from calving (DFC) in Holstein (HOL), Brown Swiss (BRO), and Simmental (SIM) dairy cows managed identically. Br is the breed effect, Time is the time effect, and Br x Time is the interaction effect (superscript letters denote differences for $p < 0.05$ at each time point in pairwise comparisons). Time points consider single days for BCS and weekly averages for milk yield. *Figure 2.* Trends from -21 to 28 days from calving (DFC) of plasma concentrations of P (**a**), bilirubin (**b**), glutamate-oxalacetate transaminase (AST-GOT) (**c**), ceruloplasmin (**d**), paraoxonase (**e**), and reactive oxygen metabolites (**f**) in Holstein (HOL), Brown Swiss (BRO), and Simmental (SIM) dairy cows managed identically. Br is the breed effect, Time is the time effect, and Br x Time is the interaction effect (superscript letters denote differences for $p < 0.05$ at each time point in pairwise comparisons)

Whole blood stimulation assay

After WBA, the fold change of IL-1β was the highest in HOL cows ($p < 0.05$ for both BRO and SIM; Table 3). No effect was detected in IL-6-fold changes.

4. Discussion

The transition period is characterized by an overt systemic inflammatory response which occurs immediately after calving even without any sign of infections (Trevisi and Minuti 2018), as confirmed in the present study by the dramatic increase in the positive APP (i.e haptoglobin and ceruloplasmin) paired with the decrease in the negative APP (i.e. cholesterol, albumin, and paraoxonase). Nevertheless, the magnitude of this response varied among the explored breeds, even though all cows were managed identically and fed the same diet. This unusual setting allowed us to compare different breeds, mitigating the environmental variability. Nevertheless, the facilities used to perform the present study did not enable us to record individual feed intake. Therefore, we were unable to fully distinguish between the effects of a different adaptation to the acute phase response from those that could have been driven by a different feeding behaviour (and likely by a different nutrient intake) among the three explored breeds. Another limitation is represented by the limited sample size. Thus, results of this observational study should be interpreted accordingly.

During the transition period, SIM cows had the lowest ceruloplasmin concentration, suggesting that this breed had the least severe acute phase response at the onset of lactation. Ceruloplasmin is a positive acute phase protein, whose recovery is slower than that of haptoglobin (Bertoni et al. 2008). This was likely driven by the lower metabolic demand faced by those cows, as reflected by their higher BCS and plasma fructosamine concentration compared with the other breeds. Fructosamine is a stable glycated protein formed by an irreversible non-enzymatic reaction between glucose and proteins (mainly albumin) (Armbruster 1987). Fasting plasma glucose concentration is finely reflected by fructosamine within one to three weeks (Caré et al. 2018), and higher plasma fructosamine in early lactation suggested there was a lower amount of energy diverted to galactopoietic

processes in SIM cows compared with BRO and, especially, HOL cows. This effect could have been driven by the least milk yield characterizing SIM cows while they were fed the same diet as the other breeds. In fact, SIM cows have greater attitude toward gaining muscle mass rather than prioritizing milk synthesis during energy partitioning, as reflected by the higher creatinine concentration detected in their plasma throughout the study, which can indicate greater utilization of phosphocreatine by exercising muscles (Hayden et al. 1992; Finco et al. 1997) compared with the other breeds. The lower milk yield in SIM compared with other breeds can also account for the difference in P concentration, as mammary gland uptake contributes to P losses, particularly at the onset of lactation (Goff 2000; Grünberg 2014). Previous research highlighted how the shift of metabolic functions toward homeorhetic regulation at calving is smoother in SIM compared with HOL cows (Lopreiato et al. 2019) and could also result in different leukocyte function, with SIM cows seeming to have a heightened immune response (Lopreiato et al. 2020).

As milk volume is mainly driven by the osmotic effect exerted by lactose (Sadovnikova et al. 2021), a higher glucose diversion to lactose synthesis accounted for the higher milk yield that BRO and HOL cows had during the first week of lactation compared to SIM cows. Furthermore, HOL cows had the highest milk yield from the second to the fourth weeks of lactation, suggesting the mammary demand for milk synthesis at the onset of lactation was the highest among the explored breeds. This is consistent with their lowest BCS at 28 DFC, suggesting that milk yield in HOL cows was supported a greater extent by mobilizing body reserves compared with the BRO cows, as previously reported (Gruber et al. 2014). Genetic selection in HOL has prioritized milk yield, promoting a metabolic asset that favours more nutrient partitioning towards the mammary gland and body reserve mobilization (De Koster and Opsomer 2013) compared with other less-selected breeds (Yan et al. 2006; Lucy et al. 2009; Friggens et al. 2013).

The highest concentrations of cholesterol and paraoxonase during the week after calving measured in HOL compared with other breeds suggest that HOL cows benefited from the fastest recovery from the inflammatory condition. Cholesterol decrease can be considered an indicator of reduced lipoprotein synthesis by the liver, suggesting an acute phase response occurring (Bertoni and Trevisi 2013). Paraoxonase is a liver enzyme whose

concentration is decreased by diseases and inflammatory states (Bionaz et al. 2007). In contrast, BRO cows had the highest concentration of ceruloplasmin throughout the experimental period and the lowest concentration of paraoxonase at 7 DFC. These results suggest that, in this study, BRO cows faced the most severe acute phase response. A possible explanation for the different adaptations to lactation could be due to the varying functionality of the leukocytes between these two breeds. This was supported by finding the greatest IL-1β production in leukocytes from HOL cows following *ex-vivo* stimulation, consistent with the results obtained by Gibson et al. (2016) in HOL stimulated macrophages compared with those from BRO cows. These results suggested that, in HOL, immune cells rely on a greater cytokine production in response to harmful stimuli, likely accounting for a faster resolution of systemic inflammatory conditions at the onset of lactation. Conversely, the intense acute phase response observed in BRO cows could have been driven by a stronger activation of leukocytes following parturition, as reflected by the highest concentration of ROMt detected in the plasma from these animals in the first week of lactation (Celi and Gabai 2015). The latter could be consequential to a stronger leukocyte killing capacity, as already documented by Gibson et al. (2016), who found macrophages from BRO cows had a greater ROMt production compared to those from HOL following a bacterial *ex-vivo* stimulation with LPS.

Nevertheless, trends of acute phase biomarkers in BRO cows were also accompanied by the highest concentration of bilirubin, AST-GOT, and GGT at the onset of lactation. Elevated bilirubin is mainly attributable to the lower synthesis of enzymes responsible for its clearance, while AST-GOT and GGT are markers of liver damage (Bertoni et al. 2008). Thus, liver function was somewhat impaired in BRO cows. Furthermore, although the loss in BCS was lower in BRO compared with HOL cows, they had higher plasma BHB concentration, suggesting a lower oxidizing efficiency of liver against mobilized NEFA (Herdt 2000). Both liver damage and impaired liver oxidation have been reported in dairy cows facing a remarkable acute phase response (Bertoni et al. 2008), and trends of the aforementioned plasma analytes detected in BRO cows during the present study were beyond the physiological ranges reported for HOL dairy cows at the onset of lactation (Premi et al. 2021). Of interest, BRO cows did not show any clinical metabolic disorder

and maintained their milk yield throughout the experimental period. Thus, these data could suggest that, despite severe inflammatory conditions, BRO might be more resilient to the intense metabolic changes typical of the transition period. However, the metabolic stress caused by nutrient mammary uptake is likely lower than that occurring in HOL. Analysing the impact of heat stress on BRO cows, Maggiolino et al. (2020) found that, as temperature and humidity rise, BRO does not reduce milk yield. In our study, we observed that, in another stressful condition (as pointed by the blood markers investigated), BRO cows had a similar response. Nevertheless, it may be possible that reference ranges based on HOL are not suitable for BRO, and specific values should be calculated.

5. Conclusions

This study suggests that the three explored breeds have different adaptations to the systemic inflammatory state occurring at the onset of lactation. The acute phase response observed in SIM cows was mitigated by their lower metabolic load (a consequence of their lower milk yield). Moreover, HOL cows produced more milk and relied on the most efficient activation of the immune system, resulting in a faster resolution of the acute phase response. BRO cows maintained systemic inflammatory conditions longer than the other breeds, but negligible effects were detected regarding their general performances, suggesting this breed might have the greatest ability to cope with the metabolic shifts driven by the acute phase.

We speculate that the immune system function of the three explored breeds could have been affected by the selection procedures aimed at maximizing the different productive traits, possibly accounting for the different metabolic adaptations to the transition period detected here. Nevertheless, these should be considered preliminary results due to the limitations represented by the facilities where this study was carried out and the limited sample size. Further research is required to fully elucidate the adaptive strategies adopted by different dairy breeds to cope with the dramatic changes occurring at the onset of lactation.

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Chapter 2

Physiological adaptations during first two transition periods in Brown Swiss and Holstein breeds: feeding behavior and metabolic status.

It will be submitted to Journal of Dairy Science soon.

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Abstract

The most widespread dairy breed in Europe as in the world is the Holstein breed (HO) (because of its high milk production. Brown Swiss breed (BS) is also wide managed by dairy breeders especially in country across the Alps. The two breeds are different for a lot of dairy traits and the most investigated is milk quality. Despite this general information, few studies have been reported regarding the differences between two breeds in feeding behavior, feed efficiency and apparent nutrient digestibility, especially during the transition period. For that reason, we conducted a trial with 10 Holstein dairy cows and 9 Brown Swiss dairy cows monitored at transition period (from -21 to 28 days in milk DIM), in both the first and second lactations. Cows were housed at the experimental dairy farm CERZOO s.r.l. and fed with TMR ad libitum though the roughage intake feeding system (RIC, Hokofarm Group, Netherland) for measuring daily dry matter intake (DMI). Further, milk quality was monitored daily at each milking though a NIRS instrument in the milking parlour (Afifarm, Israel). Additionally, milk samples were weekly collected for urea determination. The DMI was similar between the two breeds for the first lactation, but the trend of the intake during the transition seems to be different (P=0,05) around second calving starting from one week before calving until the 4 week of lactation. A significant breed effects in both lactations was reported for the parameter DMI on body weight (DMI/BW %), with greater ($P < 0.05$) values for HO compared to BS. Concerning the feeding behavior of the first transition period, there are no differences between HO and BS. Contrarily, a different feeding behavior was observed between two breeds ($P< 0,05$) in the second lactation: the daily eating time was higher for HO than BS around calving (from -14 to 7 DIM) and the DMI for each meal as well as the time spent for each meal were higher for HO than BS during the whole transition period. Daily meals are equal for both breeds. Lying time, milk yield, ECM and FCM were higher in HO ($P<0,05$) in lactation, but the feed efficiency did not differ. As reported in previous studies, milk protein and urea contents were higher in BS than HO, whereas fat and lactose contents were similar between breeds. Data from this study suggested the adaptation of each breed to the transition period is different, for traits related to feeding behavior, performance, and milk quality.

Key words: *blood parameters; milk quality; animal welfare, peripartum*

1. Introduction

All over the world dairy breeders manage different dairy breed for the milk production. Each breed is selected for different production traits (Back et al., 2006; Bland et al., 2015; De Marchi et al., 2008a; Gustavsson et al., 2014), depending on the breeding environment (Piccand et al., 2013; White et al., 2002). The most widespread dairy breed in Europe as in the world is the Holstein breed (HO) (Cunningham, 1983) because of its high milk production. Brown Swiss breed (BS) is also wide managed by dairy breeders particularly in country across the Alps (Cunningham, 1983; Mattiello et al., 2011; Zanon et al., 2020) for its milk quality. Both breeds were genetically selected for milk production. The most investigated traits among the two breeds over the years were the milk quality parameters. In literature we can find many studies regarding the milk quality evaluation between HO and BS. HO produce more milk (Carroll et al., 2006; Cecchinato et al., 2015; De Marchi et al., 2007; Dechow et al., 2007a; Gottardo et al., 2017) than BS cow. Nevertheless, BS produce more fat, protein and casein milk content than HO (Bittante et al., 2021; Carroll et al., 2006; De Marchi et al., 2007, 2008b; Franceschi, Malacarne, Formaggioni, et al., 2020a; Gottardo et al., 2017). P.J. Boettcher had shown different proportions in casein haplotypes between HO and BS, higher number of genotypes in BS (33) than HO (15) with big differences in α_{s1}, β and k casein (Boettcher et al., 2004). BS yielded 15% more cheese per kilogram of milk on average than HO according to M. De Marchi (De Marchi et al., 2008a) and this trend is confirmed from other studies (Bittante et al., 2021; Cecchinato et al., 2015; Franceschi, Malacarne, Faccia, et al., 2020; Franceschi, Malacarne, Formaggioni, et al., 2020b). Moreover, several studies showed better coagulation properties of the BS milk compared to HO milk (De Marchi et al., 2007, 2008a; Malacarne et al., 2006). Gottardo et al. had found differences in the fatty acid milk profile between two breeds: BS showed a higher content of saturated fat acids and lower content of unsaturated fat acids, higher C14:0 and lower C18:1(Gottardo et al., 2017). Besides, Carrol et al demonstrated that trans-fat acid was higher in BS milk than HO milk (Carroll et al., 2006). Dechow et al pointed out an equal cell somatic count between HO and BS (Dechow et al., 2007b) in accordance with De Marchi et al (De Marchi et al., 2007,

2008b). Otherwise Bendet et and Gibson et al showed a higher cell somatic count in HO milk compared to BS milk (Benedet et al., 2020a; Gibson et al., 2016). In a Swiss study BS dairy cows had higher BCS during the early lactation and similar BCS loss from calving to 100 DIMS to HO (Piccand et al., 2013). This trend was like which noted by Gruber et al (Gruber et al., 2014). In the same comparison there were differences in reproduction parameters between breeds. We have found few studies relating the differences in feeding behavior, nutrition, feed efficiency and energy metabolism between HO and BS cows. In 1969 Dickinson et al noted a different feed efficiency between HO and BS, where BS cows were less efficient compared to HO (Dickinson et al., 1969). Anyway, in the years the genetic selection had improved the performance of the two breeds. According to Gruber et al (Gruber et al., 2014) dry matter intake was similar during the dry period between BS and HO both as daily total dry matter intake as % referred to the body weight, despite the equal body weight of the two breed groups. However, in late lactation and post-partum period HO had a higher dry matter intake both as total as % referred to body weight (Gruber et al., 2014). Relating to nutrition performance Samuelson et al have settled that BS seems to be more responsive than HO to supplemental rumen protected methionine, replying at lower inclusion levels (Samuelson et al., 2001). Carrol et al had demonstrated that increasing the dietary fat impacted the proportion and yield of fat acids both in BS and HO (Carroll et al., 2006). BS in different study based on pasture system had showed less rumination time than HO (Braun et al., 2015b; Grodkowski et al., 2023). The milk urea content is strictly connected with the blood urea (Baker et al., 1995; Broderick & Clayton, 1997; Burgos et al., 2007) and in relationship with the protein metabolism, rumen fermentation pathway (Bastin et al., 2009; Hof et al., 1997; Nousiainen et al., 2004) and energy-protein balance (Broderick & Clayton, 1997; Wattiaux & Karg, 2004). Several studies pointed out the higher milk urea content in BS versus HO among different dairy farms (Benedet et al., 2020b; Bittante, 2022; Carroll et al., 2006; Doska et al., 2012; Wattiaux et al., 2005) and in the same farm with identical feeding (Kessler et al., 2020). Caccamo et al showed that BS and HO cows seems to have different lactation curves: HO showed a higher milk peak and less persistence, BS had an opposite trend

(Caccamo et al., 2010). Alvarado et al had shown similar fertility traits between HO and BS in alpen mountain region as day open, non-return rate at 56 days and pregnancy rate at first service (Toledo-Alvarado et al., 2017).

The transition period of dairy cows is one of the most investigated and most debated for several decades (Grummer, 1995; Drackley, 1999) for different challenges occur. Most of the studies take into consideration HO dairy cows as the model cow. To the best of our knowledge, few studies have reported the differences concerning feeding behavior, digestibility, milk quality, energy balance status, resting time and other aspects during the transition period between HO and BS under the same environmental conditions. A previous study of our research groups had pointed out a different adaptation to transition period between BS, HO and Simmental (SI) dairy cows in a commercial dairy farm with same environmental and feeding conditions (Catellani et al., 2023), especially regarding the immune-metabolic profile. Unfortunately, due to the technology level of the commercial farms we had not the possibility to detect some parameters, like DMI, rumen liquid profile and others. Based on these results, we wanted to better understand the different adaptation. In view of that, the aim of our study is to examine the physiological responses during the transition period of two groups of BS and HO cows and for two following lactations (first and second) under the same breeding conditions and identical feeding.

2. Material and Methods

2.1 Animal management and diets

The study involved 10 Italian Holstein dairy cows and 9 Italian Brown Swiss dairy cows during the transition period at first and second lactation: from 3 weeks before expected calving (-21 d) through 4 weeks of lactation (+28 d). Cows had first calving from October 2020 to May 2021, and then they calved again for the second time from November 2021 to May 2022. Age at first calving didn't differ between HO (27,3 months) and BS (28 months). The animals involved in this study were housed in a free stall barn at the experimental dairy farm Cerzoo of the Università Cattolica del Sacro Cuore (San Bonico, Piacenza, Italy). The trial was performed according to the Italian laws on animal experimentation with authorization n° 114/2021-PR. Pregnant heifers and dry cows were moved in a straw-bedded maternity pen 3 weeks before expected calving day. After calving cows were moved in the milking group pen with cubicles as resting area until 4 weeks post-partum. Fresh water was available at libitum. Cows were fed with TMR ad libitum though the feed bin of RIC (roughage intake control) feeding system (Hokofarm Group, Netherland) both in the dry and milking pen. TMR was prepared though a self-propelled mixer wagon (Rotomix, Bravo, Cuneo, Italy) and daily distributed in the feed bin at 8.00 am. Dry and milking TMR diets composition are reported in table 1. Cows were milked twice daily (4.30 and 16.30 h). Representative samples of dry and milking TMR were collected twice monthly. All samples were dried in a forced-air oven (60°C, 48h) for DM determination. After that were ground at 0.5 mm for chemical analysis with a near-infrared instrument by Foss (Hilloreroed, Denmark) NIR systems 5000 spectrophotometers equipped with a monochromator and transport module, scanning over the wavelength range 400-2500nm, every 2mm. Samples were analyzed for crude protein (CP), NDF, ADF, ADL, Ash, ether extract (EE), starch and sugar. Energy evaluation of the diets was calculated according to NRC 2001 formulas (NRC, 2001).

Table 1 Milking and dry diet offered to cow during the trial. Each feed component is expressed in % of total DM.

Item % DM	*Milking*	*Dry*
Corn silage	*34,88*	-
Barley silage	*5,13*	*79,4*
Alfalfa hay	*20,96*	-
Wheat straw	-	*11,96*
Soybean meal	*10,54*	*4,43*
Sunflower meal	*3,66*	*3,1*
Corn grain ground dry	*13,4*	-
Barley flaked	*8,91*	-
Hydrogenated fat	*0,8*	-
Milking premix	*1,04*	-
Dry premix	-	*1,1*
Sodium bicarbonate	*0,32*	-
Calcium carbonate	*0,32*	-
Sodium chloride	*0,04*	-

2.2 Health status

Health status of dairy cows was daily monitored during the study. Retained placenta was diagnosed when fetal membranes weren't expelled within 24 h after calving. Metritis and endometritis were diagnosed with ultrasound check after calving. Milk fever, ruminal problems, lameness were exanimated by a veterinary practitioner. Mastitis was diagnosed by visual evaluation of abnormal milk, somatic cell count and on farm culture bacterial test were performed for suspicious cases. Diarrheic feces were defined as those with fecal score less than 2.

2.3 Feeding behavior and lying time.

Each cows had free access to feed bin of the RIC feeding system who recorded individual daily TMR intake. Dry matter intake (DMI) was reported as total daily dry matter intake for the entire experimental period and in addition as referred to BW after calving (DMI/BW). Rumination time (RT) was measured using a rumination monitoring system by a neck collar (RuminAct, SCR Heatime, Netanya, Israel) and data were processed with its own software (Data Flow software, Netanya, Israel). Rumination data was also referred to DMI (RT/DM) and kg of NDF ingested by day (RT/NDF). Feeding behavior was monitored through data and records by the RIC system to obtain the daily meal frequency, meal size, meal duration, feeding time and feeding rate of the cows. In our trial we considered that the access to the RIC bins within 20 minutes were considered as the same meal. Daily lying time (LT) and daily activity time were recorded by a pedometer sensor (Afimilk, Afikim, Israel). Body Weight (BW) was measured daily after each milking though an electric weigher (Afimilk, Afikim, Israel).

2.4 Ruminal liquid profile, fecal samples, and apparent digestibility

Rumen fluid samples were collected with an orogastric probe (Ruminator; profs-products.com, Germany) before the morning TMR distribution. To reduce the buffer effect of saliva the first 1 L of rumen fluid was discarded. Rumen samples were taken at – 21 and + 28 d from calving. A 10-mL aliquot of the supernatant was transferred in to 15-mL tubes and immediately transferred to the lab in ice-water. Samples were analyzed for Total VFA concentration and molar proportion of acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, caproic, iso-caproic, and enanthic acids. Single VFA were expressed as relative amounts compared with total VFA concentration. Urea nitrogen and Lactate was analyzed with a spectrophotometric clinical auto-analyzer (ILAB-650, Instrumental laboratory, Lexington, MA, USA) and commercial kits. Fecal samples were individually collected from the rectum of each cow al -21 and +28 d and then frozen at -20 °C. De-frozen samples were analyzed for chemical composition and fermentative fecal profile. A subsample of 1 kg was dried at 60°C in a forced draught oven for 48 h for dm determination. After that

samples were ground with knife mill (mm screen) at 0,5 mm and analyzed for CP, NDF, ADF, ADL, starch and ash with a NIR instrument by FOSS (Hillrod, Denmark). NDF, ADL, starch and CP were used to obtain apparent digestibility coefficients using ADL as internal marker according to the following equations:

$$X\ digestibility = (1-([ADL]_{diet} \times [X]_{faeces})/([ADL]_{faeces} \times [X]_{diets})) \times 100\%$$

with: X digestibility = digestibility coefficient of specific nutrient X (starch, NDF or CP). The following superscript indicated samples (with diet or faeces) in which the nutrient or internal marker were quantified, as described above.

A wet samples of 50 g was extracted using a Stomacher blender (Seward Ltd. West Sussex, UK) for 3 min in distilled water (proportion water:samples 3:1. The mix was flirted through gauze, and then an aliquot of 2 mL of the solution was added to 1 mL of an oxalic acid solution (15.2 g/L) and 1 mL of a mixture of pivalic acid (internal standard, 1 g/L and formic acid (50 mL/L). The mixture was shaken for 10 min, centrifugated at 4500 x g for 10 min, and the liquid phase was retained for determination of VFA using the GC equipment according to Sigolo et al., (2023).

2.5 Milk yield and composition

Milk yield was recorded by a milk meter at each milking (Afimilk, Afikim, Israel). Milk quality was checked into different way. Fat, protein and lactose content were daily recorded for each milking by a milking parlour infrared measurement instrument (Afilab, Afimilk, Afikim, Israel). In addition, representative daily milk samples were taken each week and analyzed for urea content using a laboratory infrared measurements instrument (Milk Oscan FT 120, Foss electric, Hillerod, Denmark) according to Chessa et al., (2014) and cell somatic count was determined using an optical fluorometric method with an automated cell counter (Fossomatic 180, Foss Electric). The production and quality data were used to calculate the fat corrected milk (FCM), energy corrected milk (ECM) and feed efficiency (FE). The amount of daily production of kg fat + kg protein (FAT+PROT kg/d) and the

efficiency of fat-protein production referred to each kg of DM ingested (FAT+PROT/DMI) were calculated.

2.6 Metabolic profile and Body Condition Score and Energy Balance

Blood samples were taken from the jugular vein and collected in a 10-ml lithium heparin tubes (BD Vacutainer, BD Diagnostics, Franklin Lakes, New Jersey, United States) before the morning feeding distribution at -28, -14, -7, +1, +3, +7, +14, +21, +28 d from calving. Samples were immediately placed in ice-water and brought to the laboratory A small amount of blood was used to calculate packed cell volume (Centrifugette 4203; ALC International Srl, Cologno Monzese, Italy), the remaining blood was centrifuged (3,500 × g for 16 min at 4°C), and the resulting plasma was separated into aliquots and stored at −20°C until analysis. Plasma metabolites were analyzed at 37°C using an automated clinical analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA) as described by Calamari et al. (2016). Commercial kits from Instrumentation Laboratory SpA (Werfen, Italy) were used to measure glucose and urea (BUN). Kits from Wako (Chemicals GmbH, Neuss, Germany) were used to measure nonesterified fatty acids (NEFA), BHB. At the same time rectal temperature was recorded with a rectal thermometer at 8.00 am. Body condition score was visual performed by the same operator with a 1 to 5 scale according to Edmonson et al., (1989) after the morning feed distribution. First the evaluation of the energy balance was calculated daily for the first 4 weeks of lactation after calving according to (NRC, 2001) to detect the possible difference in energy balance between BS and HO. Energy balance (EB_{NRC}) (Mcal/d) was calculated as = intake of NE_L – (NE_L for milk synthesis + NE_M). The net energy content in milk (NE_L) was calculated as NE_L (Mcal/kg) = [0,0929 x fat (g/100 g)] + [0,0547 x protein (g/100 g)] + [0,0395 x lactose (g/100)] and multiplied by milk yield to calculate the daily amount of produced NE_L. Net energy required for maintenance (NE_M) was calculated as (Mcal/d) = 0,08 x $BW^{\wedge 0,75}$. Furthermore, we calculated the net energy balance (NEB_{NRC}) considering tissue mobilization and repletion after calving. NEB_{NRC} was calculated as = EB_{NRC} (previously evaluated) + NE_L from body reserve loss (Mcal/kg) - $_{NEL}$ for BW gain (Mcal/kg). NE_L from body reserve and for BW gain were calculated as reported in NRC (2001).

2.7 Statistical Analysis

Data were analyzed using SAS software, version 9.4 (SAS Inst. Inc., Cary, NC, USA) and are presented in graphs and tables as the least squares mean and pooled standard error for individual means of breeds over time. Data underwent ANOVA testing using a mixed model for repeated measures (Glimmix Procedure, SAS Inst. Inc.). Parity was firstly included and resulted always significant then was removed, and the first and second lactation was analyzed separately. The statistical models included the fixed effect of breed (Br), time (DFC for BCS and plasma analytes, weeks from calving for milk yield), their interaction (Br × Time), and the random effect of the individual cow. The pairwise comparison was done using the least significant difference test. Significance was declared $P \leq 0.05$, and differences for $P \leq 0.1$ were discussed in the context of tendencies.

3. Results

3.1 Diets and animal health

Results of weekly diet analysis are reported in tab n° 2. Diets composition had a lower variability for each nutrient parameter during the experimental period both for dry groups and milking group. Table 3 showed the number of health disorder detected during the trial. Each disorder hadn't severe compromised the physiological and health status of cow enrolled in the study because they weren't severe health disorder. No antibiotics were used for the above disorders. In case of retained placenta cows were treated with oxytocin, whereas for metritis has been used and prostaglandin treatments. The health problem incidence was quite balanced between HO and BS in both transition periods.

Table 2 Milking and dry diet offered to cow during the trial. Each feed component is expressed in % of total DM.

% DM	*Milking*	*Dry*
DM	*0,52 ± 0,03*	*0,42 ± 0,03*
CP	*15,70 ± 0,76*	*11,99 ± 0,38*
NDF	*34,05 ± 1,85*	*53,40 ± 2,45*
ADF	*20,99 ± 1,73*	*34,9 ± 1,85*
ADL	*3,40 ± 0,21*	*5,00 ± 0,44*
Ash	*7,00 ± 0,12*	*6,55 ± 0,18*
EE	*3,2 ± 0,17*	*2,4 ± 0,28*
Starch	*26,5 ± 1,03*	*7,20 ± 0,5*
Sugar	*4,3 ± 0,30*	*4,2 ± 0,29*

Table 3 Number of health disorder detected during the entire experimental period for first and second lactation between HO and BS breed.

Health problem	*1° lactation*		*2° lactation*	
Breed	BS	HO	BS	HO
Retained placenta	*1*	*3*	*1*	*1*
Metritis	*2*	*2*	*1*	*3*
Lameness	*2*	*1*	*0*	*0*
Mastitis	*0*	*0*	*0*	*1*
Rumen disorder	*1*	*1*	*1*	*1*

3.2 Feeding behavior and lying time.

The table n° 4 reported the results feeding behavior traits and lying time of the two breeds around first and second transition period. During the first transition there wasn't any difference in the amount of daily DMI between BS and HO. The trend of DMI became different (P<0,05) in the second transition as reported in the figure n° A2 starting from the last week of dry period (P<0,05) to the 1st, 3rd and 4th weeks of lactation with higher levels of DMI for HO in comparison of BS. BS was heavier than HO in the first lactation (P<0,05). Then in the second lactation there was no difference in BW between the two breed groups, even though numerically differences. The DMI referred to BW was significantly different between two breed both in first and second experimental period. BS cows compared to HO (P<0,05) had lower DMI in percentage of BW. The two breeds showed a similar feeding behavior around calving for daily meal frequency and feeding rate in both lactations, while meal size, meal duration (P<0,05) and feeding time (P<0,10) were different in the second lactation. HO compared to BS shown higher feeding time from last week of dry period to 2nd week of lactation and bigger meal size (P<0,05) in the 1st, 3rd and 4th milking week. Daily rumination time was different between breeds in both experimental period (P<0,05). BS had lower rumination time compared to HO. In addition, HO had more rumination time for each kg of NDF ingested than BS after first calving (P<0,05). The time

spent for each daily meal was lower for BS than HO (P<0,05) during the entire transition period, with exception of the 2nd and 3rd weeks. Finally, HO showed higher lying daytime than BS (P<0,05) in the second transition period. No breed difference was detected in the first lactations. Furthermore, the activity time spent every day in the barn was similar between HO and BS.

3.3 Ruminal liquid profile, fecal samples, and apparent digestibility

Table n° 6 reported the results of apparent digestibility, fecal sample composition and ruminal liquid profile analyzed. About apparent digestibility no breed effect was detected in both lactation either for starch, protein and NDF. BS showed a higher Dig-PG at +28 dim after first calving (P<0,05). Dig-PG was over 90% among two breeds in both two dry periods and over 70% after calving. Dig-Starch ranged from 96,74% (HO, 1° lactation) to 98,02% (HO, 2° lactation) after calving and from 92,48% (BS, 1° lactation) to 98,65% (HO, 2° lactation) before. Finally, Dig-NDF ranged from 63,93% (BS 1° lactation) to 77,96% (HO 2° lactation) before parturition and from 68,27% (HO, 1° lactation) to 76,79% (HO, 2° lactation). Additionally, the fecal nutrient profile was similar between breed in both transition period. About VOC fecal profile we only detected a higher acetaldehyde and aldehydes in BS feces in comparison with HO at first transition period.

HO compared to BS had a higher percentage of propionic acid in the ruminal fluid at 4th weeks after first calving (P<0,05) and BS compared to HO showed a higher VFAt concentration at 28 d after second calving (P<0,05). Butyric acid percentage was higher in BS compared to HO in both experimental periods (P<0,05), especially after calving at 28 DIM (P<0,05). Iso-butyric and iso-valerianic acid percentage were higher ad 28 DIM in BS compared to HO during first experimental period. D-lactic acid, L-lactic acid and urea concentration in the ruminal fluid was higher in BS compared to HO in both lactations (P<0,05). No breed effect was detected about VOC fecal profile (table n°6-7).

3.4 Milk yield and composition

Milk yield was significantly higher in HO in both lactations (P<0,05). Otherwise, BS have a higher protein milk content in the first lactation (P<0,05) and the difference tend to continue after the second calving (P<0,10). No breed effect was found between HO and BS for milk fat and lactose content. Milk urea (MUN) was higher in BS compared to HO for both lactation (P<0.1 and P<0.05 for first and second lactation respectively). Higher SCC was detected for HO during the post-partum period after second calving (P<0,05) and in the 1st (P<0,05) week after first calving. The ECM was similar between BS and HO during first lactation but became higher in HO compared to BS in second lactation (P<0.05). HO had a higher FCM production for both lactation (P<0.1) The daily production of fat and protein was higher in HO compared to BS in the second lactation (P<0,05), especially from the 2nd week of lactation. Regarding the feed efficiency not statistically difference we noted between the two breeds both in first and second lactation. Same trend was observed for FAT+PROT/DMI with no difference between breeds.

3.5 Metabolic profile, Body Condition Score and Energy Balance

The BCS was different between breeds in both lactation (P<0,05). BS showed the higher value, especially after calving. Moreover, the delta BCS between dry period and 28 DIM was lower in BS compared to HO. Blood glucose was similar between breeds in the first calving, while during second experimental period glucose showed higher concentration in HO compared to BS (P<0.1). Uremia was similar between two groups without statistically differences in both lactations. In the first lactation BS compared to HO showed lower plasmatic NEFA concentration after calving and no difference was observed for BHOB. During second lactation BS showed higher concentration of BHOB compared to HO (P<0,05). The energy balance in the first 4 weeks of lactation (tab 8) sowed no difference related to breed.

4. Discussion

4.1 Feeding behavior and lying time.

Our data pointed out many differences in the BS feeding behavior around calving compared to HO when they are feed with the same diet and breed in the same environmental conditions. These differences range from 1st to 2nd calving. DMI had a different trend according to parity. Through the first transition period BS eat similar quantity of feed before and after calving. However, BS involved in our trial were heavier than HO. Therefore, the DMI referred to BW (DMI/BW) was higher in HO than BS. At the same time, no one traits of their feeding behavior were significantly different compared to HO. Thus, around first calving BS showed an equal feeding behavior to HO, but they eat less dry matter referred to BW. This difference could explain a different DMI capacity linked to the breed. In the second transition period BS had similar DMI to HO at dry period until the last week of pregnancy, as reported by Gruber et al., (2014). From -7 d HO started to eat more DM until the end of the experimental period at + 28 d in comparison with BS. The BW difference between breeds wasn't' so significant as around 1st calving. Given that BW differences, BS had a lower DMI/BW than HO in accordance with Gruber et al., (2014). Another study which compared these two breeds detected a lower DMI in BS when fed with the same diet of HO (Carroll et al., 2006). In addition, few feeding behavior traits related to DMI were different in BS and HO around second calving. HO seems to eat more kg of DM for each meal with greater meal duration even though the same daily meal frequency. These data are in accordance with the difference of DMI previously described. RT showed interesting data between BS and HO. About BS, we detected higher daily RT compared with previous Swiss studies that recorded RT for BS cows (Braun et al., 2015a; Graf et al., 2005) This difference was probably due to the different diet and feeding system in the Swiss studies (extensive farm system based on pasture) compared to our trial conditions (intensive farm system with TMR). Few studies on the best of our knowledge evaluated the rumination activity of BS in comparison with other breeds. We noted that BS spent significantly less time to ruminate during the day in comparison with HO, from dry to

milking period and for both lactations. Our data are in accordance with Braun et al., (2015b) that evaluated the rumination activity of 100 BS in comparison with other breeds (HO and SI) to determine RT standards for BS. Despite different stage of lactation and diets (TMR or pasture) but similar MY, in our study we noted that BS had lower RT than HO in agreement with Braun et al., (2015b) and Grodkowski et al., (2023). Towards these authors that reported RT and eating time, we had also recoded the individual DMI of each cow. The RT/DMI was lower in BS compared to HO only around first calving, but resulted similar at the second calving, though when DMI was different between breeds. The trend of RT/DMI and RT/NDF were comparable in both experimental periods, and we detected higher levels of the two parameters in comparison to data recoded by Braun et al., (2015a) for BS cows. This data about RT and RT/DMI suggests that the lower total daily RT of BS could be due to the lower feed intake and not to a lower rumination behavior. We probably think that BS has a lower DMI capacity than HO when fed with the same diets and the same breeding environment after calving, as reported by other studies (Carroll et al., 2006; Gruber et al., 2014). In addition, as suggested by other authors (Braun et al., 2015b), BS has the proper RT standard, different to other dairy breeds (like HO and SI).

4.2 Ruminal liquid profile, fecal samples, and apparent digestibility

Apparent digestibility of starch, NDF and protein seems to not differ between BS and HO cows before and after calving. Only BS primiparous had a higher Dig-pg at +28 d in comparison with HO primiparous. In addition, fecal composition among breed was very comparable in both lactations. In opposite, we pointed out many differences on the ruminal liquid profile among breeds. We detected more VFAt at 28 d in BS rumen liquid even though the lower RT of BS previously described. This difference could be due by a lower passage rate (Roman-Garcia et al., 2021) - kp - linked to the lower DMI after calving compared to HO as reported above. Moreover, the proportion of the main VFA differs from BS to HO. BS showed a ruminal liquid with more butyric content in both milking periods. Furthermore, BS had more isobutyric, isovalerianic and lower propionic in the rumen liquid after first calving. Iso-FA are fermentation products correlated to protein degradation, as they originate from protein and branched-chain amino acid deamination (Kaneda, 1991;

Roman-Garcia et al., 2021). An interesting result is the urea rumen content, wherever we detected a significant breed effect in both lactations. BS had more urea content when fed with either dry or milking diets compared to HO. Despites both breeds were fed with the same level of protein, energy and NDF, BS presented a higher urea concentration (with at times 50% higher concentration) in the rumen. The rumen is the most important compartment where urea can be upcycled (Lapierre & Lobley, 2001) and support the microbial protein synthesis (Virtanen, 1966). Urea recycles into the rumen append through salivary secretion and by transport throught the rumen epithelium (Marini & Van Amburgh, 2003) and the proportion of urea transferred through saliva versus rumen varies based on the diet composition (Huntington, 1989). Thus, the differences of urea detected into the rumen didn't be explained by the diet composition. Saliva has been estimated to supply the 70-90% of the buffering capacity of the rumen (*Citations-20230717T092305*, n.d.). Many studies in the literature (Beauchemin et al., 2008; Bowman et al., 2003; Cassida & Stokes, 1986; Jiang et al., 2017; Maekawa et al., 2002) detected correlations between salivary secretion and chewing activity, eating time and resting time, but in our studies, we noted that BS had similar rumination for each kg of feed ingested to HO, same daily eating time and less resting time around second calving. Taking into consideration this behavior results from studies above, BS may produce less salivary. However, we detected more rumen urea in BS, so probably a higher amount of urea from salivary arrived in the BS rumen, leading a higher buffer capacity. In our study both BS and HO were fed with the same diets, with the same protein and energy level. Thus, the difference in rumen urea content could be also related to a different protein degradation by the rumen microflora in the two breeds. In any case the urea synthesis occurs into the liver (approximately 1.5/2.0 mol urea N/g liver per min; Symonds et al., 1981) mainly as a metabolic priority for ureagenesis from ammonia that arrive from the rumen (and to avoid hyperammonaemia) and also by the ureagenesis that arrive from the oxidation of amino acids used to cover the body requirements (Lobley & Milano, 1997). For this reason, the higher concentration of urea in the BS rumen fluid might be a consequence of a different and higher nitrogen recycling pathways to avoid mainly hyperammonaemia. To confirm these explanations more studies on the BS rumen environment and BS nitrogen metabolism must be performed. During the milking period,

this rumen urea data had the same trend observed in MUN content described. To the best of our knowledge no studies have evaluated the difference in rumen liquid composition between BS and HO fed with the same diet and in the same environmental breeding conditions. Moreover, few studies investigated the possible different rumen population of BS cows in comparison with other breeds, especially HO. Gonzalez-Recio et al. (2018) pointed out that the host genetic component could partially regulate the composition of the microbiome and indirectly some metabolic pathways, between HO and BS. We probably could assert that the BS rumen environment have a different fermentation activity because we detected a different proportion of VFA even though experimental animals were fed with the same diet.

4.3 Milk yield and composition

Milk quality and production are two of the most investigated traits between HO and BS in literature for decades. In our transition trail we also recorded the milk performance of the two breeds fed with the same diets and in the same environmental conditions. We detected that HO produced more milk for the first 4 weeks of lactation as reported by many studies in different stage of lactation (Benedet et al., 2020a; Bittante et al., 2021; Carroll et al., 2006; Cecchinato et al., 2015; De Marchi et al., 2008b; Gottardo et al., 2017), included in post-partum period (Catellani et al., 2023). Otherwise, BS showed an higher milk protein content and equal lactose content in comparison with HO, as suggested by different authors considering milk from different farms (Bittante et al., 2021; De Marchi et al., 2008b; Gottardo et al., 2017) or under the same farm (Kessler et al., 2020). In contrast to different studies with the same environmental conditions (Kessler et al., 2020) or different (Cecchinato et al., 2015; De Marchi et al., 2007, 2008b; Gottardo et al., 2017) for the two breeds, we didn't find a higher fat content in BS milk compared to HO milk in both lactations. Probably our data are in contrast with previous studies for the different stage of lactation considered. About SCC our data agree with other studies (Benedet et al., 2020a; Cecchinato et al., 2015; Gibson et al., 2016) where BS showed less SCC compared to HO. Our data of milk urea agree with Kessler et al., (2020) who noted that BS had higher milk urea content than HO under the same farm conditions. Moreover, same urea trend between

BS and HO was detected by many studies of comparison breed under different farm conditions (Benedet et al., 2020a; Bittante, 2022; Carroll et al., 2006; Doska et al., 2012; Wattiaux et al., 2005). Bittante, (2022) and Doska et al., (2012) founded higher milk urea in BS cows in a population of different herds. However, they didn't include the herd effect in the model because most of the herds considered were multibreed farm. These Authors didn't attribute the urea content difference between breed to environmental breeding conditions. For these reason Kessler et al., (2020) attribute the reason of these breed difference in the urea content to a genetic heritage. Taking into consideration the milk production and quality, HO produced more FCM and ECM after second calving, probably due to the greater MY difference between breed in early lactation. The same trend we observed for the FAT+PROT daily production. About FE we obtained that two breed seems to have the same production efficiency: BS produced low milk meanwhile eat less DM, especially after second calving. This data contrasts with Dickinson et al., (1969). Only a slightly tendency was detected after first calving. About BS cows, we must also consider milk quality due its genetic selection through the years. Considering the production efficiency of kg fat and protein for each kg of DMI, BS wasn't less efficient than HO in both lactations. Our results of milk production and composition of BS and HO in the first 4 weeks of lactation confirmed the different genetic selection goal of the two breeds: higher milk production for HO and higher quality for BS, as suggested by many authors previously described. Few studies in literature reported the differences in feed efficiency between two breeds in the same environmental conditions. With our data we could assert that the two breed in early stage of lactation had the same FE. Other studies taking into consideration different stage of lactation must be performed to better understand the FE of HO and BS.

4.5 Metabolic profile, Body Condition Score and Energy balance

In our study we detected some differences between BS and HO during transition period of first and second calve for metabolic profile and BCS. We noted a breed effect on the BCS around calving: BS showed higher BCS than HO, and confirmed results of Gruber et al., (2014). The interaction breed*week was always significant (P<0,001) as can be appreciated in the fig n°F. During the first transition period the difference of BCS among BS and HO starting from 7 d after calving. In the figure, it is also evident that the postcalving decrease of BCS resulted lower in BS vs HO. This trend was in accordance with a study performed in a commercial farm (Catellani et al., 2023). To investigate the metabolic response we analyzed blood glucose, NEFA, BHB and BUN immediately before the most important meals of the day. To the best of our knowledge, few studies had analyzed these parameters comparing BS and HO during the transition period. Blood glucose tended to be higher in HO in contrast to data obtained in a a previously study (Catellani et al., 2023). In this trial we confirmed numerically differences in the BUN content, as observed in a previous experiment (Catellani et al., 2023) and by a Swiss study (Urdl et al., 2015). In particular, the BUN content resulted numerically higher in BS vs HO in both lactations. Data agrees with Benedet et al., (2020a) who detected higher predicted BUN in BS in comparison with HO, even though in different farm environment (Alpen region) based on summer pasture toward our trial condition (Po valley land) based on TMR feeding system. NEFA was higher in HO after first calving in agreement with Benedet et al., (2020a). The numerically difference in NEFA didn't still be significant around a second calving as we have previously observed in multiparous (Catellani et al., 2023). BHB resulted similar in the two breeds in the first calving, in agreement with Benedet et al., (2020a). Nevertheless, in the second lactation BHB concentration resulted higher in HO vs BS cows, as already found by other authors (Catellani et al., 2023; Urdl et al., 2015). Our data suggested that the breed influences the variation of blood metabolites in the early lactation and confirmed data of Benedet et al., (2020a). Due to the difference in the metabolic profile and BCS trend during the transition period we noted some numerically differences in the NEB calculated after calving among breeds, but without a statistical relevance. Our data suggest that BS and HO cows have likely a different energy metabolism around calving. To sustain the NEB

condition, BS seems to show a different use of body reserves and mobilization during the transition period in comparison with HO. Data obtained in this research suggest that BS uses more protein body reserves around calving instead that fat body reserves, whereas HO showed the opposite tendency. About EB we didn't detect any difference between BS and HO, considering NRC.

5. Conclusions

Few studies have investigated nutritional and metabolic specific traits of the BS breed in comparison with HO. In addition, to the best of our knowledge, this is the first experiment that have compared BS and HO during first two transition periods, investigating their physiological adaptive mechanisms around calving and maintaining both breeds under the same environmental and management conditions. Our results highlight that the different strategy of genetic selection in the BS has led changes in some digestive adaptations and energy-protein pathways. We noted that BS had a different feeding behavior, DMI capacity, rumination patterns, ruminal environment, milk performance, energy-protein metabolism, and utilization of body reserves during NEB condition. These differences led us to affirm that the BS diet must be different in comparison with HO, at least at the beginning of lactation.

Interestingly, our results support a difference management of body reserves during NEB conditions among the breeds. BS in comparison with HO showed a higher use of protein than fat body reserves. This ability seems to limit the weight losses of BS after calving and have consequences also at the mammary gland synthesis. Metabolic and digestive differences among breeds suggest the need to a better investigation of the immune functions.

In conclusion, our data led us to affirm that the gold standard for many nutritional and metabolic parameters presented by literature through the years for HO cannot be used for BS. Thus, the nutrition and management of BS cows should consider these differences and should be modified to better cover the nutritional requirements in the transition period. Future studies should confirm these findings with more cows, extending the controls in the whole lactations and in cows up to 2 parities.

Tab 4: Least squares means and associated SEM for dry matter intake (DMI), feeding behavior parameters, lying, activity and rumination time for BS and HO cows during the first (I° lactation) and second (II° lactation) transition period.

DMI (kg)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	10.04	10.00	0.965	0.819	0.856	<0.0001	0.058	13.46	14.13	0.607	1.291	0.050	<0.0001	<0.0001
-2	9.56	10.00	0.592					12.02	14.19	0.105				
-1	8.89	9.58	0.410					10.53	13.41	0.036				
1	11.68	11.72	0.959					14.23	17.81	0.011				
2	14.65	15.49	0.315					18.69	20.12	0.280				
3	17.57	16.61	0.251					19.46	23.50	0.005				
4	17.96	17.87	0.916					20.66	23.53	0.037				

Meal frequency (n°/d)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	7.59	7.35	0.641	0.515	0.146	0.029	0.891	8.49	7.90	0.280	0.541	0.355	<0.0001	0.153
-2	7.78	6.93	0.101					8.30	7.94	0.511				
-1	8.11	7.41	0.172					8.72	8.37	0.525				
1	7.83	7.08	0.145					9.14	9.53	0.472				
2	7.98	7.33	0.208					9.38	8.86	0.344				
3	8.11	7.73	0.462					9.41	9.14	0.627				
4	8.41	7.76	0.208					9.79	8.63	0.041				

Meal size (kg DM/meal)

	I lactation							II lactation						
WEEK	BS	HO	P<	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	1.43	1.45	0.920	0.184	0.265	<0.0001	0.137	1.67	1.90	0.270	0.198	0.026	<0.0001	0.053
-2	1.29	1.55	0.160					1.57	1.93	0.076				
-1	1.19	1.36	0.351					1.28	1.72	0.032				
1	1.62	1.78	0.373					1.78	2.04	0.200				
2	1.99	2.39	0.033					2.13	2.44	0.127				
3	2.40	2.31	0.619					2.15	2.72	0.007				
4	2.26	2.47	0.251					2.16	2.85	0.002				

Meal duration (min/meal)

	I° lactation							II° lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	28.83	25.56	0.137	2.154	0.855	<0.0001	0.084	22.82	26.64	0.005	1.339	0.001	<0.0001	<0.0001
-2	26.12	27.68	0.464					19.85	26.93	0.000				
-1	22.96	24.60	0.439					16.72	23.20	0.000				
1	16.39	15.92	0.826					12.33	15.10	0.045				
2	18.07	19.80	0.421					17.41	18.82	0.300				
3	20.41	22.40	0.352					19.80	20.49	0.614				
4	22.72	21.67	0.627					20.95	23.25	0.096				

Feeding time (min/d)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	186.78	162.60	0.167	17.129	0.654	<0.0001	0.012	185.86	200.71	0.248	12.697	0.075	<0.0001	0.0001
-2	196.22	170.13	0.137					159.92	205.27	0.001				
-1	178.60	173.49	0.767					138.78	187.50	0.000				
1	118.40	102.17	0.350					105.33	131.03	0.048				
2	129.92	140.16	0.554					159.46	159.21	0.985				
3	150.81	166.76	0.359					180.02	181.90	0.885				
4	165.22	164.43	0.963					203.05	195.27	0.550				

Feeding rate (min/kg DM)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	18.86	16.70	0.134	1.414	0.401	<0.0001	<0.0001	13.98	14.69	0.616	1.3782	0.982	<0.0001	0.001
-2	20.95	16.90	0.007					13.60	15.16	0.271				
-1	20.37	18.23	0.138					13.84	14.45	0.663				
1	10.55	8.96	0.268					7.63	7.83	0.886				
2	8.63	9.25	0.665					8.55	8.51	0.976				
3	8.12	10.24	0.141					9.38	8.12	0.373				
4	9.00	9.27	0.849					10.16	8.60	0.271				

Lying time (min/d)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	771.40	827.82	0.284	51.615	0.269	<0.0001	0.015	776.95	848.54	0.085	39.876	0.046	<0.0001	0.104
-2	752.62	810.64	0.270					772.67	807.57	0.390				
-1	695.95	768.05	0.170					766.78	834.54	0.102				
1	588.01	704.15	0.032					584.58	655.15	0.092				
2	630.25	675.66	0.375					569.27	677.06	0.012				
3	653.65	679.29	0.615					572.97	665.93	0.029				
4	682.40	675.14	0.887					571.45	669.90	0.021				

Activity (min/d)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	188.08	185.60	0.907	21.066	0.235	<0.0001	0.101	219.24	129.03	0.030	38.858	0.124	<0.0001	0.009
-2	183.25	168.10	0.477					224.51	127.51	0.021				
-1	203.38	180.76	0.290					185.29	131.57	0.181				
1	259.84	231.71	0.181					248.49	195.93	0.190				
2	186.89	179.90	0.737					190.79	155.27	0.371				
3	197.00	171.36	0.222					197.45	150.23	0.239				
4	222.00	171.57	0.020					181.41	144.66	0.357				

Rumination time (min/d)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	517.22	568.47	0.048	265.610	0.008	<0.0001	0.089	497.53	601.08	0.000	408.41	<0.0001	<0.0001	0.247
-2	504.11	569.43	0.014					464.48	598.91	<.0001				
-1	491.14	544.81	0.039					445.59	577.80	<.0001				
1	322.83	378.74	0.032					349.78	474.03	<.0001				
2	445.13	500.01	0.035					470.90	559.31	0.001				
3	467.48	540.71	0.006					466.59	607.59	<.0001				
4	443.83	542.85	0.000					473.46	588.39	<.0001				

Rum/kg DM (min/kg DM)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	53.57	58.11	0.222	14.096	0.061	<0.0001	0.159	38.55	44.06	0.093	5.2758	0.294	<0.0001	0.055
-2	54.39	60.29	0.113					40.84	43.97	0.334				
-1	60.06	59.88	0.961					46.49	44.92	0.624				
1	30.56	42.13	0.002					27.82	31.93	0.207				
2	31.32	32.74	0.700					25.90	31.97	0.066				
3	28.26	32.87	0.215					25.02	26.66	0.615				
4	24.84	30.79	0.096					24.08	25.63	0.627				

Rum/NDF (min/kg NDF)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
-3	100.32	108.82	0.370	52.121	0.040	<0.0001	0.086	71.93	82.21	0.181	30.949	0.261	<0.0001	0.045
-2	101.86	112.90	0.245					76.19	82.03	0.443				
-1	112.48	112.14	0.971					86.74	83.80	0.698				
1	89.76	123.72	0.001					80.21	92.03	0.127				
2	91.98	96.17	0.659					74.64	92.14	0.026				
3	82.99	96.52	0.155					72.31	76.82	0.558				
4	72.96	90.41	0.055					69.60	73.86	0.571				

Tab 5: Least squares means and associated SEM for ruminal liquid profile parameters for BS and HO cows during the first (I lactation) and second (II lactation) transition period.

Ph													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	7.10	7.10	0.961	0.449	0.004	0.392	-21	7.04	7.07	0.819	0.313	0.017	0.424
28	6.92	6.80	0.233				28	6.73	6.90	0.202			
VFAt (mmol/L)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	79.86	73.54	0.461	0.849	0.016	0.227	-21	79.03	78.71	0.961	0.089	0.046	0.090
28	88.34	96.99	0.315				28	96.79	80.30	0.017			
Acetic (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	70.01	70.70	0.612	0.611	<0.0001	0.230	-21	68.12	67.02	0.495	0.516	0.001	0.109
28	63.90	62.23	0.221				28	61.56	64.23	0.116			
Propionic (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	16.00	16.76	0.583	0.022	<0.0001	0.071	-21	18.42	19.93	0.249	0.307	0.032	0.604
28	18.66	22.99	0.003				28	20.71	21.37	0.622			
Butyric (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	10.25	9.06	0.090	0.010	0.007	0.801	-21	9.20	8.94	0.673	0.008	<0.0001	0.005
28	11.95	10.50	0.041				28	12.60	9.86	0.000			

Isobutyric (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	1.07	0.98	0.574	0.036	0.497	0.203	-21	1.17	1.14	0.786	0.648	0.301	0.929
28	1.30	0.91	0.017				28	1.09	1.04	0.700			
Valerianic (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	0.83	0.86	0.833	0.754	0.000	0.530	-21	1.03	1.08	0.665	0.530	0.001	0.228
28	1.48	1.36	0.505				28	1.47	1.31	0.198			
Isovalerianic (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	1.45	1.21	0.276	0.013	0.006	0.246	-21	1.66	1.56	0.549	0.205	0.117	0.702
28	2.11	1.50	0.008				28	1.91	1.72	0.257			
Hexanoic (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	0.36	0.39	0.652	0.454	0.030	0.308	-21	0.37	0.31	0.311	0.035	0.001	0.393
28	0.55	0.47	0.204				28	0.57	0.44	0.034			
Heptanoic (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	0.03	0.03	0.572	0.625	0.004	0.355	-21	0.02	0.02	0.619	0.317	0.002	0.745
28	0.05	0.04	0.293				28	0.04	0.04	0.352			

C2/C3

I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	4.40	4.25	0.576	0.070	<0.0001	0.187	-21	3.72	3.47	0.309	0.640	0.005	0.344
28	3.48	2.86	0.024				28	3.01	3.09	0.753			

C2C4/C3

I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	5.04	4.79	0.410	0.036	<0.0001	0.177	-21	4.22	3.92	0.271	0.368	0.017	0.548
28	4.14	3.35	0.012				28	3.63	3.55	0.780			

Urea (mmol/L)

I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	6.06	3.20	0.113	0.029	0.001	0.882	-21	6.06	4.93	0.381	0.003	<0.0001	0.019
28	11.26	8.04	0.075				28	13.19	7.56	0.000			

Dlact (mg/L)

I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	41.39	27.58	0.112	0.001	0.003	0.163	-21	64.01	32.50	0.032	0.019	<0.0001	0.814
28	72.93	40.46	0.001				28	99.08	64.76	0.023			

Llact (mg/L)

I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	37.13	23.49	0.250	0.005	0.007	0.183	-21	63.33	30.87	0.034	0.020	<0.0001	0.814
28	75.00	37.64	0.003				28	98.01	62.67	0.024			

VFAt = volatile fat acids total; Dlact d = lactic acid; Llact = l lactic acid

Tab 6: Least squares means and associated SEM for apparent digestibility of nutrient and fecal composition (F_parameter) for BS and HO cows during the first (I lactation) and second (II lactation) transition period.

Dig-PG (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	90.23	91.35	0.368	0.073	<0.0001	0.006	-21	94.28	94.19	0.912	0.787	<0.0001	0.922
28	74.98	70.53	0.001				28	79.39	79.17	0.802			
Dig-NDF (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	63.93	67.87	0.146	0.631	0.097	0.140	-21	77.23	77.96	0.626	0.401	0.207	0.848
28	70.44	68.27	0.432				28	75.66	76.79	0.464			
Dig-starch (%)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	92.48	94.97	0.003	0.132	<0.0001	0.012	-21	98.53	98.65	0.861	0.693	0.158	0.876
28	97.46	96.74	0.386				28	97.76	98.02	0.699			
F_ash (% DM)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	92.48	94.97	0.003	0.132	<0.0001	0.012	-21	98.53	98.65	0.861	0.693	0.158	0.876
28	97.46	96.74	0.386				28	97.76	98.02	0.699			
F_PG (% DM)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	92.48	94.97	0.003	0.132	<0.0001	0.012	-21	98.53	98.65	0.861	0.693	0.158	0.876
28	97.46	96.74	0.386				28	97.76	98.02	0.699			

F_NDF (% DM)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	61.26	64.97	0.011	0.237	<0.0001	0.015	-21	67.79	68.34	0.651	0.178	<0.0001	0.573
28	55.70	54.62	0.451				28	58.25	59.90	0.198			
F_ADF (% DM)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	47.69	49.26	0.038	0.237	<0.0001	0.087	-21	51.45	51.33	0.933	0.500	<0.0001	0.421
28	40.32	40.08	0.758				28	39.44	40.87	0.301			
F_ADL (% DM)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	11.29	12.09	0.303	0.389	0.679	0.041	-21	17.59	17.47	0.895	0.928	0.198	0.924
28	12.80	11.06	0.035				28	16.54	16.57	0.977			
F_uNDF (% DM)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	41.29	41.24	0.973	0.134	<0.0001	0.125	-21	42.44	41.07	0.452	0.296	0.001	0.927
28	37.65	34.66	0.033				28	37.37	35.77	0.398			
F_starch (% DM)													
I lactation							II lactation						
DIM	BS	HO	P	BREED	DIM	BREED*DIM	DIM	BS	HO	P	BREED	DIM	BREED*DIM
-21	1.17	0.87	0.174	0.827	<0.0001	0.099	-21	0.59	0.51	0.826	0.494	<0.0001	0.662
28	2.48	2.71	0.309				28	2.53	2.20	0.428			

Dig-PG = protein apparent digestibility; Dig-NDF = NDF apparent digestibility; Dig-starch = starch apparent digestibility.

Tab 7: Least squares means and associated SEM for detected feces VOC (*volatile organic compounds*) for BS and HO cows during the first (I lactation) and second (II lactation) transition period.

VOC (mmol/DM)												
	I lactation						II lactation					
Items	BS	HO	SEM	BREED	DIM	BREED*DIM	BS	HO	SEM	BREED	DIM	BREED*DIM
acetaldehyde	1.93	0.89	0.54	0.07	0.61	0.04	0.59	0.51	0.83	0.49	0.00	0.66
ethanol	2.10	2.08	0.83	0.98	0.00	0.81	5.57	6.59	2.36	0.67	0.08	0.72
acetic	125.75	129.37	9.77	0.72	<0.0001	0.80	148.59	138.11	17.89	0.57	<0.0001	0.91
propionic	31.24	30.28	2.10	0.65	<0.0001	0.59	30.32	28.64	3.91	0.67	<0.0001	0.74
iso_butyric	2.58	2.50	0.26	0.76	0.04	0.26	2.30	2.23	0.23	0.77	0.02	0.88
butyric	14.52	14.80	1.29	0.83	<0.0001	0.74	17.63	16.04	3.69	0.67	<0.0001	0.75
iso_valerianic	8.15	6.53	2.26	0.48	0.03	0.58	8.27	6.54	3.09	0.58	0.03	0.49
valerianic	2.16	2.20	0.24	0.85	0.03	0.88	1.96	1.82	0.26	0.60	<0.0001	0.68
caproic	1.13	0.81	0.40	0.43	0.98	0.20	0.92	0.66	0.35	0.47	0.04	0.38
ALCOHOLS	2.24	2.16	0.90	0.94	0.00	0.90	5.69	7.01	2.44	0.60	0.07	0.76
VFA TOT	185.53	186.47	13.78	0.95	<0.0001	0.99	209.81	194.04	27.35	0.57	<0.0001	0.89
acetic %	68.13	69.61	1.05	0.18	0.05	0.20	72.19	72.92	1.54	0.64	0.00	0.16
propionic %	16.87	16.36	0.65	0.45	0.58	0.18	14.32	14.18	0.48	0.78	0.00	0.65
butyruc %	7.55	7.55	0.35	1.00	<0.0001	0.83	7.52	7.45	0.90	0.93	<0.0001	0.68
valerianic %	1.28	1.28	0.13	0.99	0.02	1.00	1.01	1.01	0.09	0.98	0.02	0.67
iso_valerianic %	4.08	3.31	0.90	0.41	0.67	0.64	3.34	2.79	0.91	0.55	0.42	0.13
iso_butyric %	1.50	1.48	0.17	0.91	0.00	0.17	1.27	1.39	0.11	0.28	<0.0001	0.16
caproic %	0.60	0.45	0.21	0.47	0.01	0.31	0.38	0.26	0.14	0.41	0.49	0.07
Ph	7.19	7.21	0.05	0.57	0.00	0.16	7.08	7.00	0.05	0.13	0.72	0.67

Tab n 8: Least squares means and associated SEM for milk production and quality, BW and production efficiency for BS and HO cows during the first (I lactation) and second (II lactation) transition period.

MY (kg/d)														
	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	15.67	17.19	0.257	1.31	0.024	<0.0001	0.004	23.39	27.33	0.097	2.27	0.011	<0.0001	0.007
2	20.91	24.50	0.012					30.45	36.86	0.010				
3	24.18	27.62	0.016					34.00	40.04	0.015				
4	25.17	28.92	0.009					34.92	43.01	0.002				
Fat (%)														
	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	5.05	4.70	0.028	0.15	0.279	<0.0001	0.001	4.73	4.49	0.135	0.15	0.484	<0.0001	0.001
2	4.60	4.51	0.591					4.65	4.65	0.985				
3	4.42	4.43	0.964					4.50	4.50	0.971				
4	4.43	4.24	0.210					4.52	4.35	0.260				
Protein (%)														
	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	3.82	3.45	0.000	0.07	0.000	<0.0001	0.004	3.39	3.28	0.254	0.09	0.060	<0.0001	0.356
2	3.56	3.38	0.015					3.50	3.30	0.043				
3	3.61	3.37	0.002					3.57	3.36	0.037				
4	3.73	3.41	0.000					3.59	3.42	0.074				

Lactose (%)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	4.99	5.31	0.008	0.11	0.208	<0.0001	0.000	5.27	5.44	0.076	0.09	0.590	<0.0001	0.000
2	5.14	5.26	0.285					5.15	5.22	0.449				
3	4.97	5.02	0.643					5.10	5.05	0.643				
4	4.85	4.90	0.646					4.96	4.95	0.926				

Milk Urea (mg/100ml)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	30.62	28.90	0.621	3.53	0.067	0.834	0.210	28.33	25.00	0.235	2.82	0.031	0.049	0.196
2	33.66	24.77	0.017					33.11	24.40	0.004				
3	32.53	27.22	0.142					31.61	27.20	0.126				
4	34.00	27.54	0.076					32.86	27.60	0.070				

SCC (.000 c/ml)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	21.11	38.60	0.005	5.8554	0.116	0.005	0.031	47.33	50.20	0.668	6.74	0.043	0.069	0.030
2	33.43	35.80	0.703					41.77	54.00	0.080				
3	23.78	23.50	0.962					36.14	51.70	0.028				
4	28.56	38.50	0.097					32.14	52.00	0.006				

Log SCC

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	1.29	1.57	0.002	0.09	0.062	0.007	0.019	1.66	1.69	0.657	0.07	0.052	0.020	0.062
2	1.48	1.52	0.650					1.61	1.72	0.123				
3	1.33	1.35	0.862					1.55	1.69	0.051				
4	1.38	1.57	0.032					1.49	1.68	0.006				

Cond (mS/cm)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	8.67	8.74	0.674	0.18	0.658	<0.0001	0.000	8.89	8.97	0.707	0.20	0.641	0.021	0.267
2	8.55	8.56	0.961					8.81	8.83	0.914				
3	8.61	8.45	0.404					8.78	8.96	0.373				
4	8.72	8.47	0.184					8.88	8.97	0.670				

BW (kg)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	601.87	539.09	0.010	21.62	0.005	<0.0001	0.025	648.97	608.99	0.122	24.56	0.116	<0.0001	0.362
2	584.37	515.71	0.005					639.06	594.16	0.085				
3	573.62	500.16	0.003					629.63	591.30	0.137				
4	568.71	497.63	0.004					631.61	592.99	0.134				

DMI/BW (%)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	1.93	2.18	0.054	0.13	0.002	<0.0001	0.143	2.21	2.94	0.005	0.23	0.007	<0.0001	0.020
2	2.51	3.01	0.000					2.94	3.39	0.071				
3	3.04	3.32	0.033					3.12	3.98	0.002				
4	3.15	3.58	0.002					3.30	3.97	0.010				

ECM (Kg/d)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	14.63	14.87	0.831	1.13	0.136	<0.0001	0.002	20.18	22.55	0.228	1.91	0.033	<0.0001	0.024
2	18.04	20.47	0.044					26.26	31.16	0.018				
3	20.42	22.69	0.059					28.98	33.30	0.036				
4	21.48	23.23	0.138					29.91	35.18	0.012				

FCM (Kg/d)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	18.14	18.98	0.569	1.46	0.063	<0.0001	0.001	25.87	29.20	0.197	2.50	0.022	<0.0001	0.008
2	22.82	26.31	0.026					33.36	40.29	0.012				
3	25.74	29.25	0.025					36.65	42.93	0.021				
4	26.81	29.89	0.046					37.77	45.17	0.008				

FE

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	1.58	1.68	0.412	0.1261	0.059	0.516	0.597	1.83	1.66	0.490	0.2354	0.639	0.464	0.001
2	1.46	1.62	0.194					1.67	2.01	0.159				
3	1.44	1.71	0.040					1.76	1.82	0.792				
4	1.42	1.67	0.058					1.74	1.92	0.449				

FAT+PROT (kg/d)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	1.38	1.40	0.896	0.1056	0.159	<0.0001	0.002	1.89	2.12	0.226	0.1786	0.035	<0.0001	0.032
2	1.71	1.93	0.049					2.48	2.92	0.022				
3	1.94	2.14	0.074					2.75	3.14	0.041				
4	2.05	2.21	0.165					2.84	3.33	0.012				

FAT+PROT/DMI

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	0.14	0.14	0.800	0.0106	0.301	0.007	0.334	0.15	0.13	0.229	0.018	0.881	0.390	<0.0001
2	0.12	0.13	0.383					0.14	0.16	0.201				
3	0.12	0.13	0.117					0.14	0.14	0.952				
4	0.12	0.13	0.288					0.14	0.15	0.677				

EBNRC (Mcal/d)														
	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	-4.54	-4.45	0.946	1.379	0.306	<0.0001	0.030	-6.94	-3.46	0.2745	2.223	0.737	0.2148	0.001
2	-3.05	-3.45	0.764					-5.59	-7.72	0.5001				
3	-0.35	-3.48	0.023					-6.76	-4.16	0.4143				
4	-0.42	-1.63	0.351					-5.78	-5.67	0.972				
NEBNRC (Mcal/d)														
	I lactation							II lactation						
WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK	BS	HO	P	SEM	BREED	WEEK	BREED*WEEK
1	-3.20	-2.65	0.688	1.454	0.409	<0.0001	0.049	-5.28	-1.94	0.306	2.279	0.836	0.0438	0.002
2	-0.98	-1.42	0.752					-3.80	-6.20	0.458				
3	0.68	-1.95	0.065					-5.05	-3.95	0.734				
4	0.22	-1.21	0.293					-5.91	-5.38	0.870				

MY = milk yield; SCC = somatic cell count; Cond = conductibility; BW = body weight; ECM = energy corrected milk; FCM = fat corrected milk 4%; FE = feed efficiency; EBNRC = Energy balance (not considering body reserve mobilization) according to NRC; NEBNRC = Net energy balance according NRC.

Tab n 9: Least squares means and associated SEM for energy metabolism parameters (Glucose. Urea. NEFA; BHOB) for BS and HO cows during the first (I lactation) and second (II lactation) transition period.

Glucose (mmol/L)														
	I lactation							II lactation						
WEEK	BS	HO	P	SEM	DIM	BREED	BREED*WEEK	BS	HO	P	SEM	DIM	BREED	BREED*WEEK
-21	4.70	4.89	0.24	0.184	<0.0001	0.108	0.961	4.17	4.40	0.168	0.167	<0.0001	0.072	0.637
-14	4.71	4.86	0.39					4.27	4.35	0.662				
-7	4.61	4.84	0.18					4.10	4.47	0.029				
3	4.41	4.43	0.87					3.77	3.85	0.643				
7	4.14	4.39	0.13					3.68	3.84	0.315				
14	4.12	4.29	0.29					3.73	4.01	0.087				
21	4.27	4.36	0.62					3.94	4.15	0.209				
28	4.32	4.39	0.63					3.89	4.22	0.050				
Urea (mmol/L)														
	I lactation							II lactation						
WEEK	BS	HO	P	SEM	DIM	BREED	BREED*WEEK	BS	HO	P	SEM	DIM	BREED	BREED*WEEK
-21	4.56	3.72	0.193	0.704	0.002	0.240	0.958	5.91	5.51	0.514	0.628	0.001	0.422	0.913
-14	4.60	4.07	0.453					6.45	6.29	0.805				
-7	4.48	4.08	0.545					6.30	5.92	0.544				
3	5.67	4.98	0.284					5.20	4.36	0.164				
7	5.07	4.85	0.738					5.91	5.30	0.316				
14	5.65	4.79	0.181					5.85	5.63	0.720				
21	5.45	5.28	0.792					6.20	6.08	0.855				
28	5.34	5.14	0.747					5.66	5.81	0.812				

NEFA (mmol/L)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	DIM	BREED	BREED*WEEK	BS	HO	P	SEM	DIM	BREED	BREED*WEEK
-21	0.36	0.50	0.272	0.136	<0.0001	0.032	0.003	0.32	0.20	0.224	0.100	<0.0001	0.749	0.526
-14	0.38	0.42	0.744					0.26	0.21	0.663				
-7	0.43	0.51	0.550					0.31	0.20	0.248				
3	0.74	0.98	0.060					0.55	0.62	0.430				
7	0.64	1.20	0.000					0.44	0.56	0.212				
14	0.44	0.79	0.006					0.44	0.41	0.730				
21	0.50	0.70	0.116					0.43	0.48	0.615				
28	0.46	0.52	0.602					0.40	0.36	0.646				

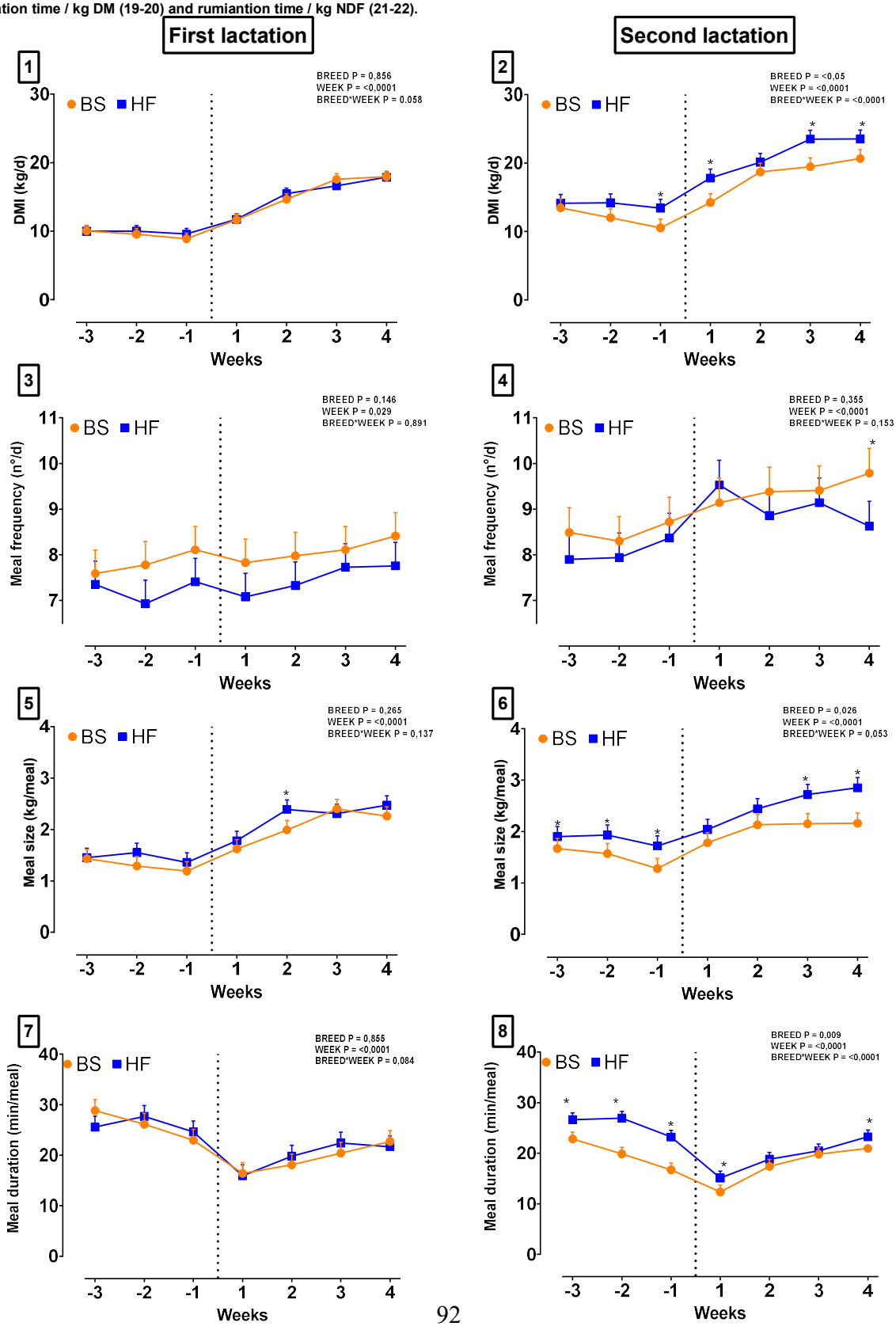
BHOB (mmol/L)

	I lactation							II lactation						
WEEK	BS	HO	P	SEM	DIM	BREED	BREED*WEEK	BS	HO	P	SEM	DIM	BREED	BREED*WEEK
-21	0.35	0.38	0.810	0.125	<0.0001	0.761	0.619	0.47	0.32	0.036	0.079	<0.0001	0.031	0.758
-14	0.42	0.33	0.482					0.44	0.46	0.780				
-7	0.39	0.35	0.689					0.41	0.38	0.722				
3	0.80	0.80	0.993					0.58	0.51	0.412				
7	0.81	0.92	0.329					0.64	0.59	0.451				
14	0.66	0.82	0.158					0.59	0.46	0.104				
21	0.66	0.72	0.545					0.49	0.43	0.443				
28	0.61	0.54	0.520					0.51	0.39	0.125				

BCS														
	I lactation							II lactation						
WEEK	BS	HO	P<	SEM	DIM	BREED	BREED*WEEK	BS	HO	P<	SEM	DIM	BREED	BREED*WEEK
-21	3,6667	3,575	0,401	0,1077	0,047	<0.0001	<0.0001	3,4722	3,1	0,002	0,1086	0,001	<0.0001	<0,0001
-14	3,5833	3,575	0,939					3,4444	3,1	0,004				
-7	3,5	3,4	0,360					3,3889	3,05	0,004				
3	3,3611	3,225	0,215					3,1944	2,875	0,007				
7	3,2778	3	0,015					3,2222	2,825	0,001				
14	3,1389	2,9	0,033					3,1382	2,75	0,002				
21	3,0556	2,775	0,014					3,2007	2,675	<0,0001				
28	3,1389	2,625	<0,0001					3,2632	2,675	<0,0001				

BCS = body condition score; NEFA = non esterified fatty acids; BHOB = beta hydroxybutyrate.

Figure A. Feeding behaviour, lying, acitivty and rumiantion time during the transition period of the first and second lacation: dry matter intake (1-2), meal frequency (3-4), meal size (5-6), meal duration (7-8), feeding time (9-10), feeding rate (11-12), lying time (13-14), activity time (15-16), rumination time (17-18), rumination time / kg DM (19-20) and rumiantion time / kg NDF (21-22).



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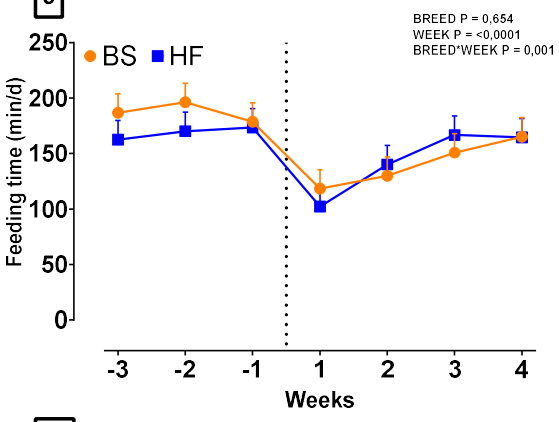

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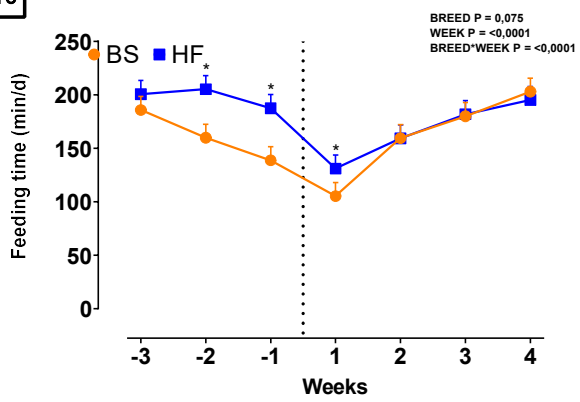

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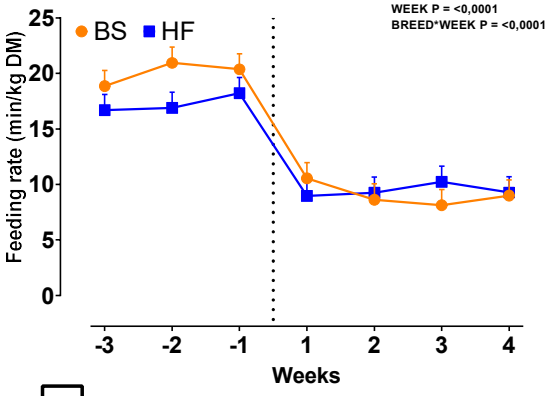

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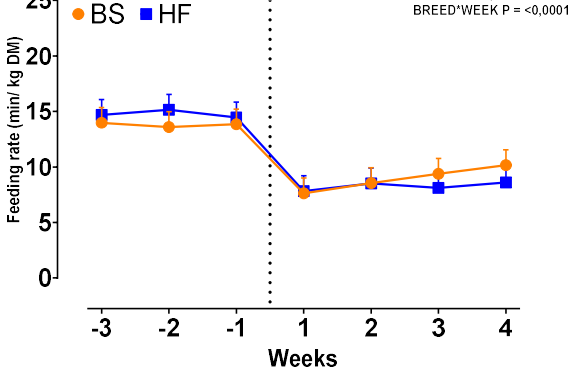

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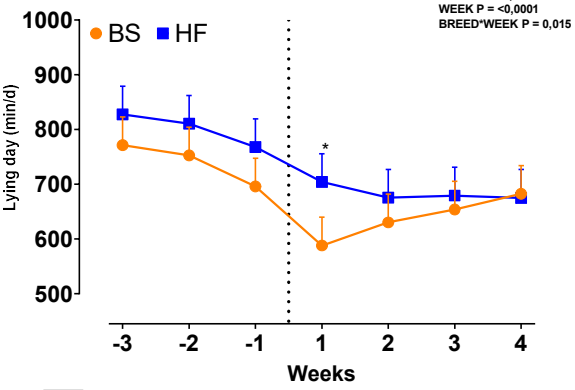

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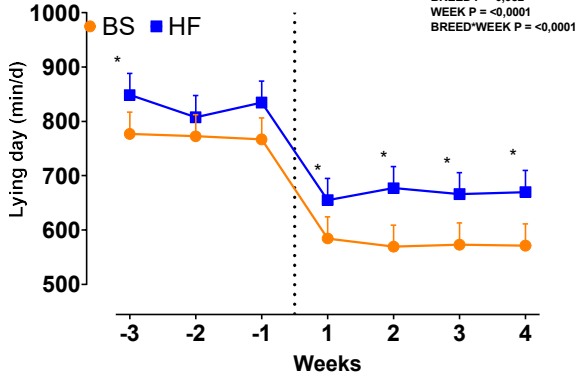

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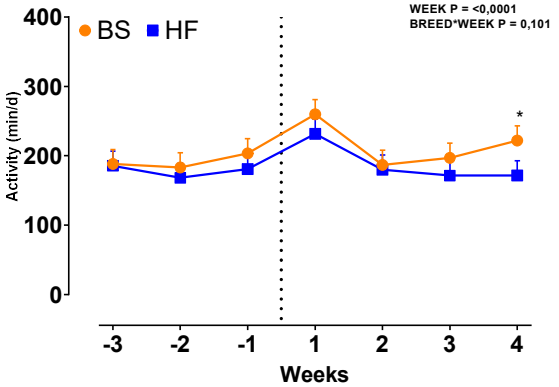

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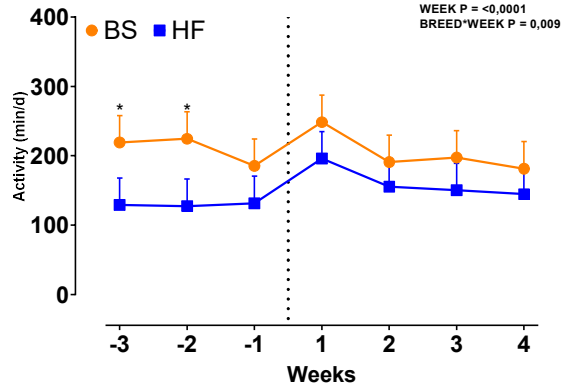


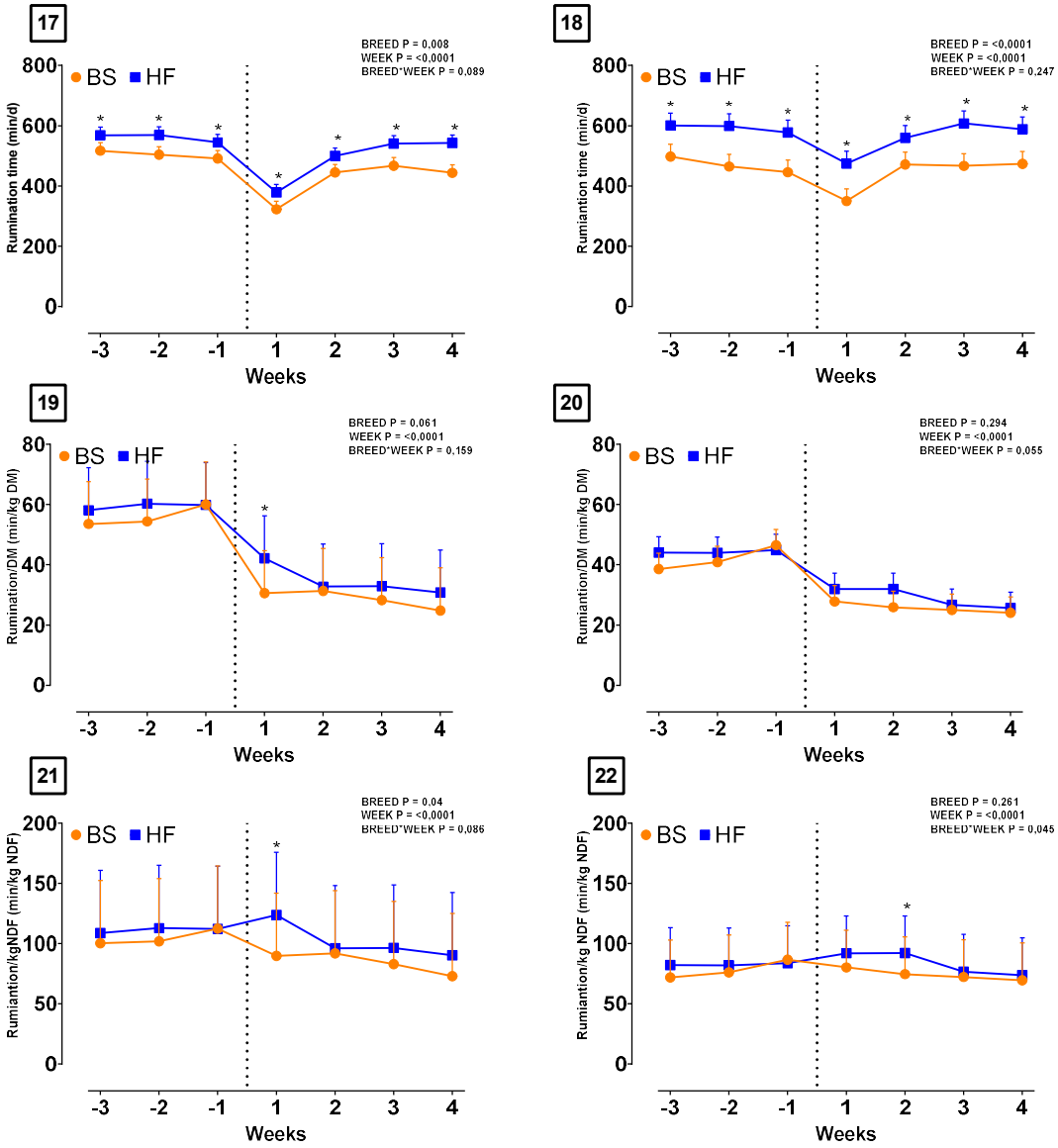

17
BREED P = 0,008
WEEK P = <0,0001
BREED*WEEK P = 0,089
BS HF
Rumination time (min/d)
800
600
400
200
0
-3 -2 -1 1 2 3 4
Weeks
18
BREED P = <0,0001
WEEK P = <0,0001
BREED*WEEK P = 0,247
BS HF
Rumiantion time (min/d)
800
600
400
200
0
-3 -2 -1 1 2 3 4
Weeks
19
BREED P = 0,061
WEEK P = <0,0001
BREED*WEEK P = 0,159
BS HF
Rumination/DM (min/kg DM)
80
60
40
20
0
-3 -2 -1 1 2 3 4
Weeks
20
BREED P = 0,294
WEEK P = <0,0001
BREED*WEEK P = 0,055
BS HF
Rumiantion/DM (min/kg DM)
80
60
40
20
0
-3 -2 -1 1 2 3 4
Weeks
21
BREED P = 0,04
WEEK P = <0,0001
BREED*WEEK P = 0,086
BS HF
Rumiantion/kgNDF (min/kg NDF)
200
150
100
50
0
-3 -2 -1 1 2 3 4
Weeks
22
BREED P = 0,261
WEEK P = <0,0001
BREED*WEEK P = 0,045
BS HF
Rumiantion/kg NDF (min/kg NDF)
200
150
100
50
0
-3 -2 -1 1 2 3 4
Weeks

Figure C. Apparent digestibility of nutrients during the transition period of the first and second lactation: protein (1-2), starch (3.4) and NDF (5-6).

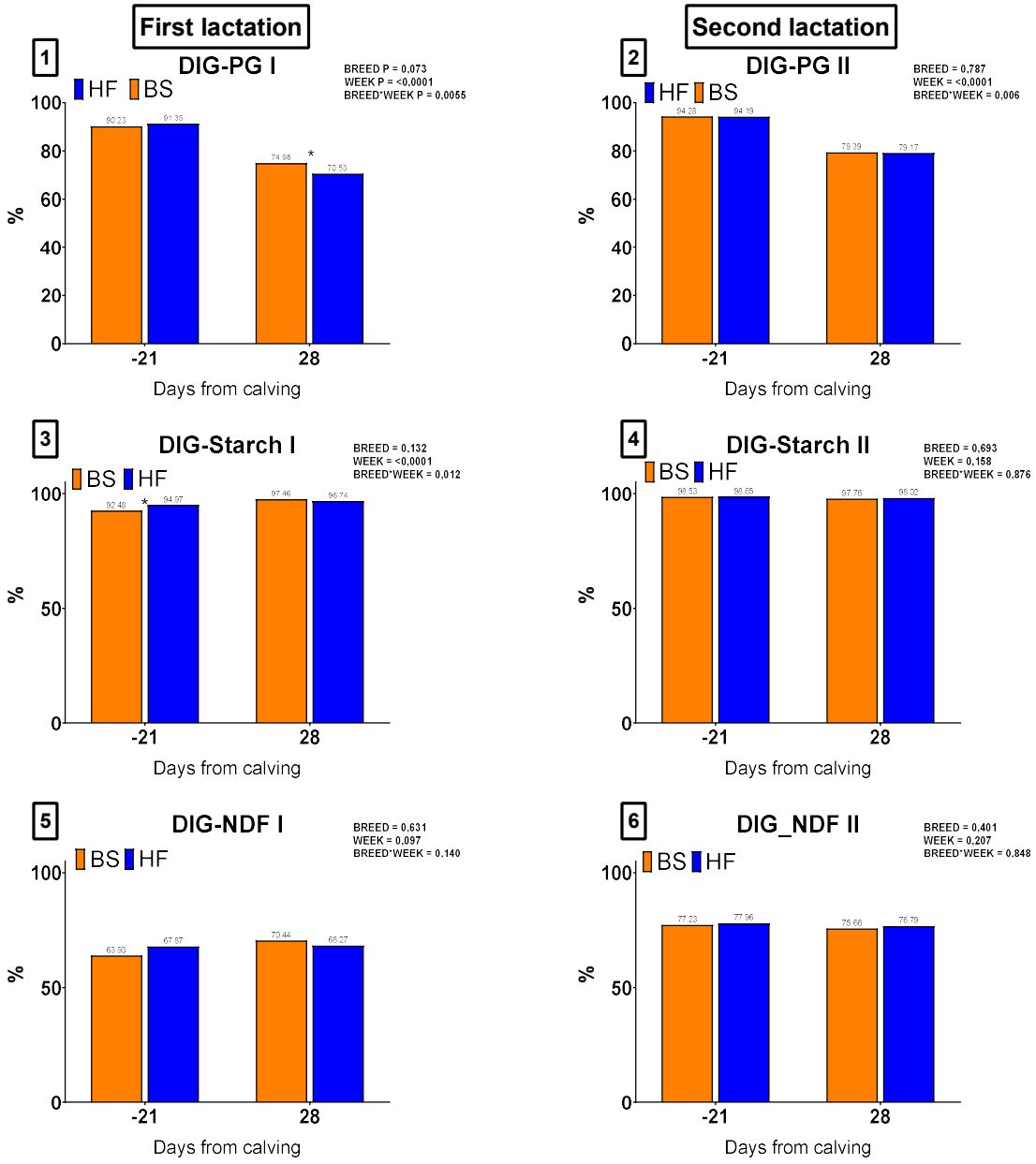
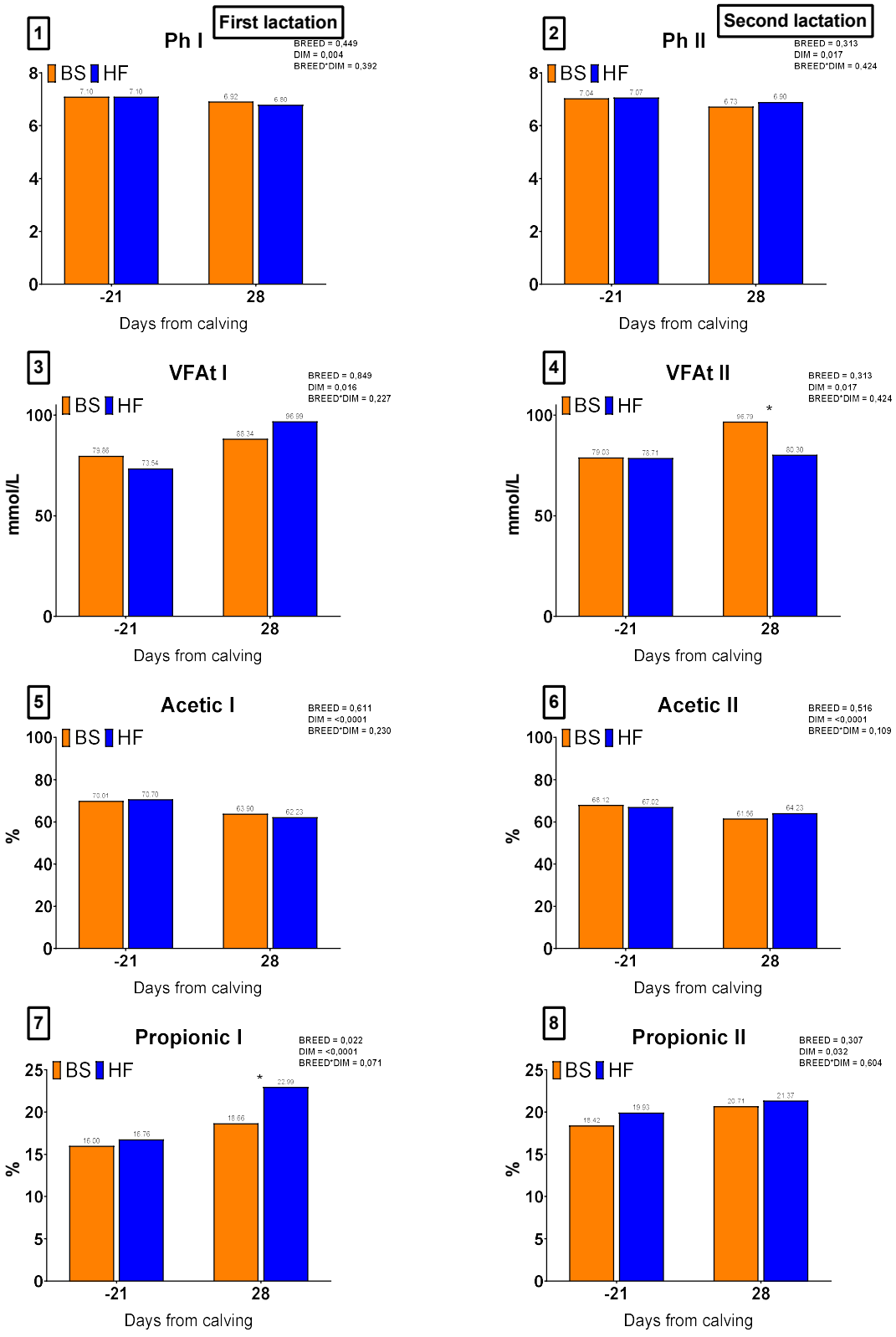
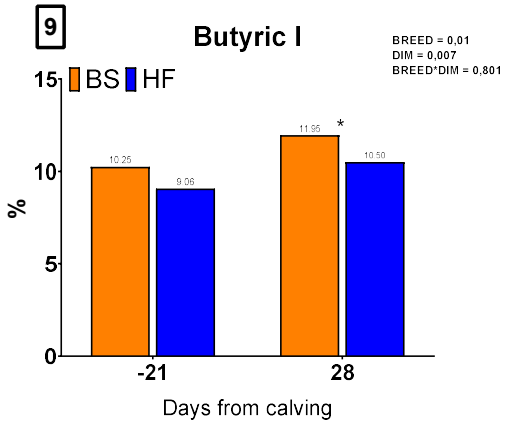
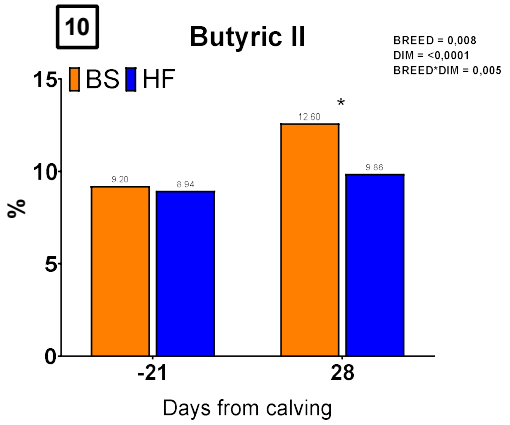
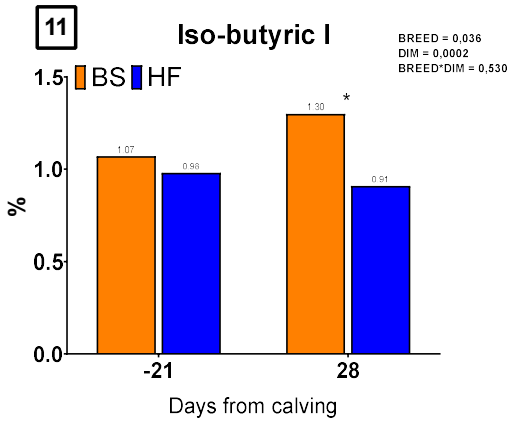


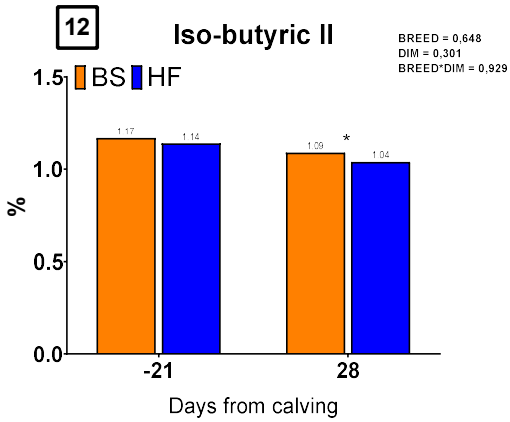
Figure D. Rumen liquid profile during the transition period of the first and second lactation: Ph (1-2), VFAt (3-4), acetic acid (5-6), propionic acid (7-8), butyric acid (9-10), iso-butyric acid (11-12), valerianic acid (13-14), iso-valerianic acid (15-16), urea (17-18), Dlact (19-20) and Llact (21-22) .

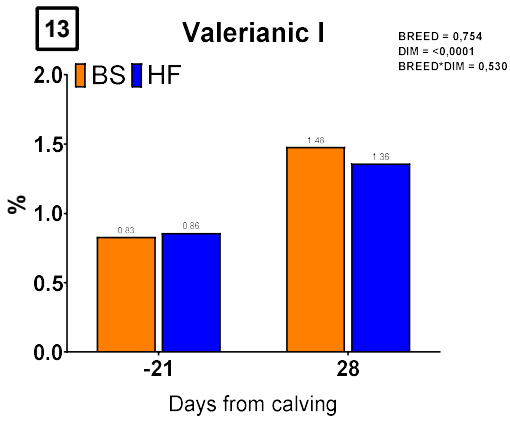


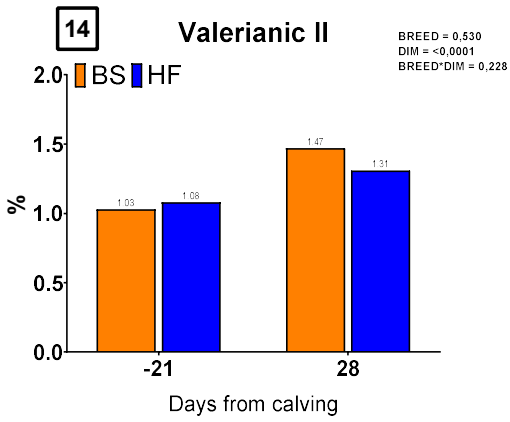

9
Butyric I
BREED = 0,01
DIM = 0,007
BREED*DIM = 0,801
BS
HF
%
10.25
9.06
11.95
*
10.50
-21
28
Days from calving

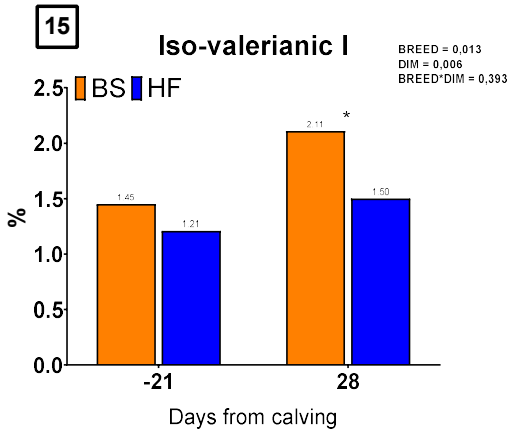

10
Butyric II
BREED = 0,008
DIM = <0,0001
BREED*DIM = 0,005
BS
HF
%
9.20
8.94
12.60
*
9.66
-21
28
Days from calving

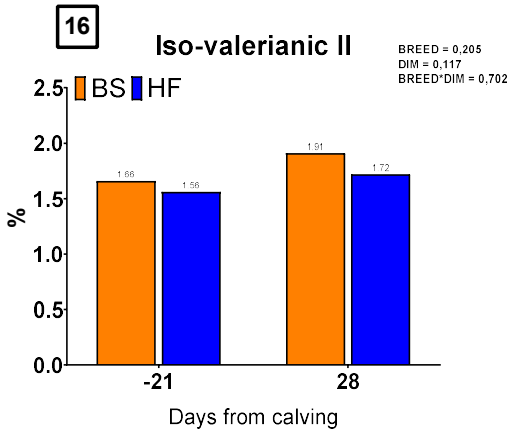

11
Iso-butyric I
BREED = 0,036
DIM = 0,0002
BREED*DIM = 0,530
BS
HF
%
1.07
0.98
1.30
*
0.91
-21
28
Days from calving

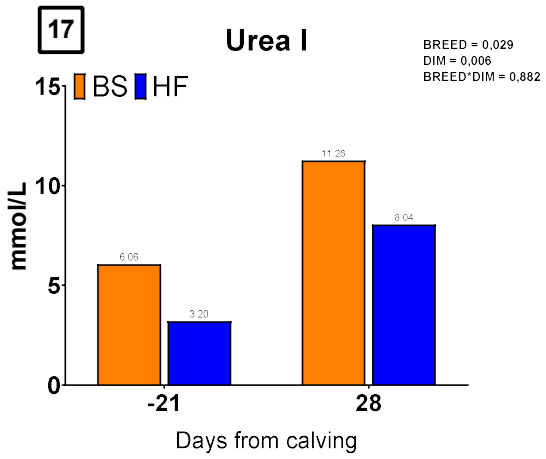

12
Iso-butyric II
BREED = 0,648
DIM = 0,301
BREED*DIM = 0,929
BS
HF
%
1.17
1.14
1.09
*
1.04
-21
28
Days from calving

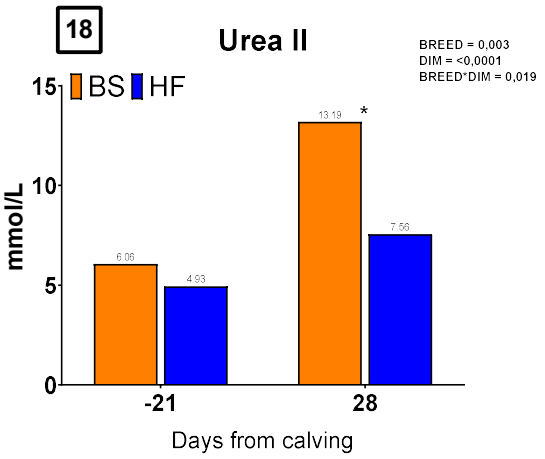

13
Valerianic I
BREED = 0,754
DIM = <0,0001
BREED*DIM = 0,530
BS
HF
%
0.83
0.86
1.46
1.36
-21
28
Days from calving

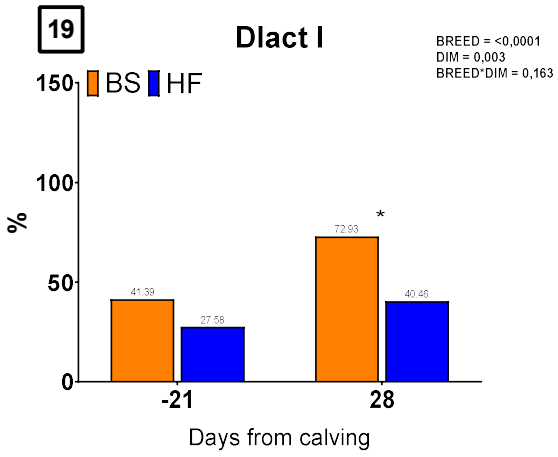

14
Valerianic II
BREED = 0,530
DIM = <0,0001
BREED*DIM = 0,228
BS
HF
%
1.03
1.08
1.47
1.31
-21
28
Days from calving

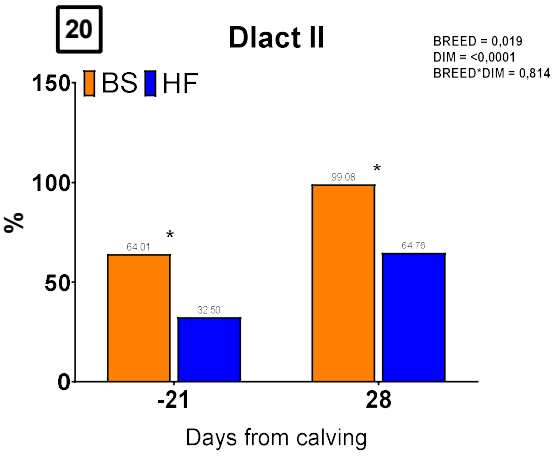

15
Iso-valerianic I
BREED = 0,013
DIM = 0,006
BREED*DIM = 0,393
BS
HF
%
1.45
1.21
2.11
*
1.50
-21
28
Days from calving

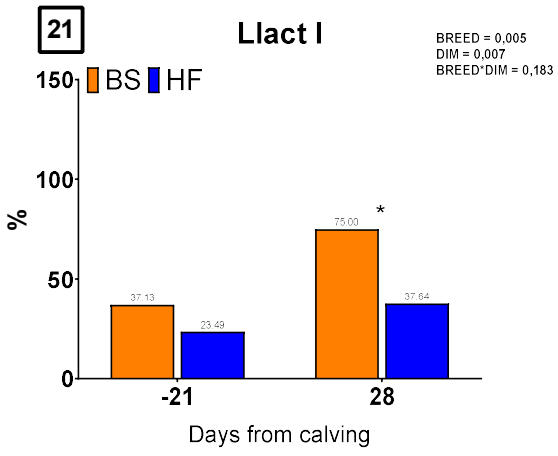

16
Iso-valerianic II
BREED = 0,205
DIM = 0,117
BREED*DIM = 0,702
BS
HF
%
1.66
1.56
1.91
1.72
-21
28
Days from calving


17
Urea I
BREED = 0,029
DIM = 0,006
BREED*DIM = 0,882
BS HF
mmol/L
15
10
5
0
6.06
3.20
11.26
8.04
-21
28
Days from calving


18
Urea II
BREED = 0,003
DIM = <0,0001
BREED*DIM = 0,019
BS HF
mmol/L
15
10
5
0
6.06
4.93
13.19
*
7.56
-21
28
Days from calving


19
Dlact I
BREED = <0,0001
DIM = 0,003
BREED*DIM = 0,163
BS HF
%
150
100
50
0
41.39
27.58
72.93
*
40.46
-21
28
Days from calving


20
Dlact II
BREED = 0,019
DIM = <0,0001
BREED*DIM = 0,814
BS HF
%
150
100
50
0
64.01
*
32.50
99.06
*
64.76
-21
28
Days from calving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Llact I
BREED = 0,005
DIM = 0,007
BREED*DIM = 0,183
BS HF
%
150
100
50
0
37.13
23.49
75.00
*
37.64
-21
28
Days from calving

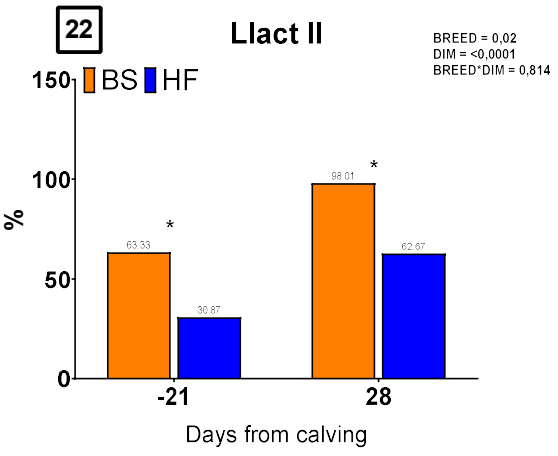
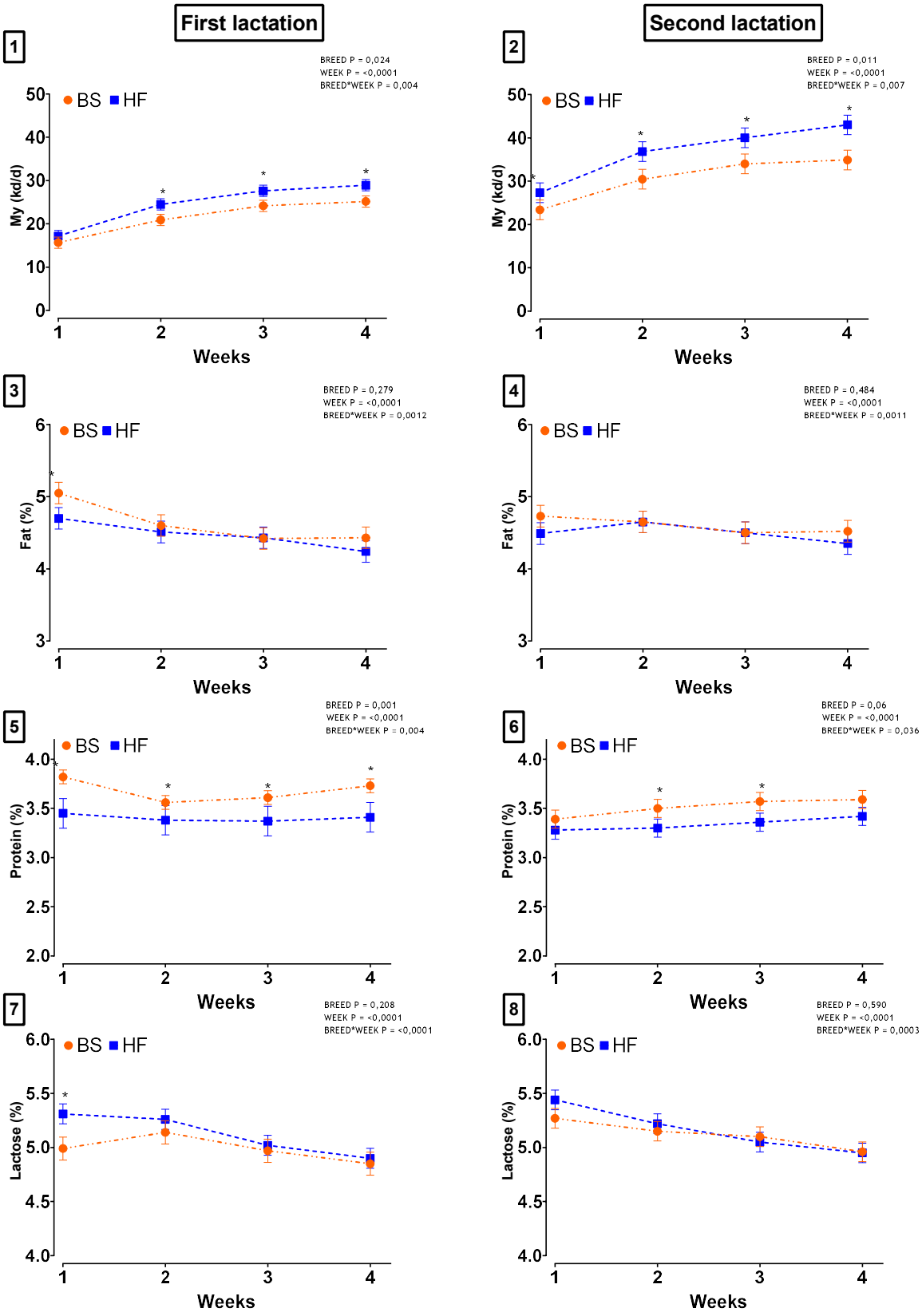
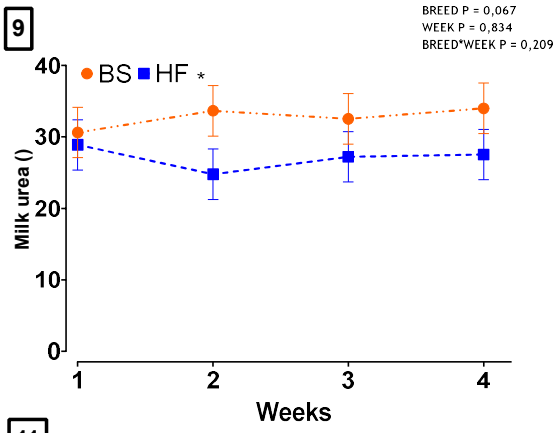
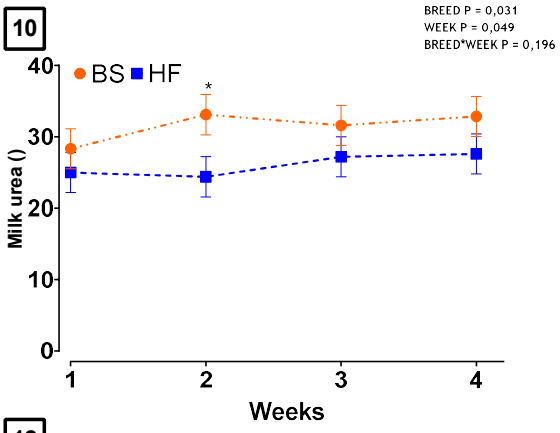
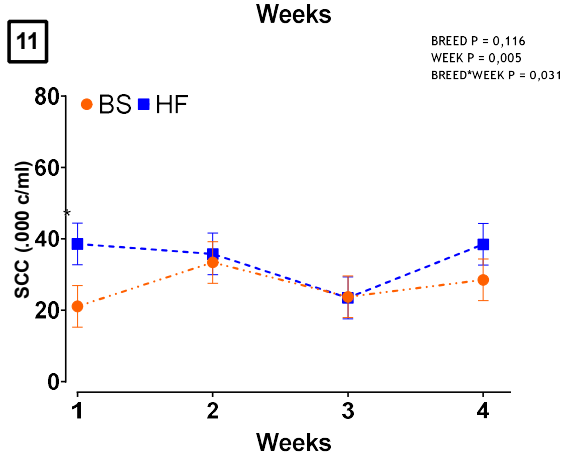

22
Llact II
BREED = 0,02
DIM = <0,0001
BREED*DIM = 0,814
BS HF
%
150
100
50
0
63.33
*
30.87
98.01
*
62.67
-21
28
Days from calving

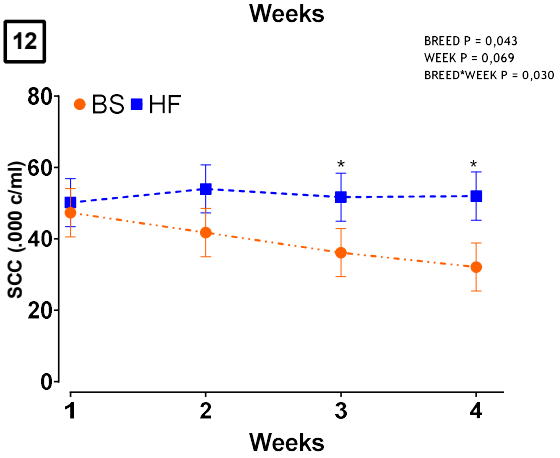
Figure E. Production, milk quality and feed efficiency in the first 4 weeks after calving of the first and second lacation: milk yield (1-2), Fat % (3,4), Protein (5,6), Lactose (7,8), Milk urea (9,10), SCC (11,12), LogSCC (13,14), COND (15,16), BW (17,18), DMI/BW (19,20), ECM (21,22), FCM (23,24), FE (25,26), FAT+PROT (27,28), FAT+PROT/DMI (29,30), EBNRC (31,32) and NEBNRC (33-34)

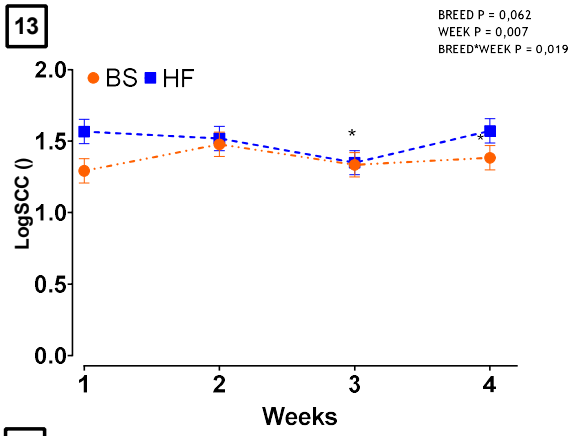


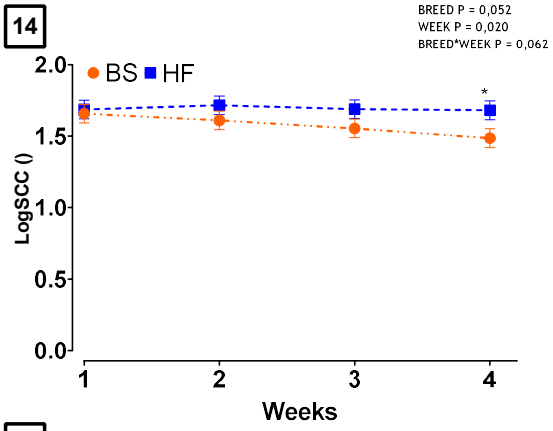

9
BREED P = 0,067
WEEK P = 0,834
BREED*WEEK P = 0,209
BS HF
Milk urea ()
Weeks

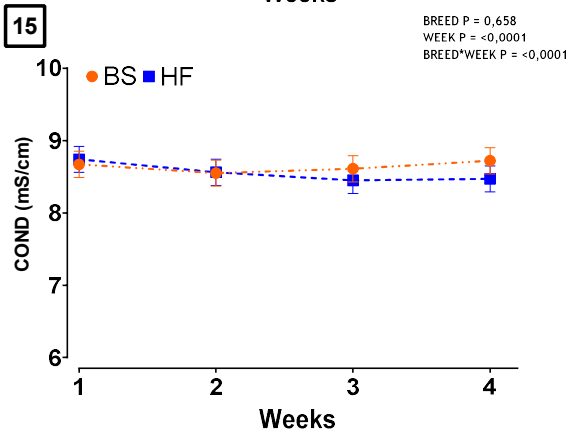

10
BREED P = 0,031
WEEK P = 0,049
BREED*WEEK P = 0,196
BS HF
Milk urea ()
Weeks

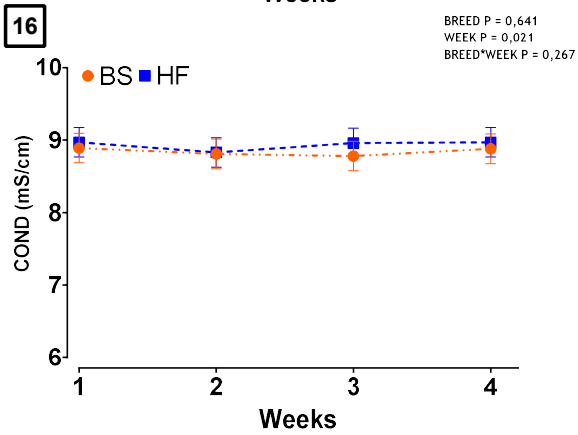

11
BREED P = 0,116
WEEK P = 0,005
BREED*WEEK P = 0,031
BS HF
SCC (.000 c/ml)
Weeks


12
BREED P = 0,043
WEEK P = 0,069
BREED*WEEK P = 0,030
BS HF
SCC (.000 c/ml)
Weeks

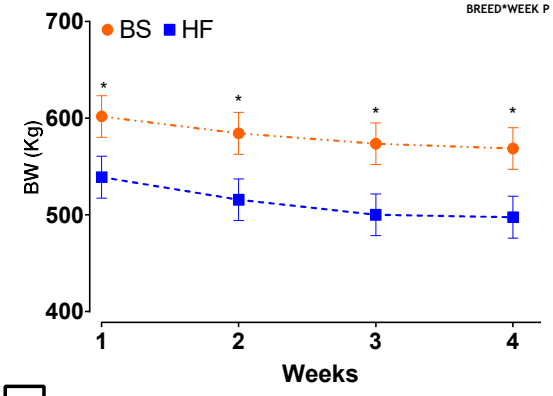

13
BREED P = 0,062
WEEK P = 0,007
BREED*WEEK P = 0,019
BS HF
LogSCC ()
Weeks


14
BREED P = 0,052
WEEK P = 0,020
BREED*WEEK P = 0,062
BS HF
LogSCC ()
Weeks


15
BREED P = 0,658
WEEK P = <0,0001
BREED*WEEK P = <0,0001
BS HF
COND (mS/cm)
Weeks


16
BREED P = 0,641
WEEK P = 0,021
BREED*WEEK P = 0,267
BS HF
COND (mS/cm)
Weeks

17



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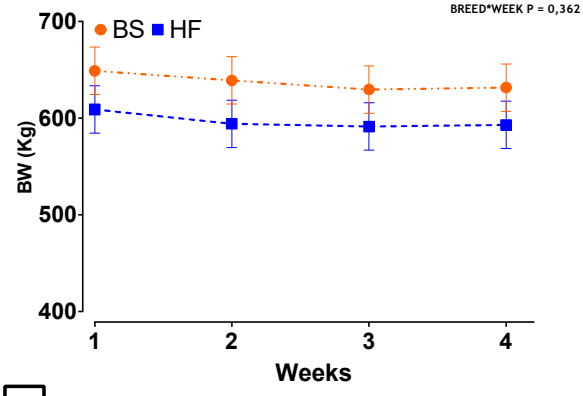

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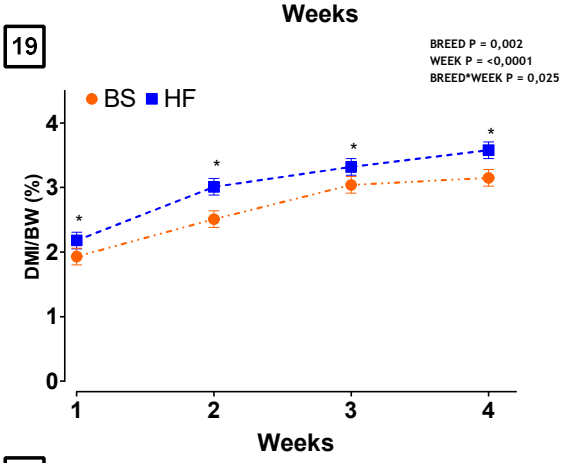

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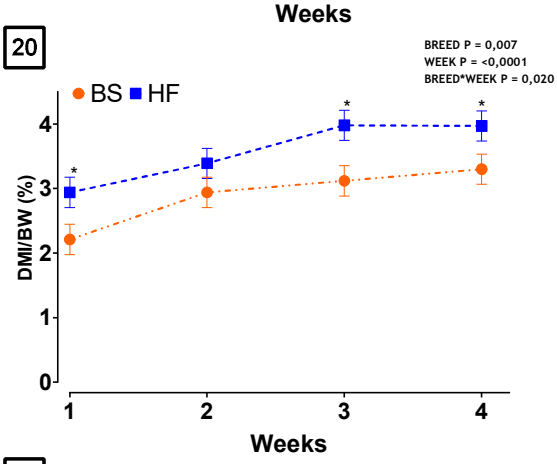

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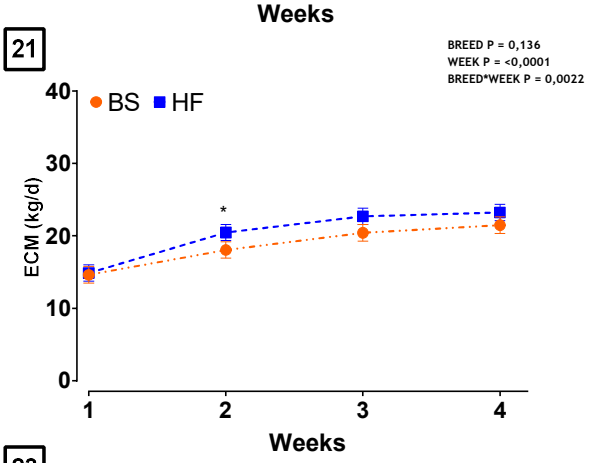

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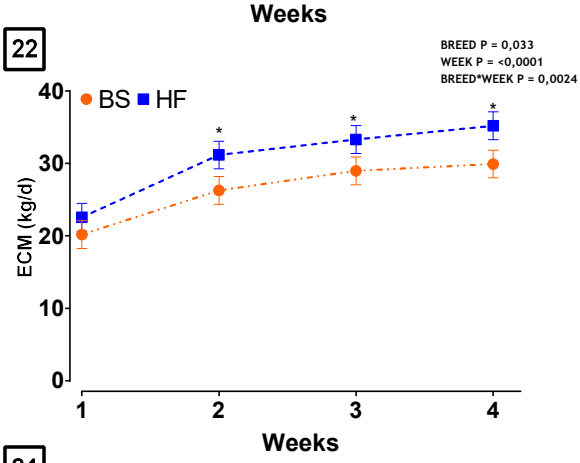

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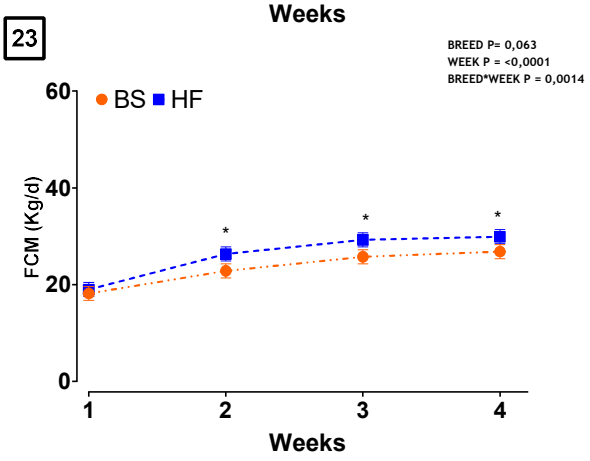

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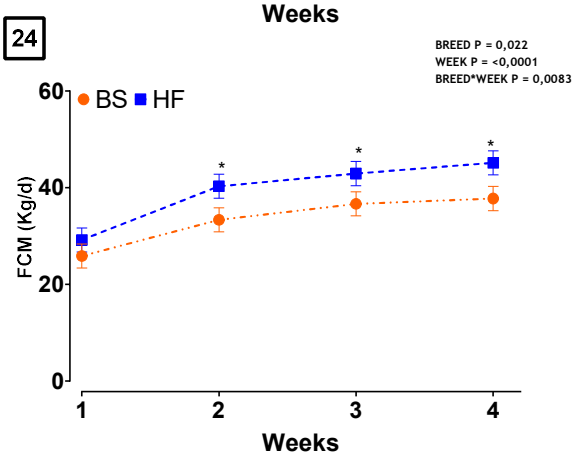

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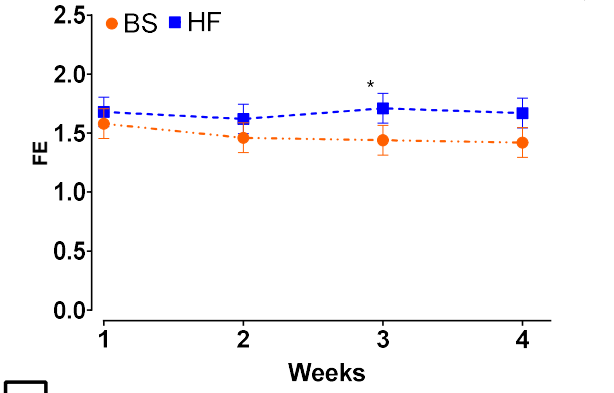

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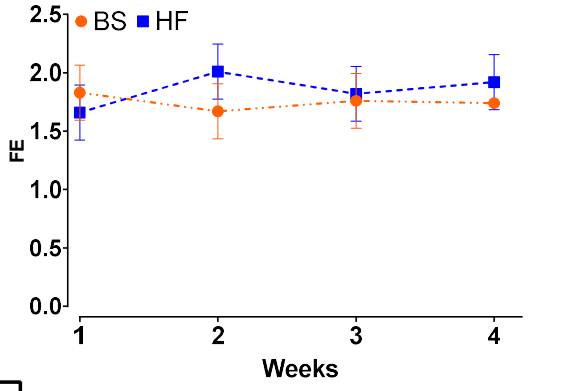

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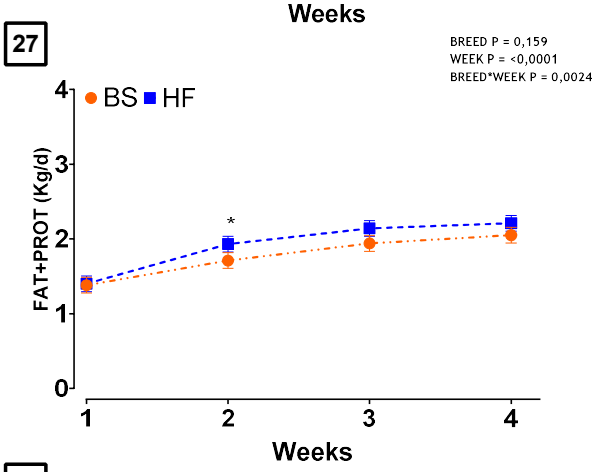

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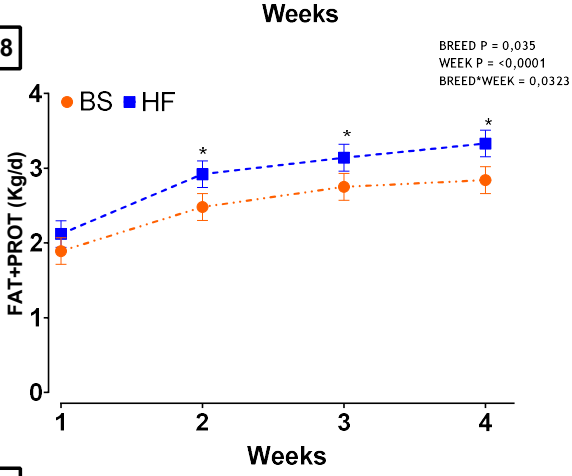

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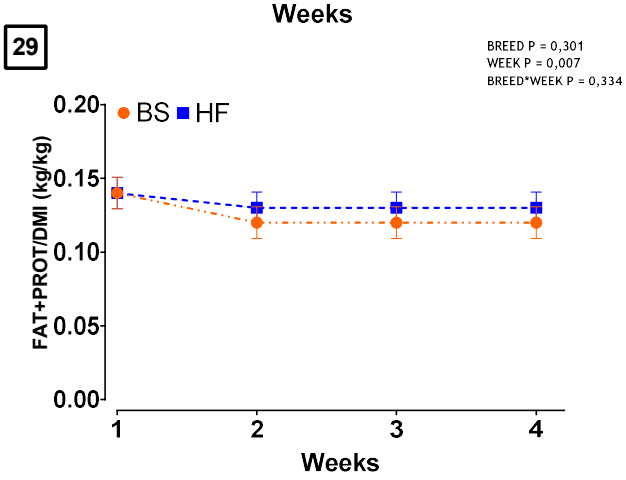

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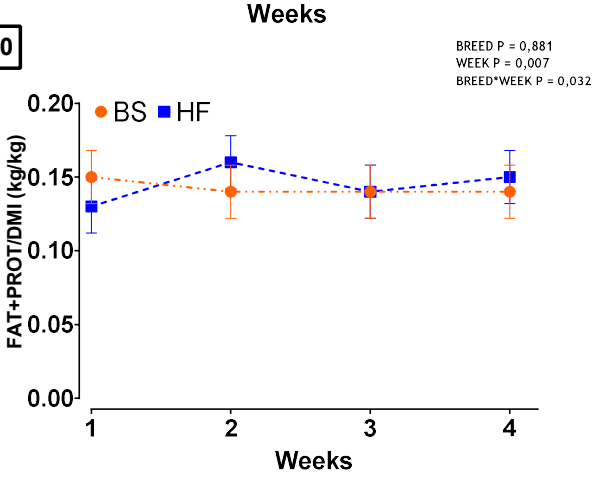


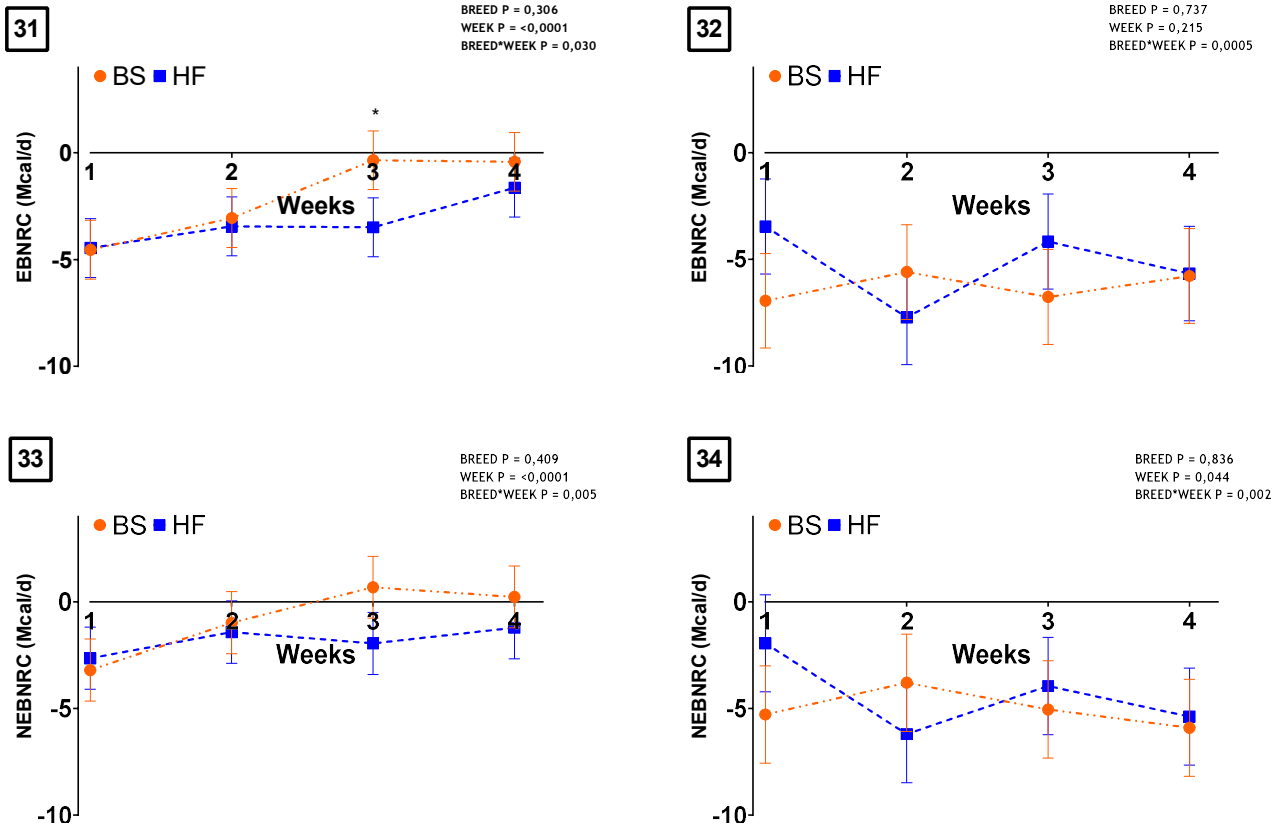


Figure F. Body condition score during the transition period of the first and second lacation (1-2)

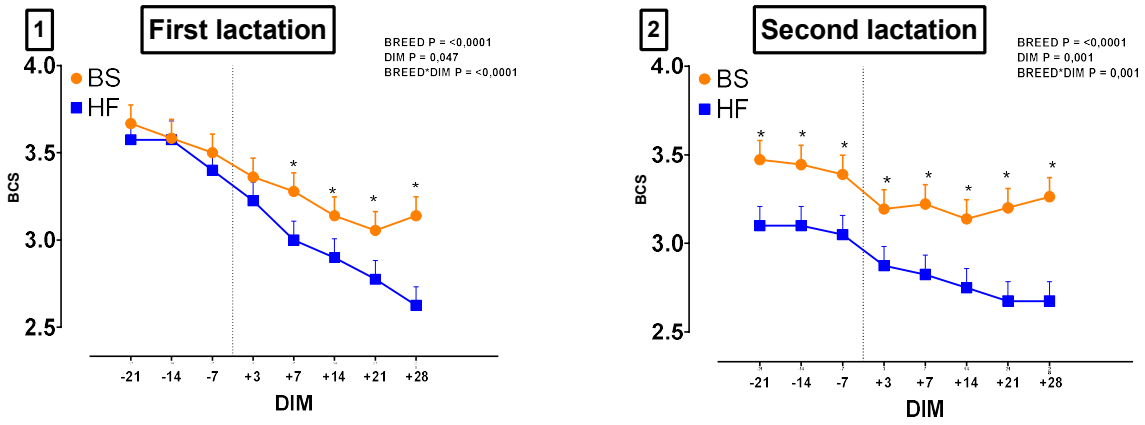
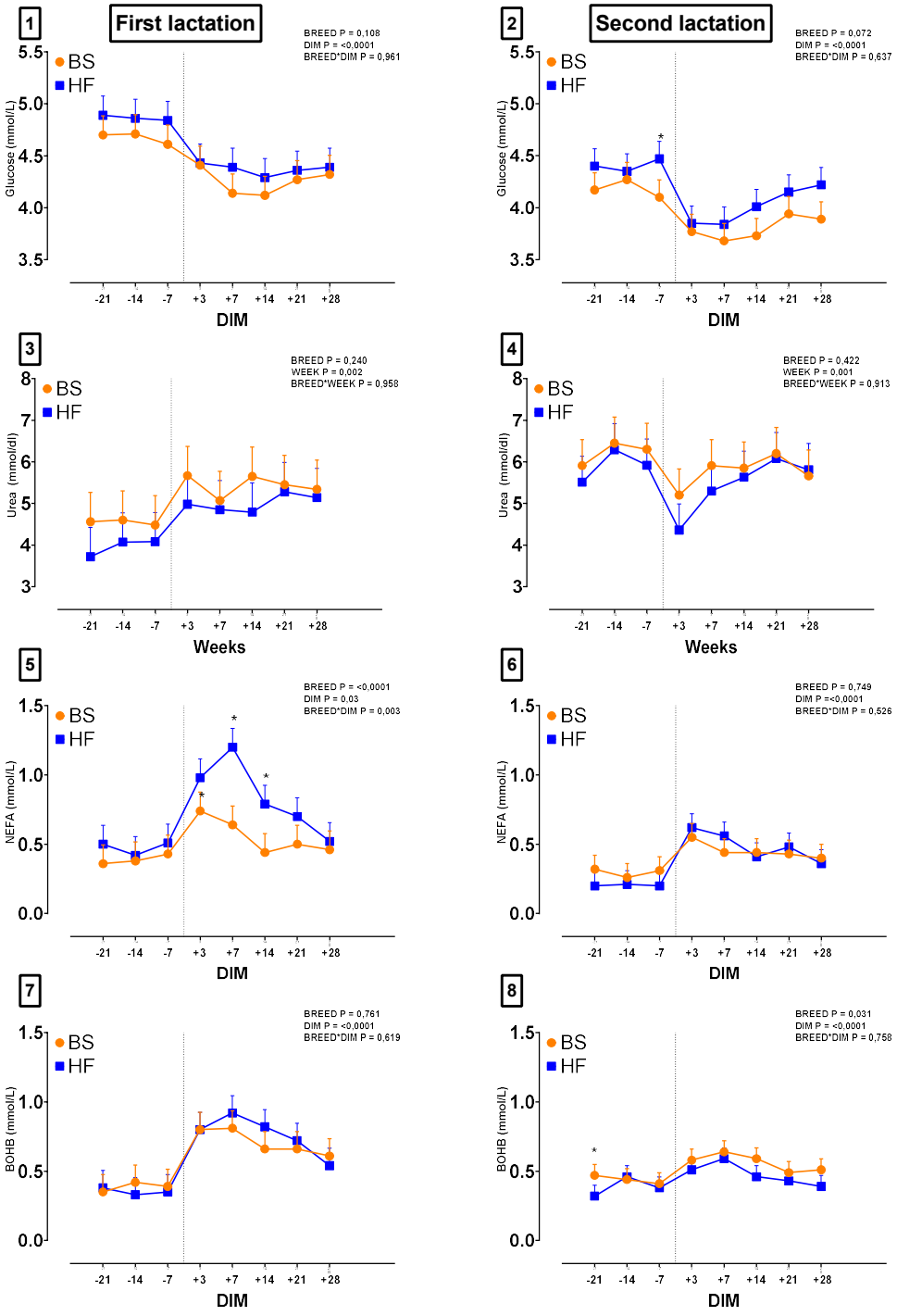


Figure G. Blood parameters of energy metbolism during the transition period of the first and second lacation: Glucose (1-2), Urea (3-4), NEFA (5-6) and BOHB (7-8).



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Chapter 3

Effects of supplementation of a mycotoxin mitigation feed additive in lactating dairy cows fed Fusarium mycotoxin-contaminated diet for an extended period.

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Abstract

Fusarium mycotoxins are inactivated by rumen flora; however, a certain amount can pass the rumen and reticulum or be converted into biological active metabolites. Limited scientific evidence is available on the impact and mitigation of *Fusarium* mycotoxins on dairy cows' performance and health, particularly when cows are exposed for an extended period (more than 2 months). The available information related to these mycotoxin effects on milk cheese-making parameters is also very poor. The objective of this study was to evaluate a commercially available mycotoxin mitigation product (MMP, i.e., TOXO® HP-R, Selko, Tilburg, The Netherlands) in lactating dairy cows fed a *Fusarium* mycotoxin-contaminated diet, and the repercussions on the dry matter intake, milk yield, milk quality, cheese-making traits and health status of cows. The MMP contains smectite clays, yeast cell walls and antioxidants. In the study, 36 lactating Holstein cows were grouped based on the number of days of producing milk, milk yield, body condition score and those randomly assigned to specific treatments. The study ran over 2 periods (March/May–May/July 2022). In each period, six animals/treatment were considered. The experimental periods consisted of 9 days of adaptation and 54 days of exposure. The physical activity, rumination time, daily milk production and milk quality were measured. The cows were fed once daily with the same total mixed ration (TMR) composition. The experimental groups consisted of a control (CTR) diet, with a TMR with low contamination, high moisture corn (HMC), and beet pulp; a mycotoxins (MTX) diet, with a TMR with highly contaminated HMC, and beet pulp; and an MTX diet supplemented with 100 g/cow/day of the mycotoxin mitigation product (MMP). The trial has shown that the use of MMP reduced the mycotoxin's negative effects on the milk yield and quality (protein, casein and lactose). The MTX diet had a lower milk yield and feed efficiency than the CTR and MMP HP-R diets. The MMP limited the negative effect of mycotoxin contamination on clotting parameters, mitigating the variations on some coagulation properties; however, the MMP inclusion tended to decrease the protein and apparent starch digestibility of the diet. These results provide a better understanding of mycotoxin risk on dairy cows' performances and milk quality. The inclusion of an MMP product mitigated some

negative effects of the *Fusarium* mycotoxin contamination during this trial. The major effects were on the milk yield and quality in both the experimental periods. These results provide better insight on the effects of mycotoxins on the performance and quality of milk, as well as the cheese-making traits. Further analyses should be carried out to evaluate MMP's outcome on immune–metabolic responses and diet digestibility.

Key words: *mycotoxin; animal; blood parameters; milk quality; animal welfare*

1. Introduction

Mycotoxins are a group of metabolites produced by several filamentous fungi in the genera Aspergillus, Fusarium and Penicillium that can induce toxic responses when ingested by humans and other living organisms [1–3]. Mycotoxins are stable and can be detected in animal feeds and forages [4–6]. Fusarium mycotoxins are widespread, and they contaminate field crops in all climatic zones where plant material is available [7]. Previous studies have described the toxicological effects of Fusarium mycotoxins in farming animals [2,8–11]. The administration of deoxynivalenol (DON)-contaminated feed to animals can lead to the exhibition of symptoms such as gastrointestinal disorders, soft feces, diarrhea, immunosuppression and decreased performance [9,12,13]. The biological mechanisms that cause these responses are not well understood, but DON can affect rumen health and damage the permeability of the rumen and/or gut epithelia [14].

Fumonisins (FBs) are mycotoxins that are cytotoxic, hepatotoxic and nephrotoxic, although their mechanism of action is unclear in dairy cows [15–18]. The animals' guts absorb FBs [19], which can lead to the alteration of some metabolic pathways. An intestinal immunosuppressive condition involves molecular systems, such as the downregulation of MyD88 and TLRs, which are important players in the first phase of pathogen recognition, ultimately leading to the inflammatory cascade [20,21]. In the inflammatory response, a parameter of particular interest is the circulating leukocytes, which is not yet well- understood in ruminants.

According to [22], some mycotoxins could be inactivated by the rumen flora (e.g., DON is partly converted into the less toxic metabolite, DOM-1, in particular rumen physiological conditions), while others pass through the rumen and reticulum (e.g., FBs) or are converted into metabolites while still preserving their biological activity (zearalenone, ZEN). Some mycotoxins can modify rumen flora due to their antimicrobial activity and negatively affect the immune system of the host animals. An example is patulin, which acts against Gram + and Gram bacteria and protozoa. In vitro studies have reported that it has a negative effect on the production of volatile fatty acids (VFA) and acetate, and on protein synthesis in the rumen fluid. Ruminoicoccus albus and Methanobrevibacter ruminatium were significantly inhibited by fusaric acid. Other mycotoxins with antimicrobial activity include mycophenolic acid, roquefortine

C, beauvericin and enniatins [6]. Impairment of the rumen microflora by mycotoxins results in a reduced filling of the rumen, less feed conversion and diarrhea; these symptoms are accompanied by decreased milk production and increasing incidences of subclinical mastitis with increased somatic cell counts [2,8].

Currently, there is limited scientific evidence in terms of mycotoxins' effects on the digestion, physiology and pathophysiology of dairy cows [2,6], especially if exposed for an extended period (e.g., more than 2 months). EFSA ranks DON as an undesirable substance of the agri-food chain [12] and it also causes undesirable effects for dairy cows: loss of appetite, reduced rumination activity and feed intake, upregulation of pro-inflammatory cytokines and immuno-suppression, but only few feeding studies with large ruminants are available, as reported in a recent EFSA scientific opinion on FBs [23]. The adverse effects of FB ingestion involve changes in organ appearance and serum enzymes and biochemistry, as well as a reduction of kidney and liver function. Mycotoxin effects on cheese-making parameters have been poorly investigated [24] when mycotoxin mitigation products (MMP) are added to diets. Consequently, an evaluation of mycotoxins' economic impact (aflatoxins and mycotoxins originating from Fusarium) on ruminant livestock production requires further investigation [25–28]. Advances in this topic may help crops/feed technicians and dairy farmers in dealing with non-specific health and performance inefficiencies on their farms. Among MMP, some commercially available products contain selected glucose biopolymers and purified ß-glucans, as well as bentonite or yeast cell walls, which are able to bind a wide range of mycotoxins and reinforce the intestinal barrier function and the immune system that are mainly affected by Fusarium mycotoxins, such as trichothecenes and fumonisins [28]. These types of MMP contain vitamins, antioxidants, yeast cell walls and bentonite clays. The objective of this study was to determine the performance effects of a commercially available MMP, TOXO® HP-R, in lactating dairy cows fed with Fusarium mycotoxin-contaminated diets at different levels of contamination and evaluate the effects on the dry matter intake capacity, rumination, immuno-physiological parameters, milk yield and quality, cheese-making traits and the overall health status of multiparous cows. The MMP tested in our trial contains four components, including bentonites, inactivated yeast cell wall fractions, β-1,3/1,6 glucans and antioxidants. It has four layers of protection. The first layer of protection,

bentonite, can adsorb a portion of certain dietary mycotoxins to prevent their adsorption in the small intestine [17]. Mycotoxins that escape from the adsorbents may damage the gut barrier function of enterocytes and, afterwards, translocate from the lumen side into the blood stream. The inactivated yeast cell wall fractions showed the efficacy of strengthening the gut barrier function [6], which helps further reduce mycotoxins' bioavailability and works as the second layer of protection. A portion of dietary mycotoxins, however, may still be absorbed in the small intestine and lead to immunosuppression, especially for the gut's innate immunity. Beta-glucans, as the third layer of protection, are recognized by the receptors of macrophages and therefore stimulate the innate immune system [6]. Lastly, most mycotoxins present in the blood stream will eventually end up in the liver for detoxification. Scientific research indicates that a dietary increase of antioxidants is beneficial because the antioxidants enhance the detoxification of ingested mycotoxins into non-toxic metabolites and help to overcome mycotoxin-induced oxidative stress, which explains the presence of antioxidants in TOXO HP-R as the fourth layer of protection. As reported above, one of the aims of this study was to evaluate the mitigation of the MMP product of some negative effects of Fusarium mycotoxin contamination through long-term exposure (54 days).

2. Results

Table 1 shows the chemical composition, digestibility and energy evaluations of experimental TMR diets fed to lactating dairy cows, as well as their average mycotoxin concentrations. The DM (% as fed) did not differ among the diets, with the highest value in the CRT group (53.8 2.4% as fed) and the lowest in the MTX group (52.7 2.3% as fed). Similarly, the CP, soluble CP and ash contents resulted in very similar levels among the experimental diets, with average values of 14.7% DM, 5.2% DM and 8.8% DM, respectively. The diets did not differ in fiber fractions and NDF digestibility when evaluated after 24 h of rumen incubation, and the differences among the diets can only be considered numerically. Similarly, the average values of aNDFom, ADF, ADL, starch and sugar were 31.7, 19.8, 3.0, 26.9 and 4.2% DM, respectively. The mean values of neutral detergent insoluble crude protein (NDICP) and acid detergent insoluble crude protein (ADICP) were 3.1 0.5 and 0.9 0.1% DM, respectively, for all the experimental diets.

Table 1. Chemical composition, digestibility, and energy evaluations of experimental total mixed ration (TMR) diets fed to lactating dairy cows in the trial: CTR diet with low contamination level, MTX with high contamination level and MMP with high contamination level supplemented with about 100/g/cow/day of MMP (i.e., TOXO® HP-R, Selko, Tilburg, the Netherlands).

Items	Experimental diets[1]		
	CTR (n=10)	MTX (n = 10)	MMP (n = 10)
Ingredients (% DM)			
Corn meal		5.9	
Barley meal		2.5	
Sunflower meal, dehulled 34%		4.3	
Soybean, solvent meal 44%		13.7	
High moisture corn		22.7	
Alfalfa hay		17.4	
Ryegrass hay		1.8	
Mineral-vitamin supplement2		1.7	
Fat (palm oil)		0.8	
Corn silage		12.0	
Sorghum silage		12.2	
Beet pulp		5.0	
Forage:concentrate ratio		49.9:50.1	
Chemical composition (% DM)			
DM (% as fed)	53.8±2.4	52.7±2.3	53.6±1.9
CP	14.8±1.0	14.6±1.0	15.1±0.8
soluble CP	5.2±0.5	5.2±0.6	5.3±0.5
Ash	8.9±0.6	8.8±0.6	8.7±0.6
aNDFom	31.9±1.8	31.9±2.4	32.0±1.2
ADFom	19.7±1.4	20.3±1.8	19.7±1.8
ADL	3.0±0.3	3.1±0.4	2.9±0.3
NDFD 24h	47.9±2.1	48.3±2.3	48.3±1.9
EE	3.3±0.5	3.3±0.5	3.3±0.4
Starch	26.9±1.7	26.8±1.9	27.1±1.9
Sugar	4.2±0.5	4.2±0.4	4.3±0.6
NDICP	3.1±0.5	3.0±0.5	3.1±0.5
ADICP	0.9±0.1	0.9±0.1	0.9±0.1
Energy evaluations (Mcal/kg DM)3			
TDN (%)	70.1±0.7	70.0±0.9	70.1±0.8
ME3x	2.54±0.05	2.54±0.06	2.54±0.05
Mycotoxin contamination4 in TMR (µg/kg DM)			
FB1 in TMR	85.3±56.3	159.5±60.9	163.8±58.1
FB2 in TMR	44.3±30.4	75.9±31.9	77.9±32.9
ZEN in TMR	43.2±13.1	196.8±75.7	248.5±139.3
DON in TMR	284.9±91.9	1021.7±234.5	1009.6±213.5

[1]treatments consisted of: i) the CTR diet, TMR with low contaminated HMC, and beet pulp; ii) the MTX diet, TRM with high contaminated HMC, and beet pulp; iii) the MMP- diet, MTX diet supplemented with 100 g/cow/day of mycotoxin-mitigationproduct (MMP, Selko, Tilburg, the Netherlands); [2]Mineral-vitamin supplement composition: sodium bicarbonate; 900000 IU of Vitamin A; 150000 IU of Vitamin D3; 3000 mg of Vitamin E; 2000 mg encapsulated Niacinamide; 20000 mg Niacinamide; 20000 mg Choline chloride; 1100 mg of Copper(I) sulfate; 1300 mg of MgO; 9400 mg of Zinc sulfate; 65 mg of Potassium iodide; 30 mg of Sodium Selenite; 18000 mg DL-methionine. [3] Energy evaluations were calculated by using equations of [54]. [4]Aflatoxins and T2 and HT2 toxins were undetected in all samples.

Very similar values among the diets were also observed for the total digestible nutrients (TDN) and metabolizable energy for lactation at three times the maintenance level (ME3x), and average values of 70.1% and 2.54 Mcal/kg DM were respectively measured or calculated. Regarding the regulated mycotoxin contaminations of the experimental diets, the highest value of FB1 in TMR was present in the MTX and MMP groups, which had mean FB1 values of 159.5 ± 60.9 and 163.8 ± 58.1 µg/kg DM, respectively, and mean FB2 values of 75.9 ± 31.9 and 77.9 ± 32.9 µg/kg DM, respectively. The CTR had the lowest FB1 and FB2 concentration values, being 85.3 ± 56.3 and 44.3 ± 30.4 µg/kg DM, respectively. The average values of ZEN and DON were 196.8 ± 75.7 and 1021.7 ± 234.5 µg/kg DM in MTX or 248.5 139.3 and 1009.6 213.5 in the MMP groups, respectively. The lowest ZEN and DON concentrations were in the CTR group, with mean values of 43.2 13.1 and 284.9 91.9 µg/kg DM, respectively. Table 2 shows the chemical and fermentative (only silage) characteristics of single feeds. The chemical compositions appeared typical for specific ingredients used in this experiment. Furthermore, no evidence of differences in the chemical composition were observed between the low-contaminated (CTR) and highly contaminated (TRT) HMC and the low-contaminated (CTR) or highly contaminated (TRT) beet pulp. The table below reports the mycotoxin contamination of FB1, FB2, ZEN and DON for each ingredient used in the in vivo trial. The main mycotoxin contamination feeds were HMC TRT, highly contaminated by FB1 (645.3 43.2 µg/kg DM), FB2 (278.3 25.9 µg/kg DM) and DON (1988.1 234.2 µg/kg DM) or beet pulp TRT, for which the main mycotoxin was ZEN (2911.4 121.4 µg/kg DM). All the feeds in Table 2 follow the specific European recommended levels for DON, ZEN, and FBs contamination levels. Only beet pulp TRT showed higher ZEN contamination, even if this feed is lower than 3 mg/kg. Table 3 shows the least squares means and associated standard error of the mean (SEM) for the feeding behavior, body weight, milk yields, feed efficiency and milk quality parameters of all the recruited Holstein cows that were fed a control diet without an adsorbent and mycotoxins (CTR) during each adaptation period. The feeding behavior, milk yields and milk parameters had a p value of the models for the groups that were not always significant ($p > 0.05$) during the adaptation period, whereas it was possible to observe a tendency towards significance with regards to body weight ($p = 0.08$).

Table 2. Chemical and fermentative (only silage) characteristics of single feeds (n=4). Nd is for not detected. Aflatoxins and T2 and HT2 toxins were undetected in all samples.

	Feed Ingredients							
Chemical composition (% DM)	**Corn Silage**	**Sorghum Silage**	**HMC CTR**	**HMC TRT**	**Beet pulp TRT**	**Beet pulp CTR**	**Alfalfa Hay**	**Ryegrass Hay**
DM, % as fed	32.68±1.9	29.4±0.1	54.7±1.4	58.13±0.4	89.1±1.3	88.8±1.1	89.0±0.4	90.0±0.3
CP	8.4±0.7	10.6±0.3	5.9±0.1	6.3±0.3	8.1±0.7	8.9±0.6	15.0±3.4	6.2±0.7
Ash	5.7±0.5	7.7±0.5	1.2±0.1	1.1±0.2	6.8±1.3	7.1±1.5	10.0±1.2	9.8±1.0
aNDFom	40.0±2.8	48.3±0.3	16.4±0.5	17.8±2.3	40.5±4.9	44.1±3.7	44.9±7.6	59.2±1.0
ADFom	25.2±1.9	31.7±0.7	9.4±0.1	8.4±2.1	20.6±2.0	23.8±1.5	33.9±2.0	40.7±1.6
ADL	3.2±0.7	4.6±0.1	-	-	1.9±0.8	1.7±0.7	7.2±0.7	6.0±0.4
$NDFD_{24h}$, %NDF	50.9±7.2	43.6±0.6	-	-	-	-	32.8±6.4	45.9±2.6
EE	3.2±0.3	3.6±0.1	-	-	0.9±0.4	0.9±0.4	1.9±0.5	1.3±0.3
Starch	21.3±0.8	20.6±1.1	59.6±2.7	60.3±4.1	-	.	2.9±0.2	3.5±0.8
Sugar	1.3±0.5	2.1±0.1	-	-	6.6±2.2	7.1±2.4	7.9±1.5	9.2±0.2
				Mycotoxins (µg/kg DM)				
FB1	96.2±8.7	106.2±13.2	<10	645.3±43.2	<10	13.1±2.4	<10	<10
FB2	38.3±4.8	21.4±1.3	<10	278.3±25.9	<10	<10	<10	<10
ZEN	<10	238.2±21.9	<10	76.1±21.1	2911.4±121.4	<10	<10	<10
DON	713.2±54.2	635.6±47.2	38.3±13.1	1988.1±234.2	45.3±5.6	27.5±3.8	519.2±78.5	355.3±43.2
Fermentation parameters (%DM)								
pH	3.68±0.05	3.83±0.02						
Ethanol	0.52±0.34	1.10±0.13						
Acetic acid	3.02±0.55	3.55±0.63						
Propionic acid	0.06±0.02	0.28±0.15						
Iso-butyric acid	0.00±0.00	0.00±0.00						
1,2 Propanediol	2.42±0.97	1.87±1.12						
Butyric acid	0.02±0.02	0.01±0.01						
Iso-valerianic acid	0.00±0.00	0.00±0.00						
Valerianic acid	0.00±0.00	0.00±0.00						
Lactic acid	5.77±0.91	6.40±0.49						
Aldehydes tot.	0.01±0.00	0.01±0.00						
Alcohols tot.	0.75±0.47	1.87±0.41						
Ketons tot.	0.00±0.00	0.00±0.00						
Esters tot.	0.02±0.01	0.04±0.01						

Table 3. Least squares means and associated SEM for feeding behavior, body weight, milk yields, feed efficiency and milk parameters of 36 Holstein cows (12 for each experimental group) fed with 3 experimental diets: CTR diet with low contamination level, MTX with high contamination level and MMP with high contamination level supplemented with about 100/g/cow/day of MMP (i.e., TOXO® HP-R, Selko, Tilburg, the Netherlands) during the adaptation period. Period 1 and 2 refereed respectively to March/May and May/July 2022.

Items		Treatment			Period		SEM	P of the model			
		CTR	MTX	MMP	1	2		Period	Treatment (T)	Day (D)	D * T
Feeding Behaviour											
DMI	kg/cow/day	25.21	25.18	25.67	24.34	26.36	1.270	0.032	0.896	0.132	0.328
DMI	% BW	4.01	3.85	4.03	3.81	4.17	0.031	0.046	0.503	0.140	0.247
Rumination time	Min	519	504	526	519	513	50.2	0.848	0.833	0,022	0.328
Body weight	Kg	622	653	638	641	635	17.6	0.651	0.080	0,016	0.284
Milk yields											
Milk yield	L/cow/day	36.9	36.7	35.8	35.6	37.4	1.08	0.465	0.928	<0.001	0.621
Milk yield	kg/cow/day	38.0	37.8	36.8	36.7	38.4	1.15	0.465	0.982	<0.001	0.621
FPCM	kg/cow/day	38.5	37.7	38.6	34.4	40.7	6.70	0.060	0.637	-	-
ECM	kg/cow/day	35.5	34.2	35.8	33.2	37.7	6.16	0.051	0.653	-	-
Milk yield/DMI	Dmnl	1.53	1.53	1.46	1.53	1.48	0.010	0.531	0.763	0.168	0.539
Milk parameters											
Fat	%	3.71	3.91	3.99	4.17	3.56	0.406	<0.001	0.522	-	-
	kg/cow/day	1.37	1.30	1.42	1.30	1.42	0.270	0.387	0.667	-	-
Protein	%	3.23	3.28	3.28	3.29	3.22	0.235	0.578	0.927	-	-
	kg/cow/day	1.20	1.10	1.17	1.02	1.28	0.199	0,012	0.618	-	-
Casein	%	2.55	2.60	2.61	2.62	2.55	0.279	0.613	0.924	-	-
	kg/cow/day	0.94	0.87	0.93	0.81	1.01	0.160	0,016	0.624	-	-
Lactose	%	4.79	4.84	4.83	4.79	4.85	0.166	0.359	0.785	-	-
	kg/cow/day	1.78	1.64	1.74	1.49	1.94	0.329	<0.001	0.703	-	-
MUN	mg/100 ml	25.3	27.5	24.7	29.5	22.1	4.30	<0.001	0.552		
LogSCC	Log10(cells/ml)	5.01	5.03	4.67	5.21	4.60	0.668	0.065	0.540	-	-

dmnl = dimensionless.

This means that before exposure periods, the cows had similar feeding behavior and milk performances, regardless of their assigned groups. The period effect was significant ($p < 0.05$) for the dry DMI (kg/cow/day and % BW), fat- and protein-corrected milk (FPCM) (kg/cow/day) and energy-corrected milk (ECM) (kg/cow/day) milk yields, fat (%), protein (kg/cow/day), casein (kg/cow/day), lactose (kg/cow/day), MUN (mg/100 mL) and Log SCC (Log10(cells/mL)); this was probably due to differences in days in milk. No differences in the milk composition (i.e., fat, protein, casein, lactose, urea, and somatic cell counts) were observed among the groups before the exposure period.

Table 4 shows the least squares means and associated SEM for the feeding behavior, body weight, milk yields, feed efficiency and milk parameters during the exposure period. The DMI did not differ among the treated groups or periods, resulting in an average equal to 26.02 kg DM/cow/day or 4.03% BW. The high intake of the cows was related to the earlier stage of lactation and high production levels. Similarly, the rumination times did not differ among the groups and experiment periods, resulting in an average equal to 514 min per day. The BCS was evaluated for the early lactation cows used in the experiment and it resulted in an average equal to 3.20, based on a 1 to 5 evaluation scale. The BW did not differ among the periods or groups, with the average equal to 647 kg. The milk yield did not differ among the experimental groups, despite numeric differences of more than 1.5 kg/cow/day between the MTX and MMP groups; this was probably related to great intra-group variability (i.e., SEM = 0.27). In particular, the CTR and MMP groups numerically produced more milk than the MTX groups, with a difference of +0.7 and +1.8 kg of milk/cow/day, respectively. Day treatment interactions were reported for 3.5% of fat-corrected milk and energy-corrected milk ($p < 0.05$). For ECM, a higher average production of +1.8/1.9 kg/cow/day was observed in the MMP group as compared to the MTX group. The numerical differences between the CTR and MTX groups for ECM was +0.6 kg/cow/day. Moreover, we detected an interaction effect D * T for ECM and FCM that could explain a variation of the data throughout the experimental weeks of mycotoxin exposure.

Regarding the milk parameters and nutrient yields, the MMP was characterized by the highest values for fat and lactose, both in concentration and yield. Increases of approximately 5–6% of these milk nutrients were reported and the differences were more pronounced after 6 to 8 weeks of exposure. Similarly, greater values for the protein and casein yields were observed for CTR and MMP as compared to the MTX groups, and the differences tended to be more evident at the end of the exposure periods (day treatment interactions < 0.05). No differences were found among the groups for MUN and SCC.

Table 4. Least squares means and associated SEM for feeding behaviour, body weight, milk yields, feed efficiency and milk parameters of 36 Holstein cows (12 for each group) fed with 3 experimental diets: CTR diet at low contamination level, MTX at high contamination level and MMP at high contamination level supplemented with about 100/g/cow/day of MMP (i.e., TOXO® HP-R, Selko, Tilburg, the Netherland) during each experimental exposure period. Period 1 and 2 refereed respectively to March/May and May/July 2022

Items		**Treatment**			**Period**		**SEM**	**P of the model**			
		CTR	MTX	MMP	1	2		Period	Treatment (T)	Day (D)	D * T
Feeding Behaviour											
DMI	kg/cow/day	25.62	26.09	26.36	26.47	25.58	0.401	0.238	0.714	<0.001	0.987
DMI	% BW	4.02	3.98	4.09	4.10	3.96	0.01	0.290	0.829	<0.001	0.986
Rumination time	min	512	505	524	518	510	14.53	0.684	0.756	<0.001	0.800
Body weight	kg	638	657	647	646	648	8.0	0.858	0.358	<0.001	0.835
Body condition Score	1-5 scale	3.17	3.24	3.18	3.10	3.28	0.023	0.212	0.456	-	-
Milk yields											
Milk yield	L/cow/day	37.2	36.5	38.2	37.6	37.1	0.27	0.834	0.839	<0.001	0.959
Milk yield	kg/cow/day	38.3	37.6	39.4	38.7	37.2	0.29	0.834	0.839	<0.001	0.959
3.5 % FCM	kg/cow/day	39.9	39.7	41.6	41.3	39.5	0.762	0.431	0.752	0.008	0.017
ECM	kg/cow/day	37.8	36.6	38.4	38.1	36.5	0.669	0.436	0.761	0.006	0.02
Milk yield/DMI	Dmnl	1.54	1.46	1.52	1.50	1.51	0,076	0.895	0.692	0.02	0.999
Milk parameters											
Fat	%	3.52	3.74	3.80	3.77	3.60	0.011	0.133	0.138	<0.001	0.565
	kg/cow/day	1.37	1.42	1.49	\ 1.47	1.38	0.002	0.245	0.374	<0.001	0.212
Protein	%	3.36	3.26	3.31	3.31	3.30	0.016	0.921	0.383	0.007	0.194
	kg/cow/day	1.31	1.23	1.30	1.30	1.26	0.0007	0.643	0.930	0.223	0.006
Casein	%	2.67	2.59	2.63	2.63	2.63	0.0018	0.993	0.775	0.003	0.274
	kg/cow/day	1.04	0.98	1.03	1.03	1.01	0.0005	0.689	0.907	0.024	0.365
Lactose	%	4.75	4.74	4.80	4.75	4.77	0.009	0.639	0.751	0.017	0.939
	kg/cow/day	1.86	1.81	1.90	1.87	1.84	0.0013	0.741	0.910	0.276	0.030
MUN	mg/100 ml	31.6	32.7	33.7	31.9	33.5	0.859	0.125	0.301	0.002	0.965
LogSCC	Log10(cells/ml)	4.68	4.88	4.60	4.81	4.63	0.006	0.400	0.506	0.119	0.142

Table 5 shows the main nutrient digestibility. The period effects in the apparent NDF, starch and CP digestibility (i.e., 2.2%, 0.7%, and 2.0%, respectively) were measured. A slight reduction in the apparent CP digestibility was observed among the groups, with the MMP diet showing the lowest values (i.e., 79.32% in MMP vs., on average, 81.73% of CTR and MTX diets; $p < 0.05$). Similar results were reported for the apparent NDF digestibility ($p = 0.126$) and apparent starch digestibility ($p = 0.060$). The pH of the fecal samples was higher in the first than the second period, without differences among the treatments. Regarding the volatilome fecal profile, the majority of the analyzed compounds differed at a $p < 0.05$ between the experimental periods, even when no differences among the treatments were measured, with the only exception of iso-valeric acid that tended ($p = 0.066$) to be higher in MTX than the CTR and MMP diets. Only the numerical differences among the treatments ($p = 0.836$) were reported for the total volatile fatty acids, with the lowest concentration being in CTR (i.e., 224.90 mmol/kg DM) and the highest in MMP (i.e., 260.13 mmol/kg DM).

Table 6 reports the effects of various diets on the hematochemical parameters. Among the indexes of energy and protein metabolism, BOHB tended to be higher ($p < 0.10$) in MTX with respect to the other diets. In addition, cholesterol and urea were numerically higher in MTX in comparison with the other diets. MTX also showed a lower concentration of transaminases (GOT and GGT; $p > 0.05$) with the respect to CTR and MMP. Overall, the presence of the mycotoxin mitigation product (MMP vs. MTX) resulted in very slight differences at the hematochemical levels, which were not statistically supported. Interestingly, the values of the plasma parameters of MMP were very similar to the CTR group, supporting the effective adsorption of mycotoxins during the transit into the gut. The results of some blood parameters (e.g., MPO and alkaline phosphatase with a significant W*T effect) showed interesting numeric differences among the treatments and deserve future investigation. Table 7 reports the milk coagulation properties of lactating cows fed one of the three tested diets. The type of treatment did not significantly affect most of the tested milk cheese- making traits; however, numerical differences indicating a lower aptitude of milk produced by MTX to become cheese was observed for several parameters, such as r, K20, a30, a45 and a60. The only significant parameter ($p < 0.05$) was Rct_eq (min), which indicated a longer rennet coagulation time. The values for this parameter, based on recommendations from

Bittante [30], can be classified as fast-coagulating samples (Rct_eq < 17 min), samples coagulating at an average rate (17 min < Rct_eq < 22 min), and slowly coagulating samples (Rct > 22 min). Milk samples that did not coagulate by the end of the recording time (30 or 90 min) were classified as non-coagulating (NC). Consequently, the milk of the MMP group is considered average, whereas milk from the MTX group is considered a slowly coagulating sample. The tendency ($0.05 < p < 0.10$) in CFmax and CFp were observed for the MTX vs. CTR and MMP groups, with the worst results reported in the MTX treatments. Furthermore, a trend towards significance ($0.05 < p < 0.10$) was observed for the parameter tmax. No other differences were observed for the CY or REC parameters among the groups.

Table 7. Least squares means and associated SEM for milk coagulation properties of 36 Holstein cows (12 for each group) fed with 3 experimental diets: CTR diet at low contamination level, MTX at high contamination level and MMP at high contamination level supplemented with about 100/g/cow/day of MMP (i.e., TOXO® HP-R, Selko, Tilburg, the Netherland) during each experimental exposure period. Period 1 and 2 refereed respectively to March/May and May/July 2022.

Items		**Treatment**			**Period**		**SEM**	**P of the model**			
		CTR	MTX	MMP	1	2		Period	Treatment (T)	Week (W)	W * T
Milk coagulation properties											
pH	Dmnl	6.46	6.49	6.47	6.51	6.44	0.008	<0.001	0.158	0.0023	0.533
Casein index	%	78.8	79.4	80.2	80.3	78.7	0.800	0.001	0.225	0.006	0.624
Milk total solid	%	12.69	12.76	12.87	13.01	12.54	0.04	0.108	0.666	0.009	0.074
Milk total solid (w/o fat)	%	9.21	9.11	9.15	9.24	9.08	0.007	0.196	0.662	0.049	0.443
r	Min	24.2	29.0	25.8	26.9	25.8	6.92	0.744	0.171	0.418	0.274
K20	Min	8.4	11.9	8.7	10.0	9.3	1.65	0.719	0.237	0.225	0.062
a30	Mm	17.9	13.2	23.7	18.1	18.5	11.98	0.927	0.338	0.313	0.460
a45	Mm	29.3	24.7	32.3	31.0	26.4	15.64	0.251	0.177	0.948	0.202
a60	Mm	29.0	26.3	28.6	31.6	24.4	28.58	0.024	0.408	0.486	0.514
Rct_eq	Min	24.9	29.0	21.3	25.5	24.6	3.69	0.716	0.025	0.781	0.112
tmax	Min	48.0	52.3	43.1	50.8	44.9	10.15	0.051	0.085	0.545	0.847
CFmax	Mm	34.3	28.5	35.5	35.7	29.8	12.39	0.074	0.079	0.957	0.082
CFp	Mm	46.0	38.2	47.5	47.9	40.0	22.25	0.074	0.079	0.957	0.082
kcf	%/min	10.3	9.2	12.7	9.5	11.9	4.17	0.081	0.793	0.216	0.544
ksr	%/min	0.9	0.8	1.3	0.8	1.2	0.09	0.085	0.829	0.077	0.410
CY parameters											
CY curd	%	18.91	18.51	19.62	20.23	17.79	1.089	0.002	0.924	0.030	0.703
CY solid	%	6.51	6.30	6.59	6.70	6.24	0.074	0.163	0.997	0.010	0.303
CYwater	%	12.41	11.95	13.02	13.53	11.38	1.010	<0.001	0.577	0.201	0.958
REC parameters											

REC protein	%	77.82	76.12	79.32	78.60	76.91	2.480	0.148	0.272	0.207	0.923
REC fat	%	82.78	80.65	83.54	82.42	82.22	16.124	0.951	0.575	0.078	0.363
REC solids	%	51.20	48.88	51.06	51.31	49.44	3.693	0.266	0.844	0.135	0.698
REC energy	%	64.75	63.04	65.63	65.18	63.77	2.835	0.470	0.855	0.017	0.457

Table 2 r = coagulation time; K20 = time interval between gelation and attainment of curd firmness of 20 mm; a30, a45, and a60 = curd firmness 30, 45, and 60 min after rennet addition; RCTeq = rennet coagulation time estimated by curd firming equation parameter modeling; tmax = time at achievement of maximum curd firmness (CFmax); CFP = asymptotic potential curd firmness; CFmax = maximum curd firmness; kCF = curd firming instant rate constant; kSR = syneresis instant rate constant; CY = cheese yields; CY curd = cheese yields curd; CY solid = cheese yields for total solids; CY water = cheese yields for water; REC protein = recovery of protein; REC fat = recovery of fat; REC solids = recovery of solids; REC energy = recovery of energy;

Table 5. Least squares means and associated SEM for nutrient diet digestibility and volatilome of feces of 36 Holstein cows (12 for each group) fed with 3 experimental diets: CTR diet at low contamination level, MTX at high contamination level and MMP at high contamination level supplemented with about 100/g/cow/day of MMP (i.e., TOXO® HP-R, Selko, Tilburg, the Netherland) during each experimental exposure period. Period 1 and 2 refereed respectively to March/May and May/July 2022

Items		Treatment			Period		SEM	P of the model			
		CTR	MTX	MMP	1	2		Period	Treatment (T)	Week (W)	W * T
Diet digestibility											
Apparent NDF digestibility	%	65.68	66.87	64.27	66.35	64.85	0.632	0.039	0.126	0,021	0.963
Apparent Starch digestibility	%	98.21	98.12	97.48	98.30	97.57	0.050	<0.001	0.060	0.610	0.974
Apparent CP digestibility	%	81.61	81.84	79.32	81.73	80.11	0.287	<0.001	<0.001	0.363	0.612
Fecal fermentation profile											
pH of feces	dmnl	6.83	6.86	6.76	6.96	6.68	0.002	<0.001	0.378	0.504	0.919
Volatilome fecal profile											
Acetic acid	mmol/kg DM	167.63	169.25	178.99	148.73	195.17	168.13	<0.001	0.683	0.04	0.513
Propionic acid	mmol/kg DM	47.39	48.85	48.27	40.31	56.03	12.201	<0.001	0.946	0.02	0.998
Butyric Acid	mmol/kg DM	22.02	22.52	24.73	19.00	27.17	3.523	<0.001	0.595	0.004	0.556
Iso Butyric acid	mmol/kg DM	2.34	2.95	2.48	2.27	2.91	0.073	<0.001	0.142	0.058	0.922
Iso valerianic acid	mmol/kg DM	2.14	2.71	2.20	1.81	2.89	0.079	<0.001	0.066	<0.001	0.578

Valerianic acid	mmol/kg DM	3.39	3.69	3.47	2.96	4.06	0.096	<0.001	0.393	<0.001	0.384
Methanol	mmol/kg DM	0.76	0.29	0.28	0.32	0.56	0.200	0.288	0.296	0.438	0.249
Ethanol	mmol/kg DM	4.83	4.01	5.81	4.22	5.54	1.906	0.086	0.696	0.476	0.836
Volatile fatty acid tot	mmol/kg DM	244.90	249.96	260.13	215.09	288.24	320.11	<0.001	0.836	0.002	0.634
Aldehydes tot	mmol/kg DM	2.51	1.85	2.33	2.60	1.87	0.528	0.070	0.392	0.002	0.740
Alcohols tot	mmol/kg DM	6.41	4.78	6.88	5.14	6.92	2.883	0,040	0.585	0.913	0.801

Dmnl, dimensionless. Capronic acid, propylene glycol, acetone, ketons and esters were analyzed but not detected

Table 6. Least squares means and associated SEM for blood parameters of 36 Holstein cows (12 for each group) fed with 3 experimental diets: CTR diet at low contamination level, MTX at high contamination level and MMP at high contamination level supplemented with about 100/g/cow/day of MMP (i.e., TOXO® HP-R, Selko, Tilburg, the Netherland) during each experimental exposure period. Period 1 and 2 refereed respectively to March/May and May/July 2022

Items		**Treatment**			**Period**		**SEM**	**P of the model**			
		CTR	MTX	MMP	1	2		Period	Treatment (T)	Week (W)	W * T
Plasma components											
Indexes of energy metabolism-protein metabolism											
Glucose	mmol/L	4.36	4.28	4.28	4.31	4.30	0.008	0.895	0.819	0.853	0.704
Cholesterol	mmol/L	4.74	4.81	4.61	4.55	4.89	0.059	0.190	0.576	0.210	0.624
NEFA	mmol/L	0.10	0.10	0.13	0.11	0.10	0.007	0.734	0.245	0.009	0.549
BOHB	mmol/L	0.31	0.38	0.36	0.37	0.34	0.003	0.450	0.051	0.148	0.074
Urea	mmol/L	5.56	6.18	5.39	5.93	5.49	0.158	0.244	0.712	0.032	0.751
Creatinine	µmol/L	84.45	82.59	82.49	80.41	85.95	1.679	<0.001	0.814	<0.001	0.202
Indexes of mineral metabolism											
Calcium	mmol/L	2.52	2.48	2.44	2.46	2.50	0.040	0.235	0.290	0.871	0.404
Phosphorous	mmol/L	1.71	1.86	1.73	1.82	1.72	0.018	0.268	0.764	0.524	0.429
Magnesium	mmol/L	1.08	1.05	1.06	1.03	1.10	0.020	0.010	0.972	<0.001	0.622
Zinc	mcmol/L	16.47	17.07	16.74	16.18	17.34	0.918	0.311	0.988	0.159	0.946

Indexes of liver functionality											
GGT	U/L	33.28	27.64	30.05	27.74	32.91	3.288	0.052	0.317	0.016	0.420
GOT	U/L	122.50	102.25	109.64	105.63	117.30	8.080	0.311	0.250	0.277	0.382
Alkaline phosphatase	U/L	51.18	57.81	61.52	57.92	55.84	26.898	0.775	0.515	0.652	0.034
Albumin	g/L	36.07	35.93	35.83	35.47	36.42	0.345	0.049	0.377	0.019	0.149
Bilirubin	mcmol/L	1.65	1.53	1.49	1.45	1.66	0.021	0.105	0.892	0.310	0.754
Paraoxonase	U/ml	90.48	85.40	90.57	90.04	87.59	17.277	0.518	0.245	0.782	0.098
Indexes of innate immune system and oxidative stress											
Haptoglobin	g/L	0.28	0.24	0.19	0.25	0.22	0.040	0.495	0.205	0.178	0.562
Ceruloplasmin	mcmol/L	2.93	2.77	2.70	2.80	2.80	0.027	0.972	0.602	0.698	0.613
Total proteins	g/L	83.19	84.18	82.49	83.52	83.05	1.672	0.669	0.270	0.181	0.342
Globulin	g/L	47.12	48.23	46.66	48.05	46.63	1.914	0.280	0.160	0.819	0.130
MPO	U/L	446.65	466.71	470.62	453.86	468.79	476.04	0.279	0.755	0.008	0.991
Total antioxidants (FRAP)	µmol/L	132.81	133.57	134.48	127.77	139.48	39.783	0.017	0.751	0.130	0.515
ROM	mgH_2O_2/100 mL	16.33	15.85	15.19	15.86	15.73	0.867	0.891	0.848	0.145	0.740

3. Discussion

3.1. Contamination of Animal Diets

The mycotoxin contamination levels found within the TMRs used in this study are similar to those reported in [31] and well below the EU regulated limits for animal feed when the regulation was declared. The CTR diet presents low contamination levels for all the mycotoxins under investigation, specifically DON (<650 µg/kg), ZEN (<107 µg/kg) and FB (<280 µg/kg). In contrast, the MTX and MMP diets showed high contamination levels for DON (<1000 µg/kg), medium for ZEN (<260 µg/kg) and low for FB (<280 µg/kg). This is quite in line with the distribution of Fusarium mycotoxins reported by [31], in that 30% and 33% of the feed samples had low and high DON contamination levels, respectively, while 43% and 33% of the samples had low and medium ZEN contamination levels. Although the authors of [31] reported that 44% of the samples had FB contamination at a medium level, a low contamination level of 20% was found in this trial. The lack of homogeneity between the different batches of mycotoxin-contaminated culture media could be the cause of the higher levels of ZEN contamination found in the MMP diets compared to the MTX diets [31].

3.2. Feed intake and rumen activities

Regarding DMI, no significant differences were observed between the groups in this trial; however, compared to the CTR group, the DMI value was numerically slightly higher in MTX and MMP, with the highest values achieved in the MMP diet. Other authors [31–34] observed that cows maintained a similar DMI despite being fed diets with different mycotoxin levels; however, the study carried out by the authors of [35] observed an increase in DMI following the ingestion of mycotoxin-contaminated diets. This could be a good explanation for higher DMI in diets with a higher level of mycotoxin contamination. The authors of [36] also found changes in DMI following mycotoxin ingestion, such as FB and ZEN, while EFSA [12] reported that DON is associated with a loss of appetite resulting in reduced feed intake.

Regarding the rumination time, no significant differences were observed; however, the values show that the animals who were fed the MTX diet had numerically shorter rumination times, whereas the animals fed the MMP diet had longer ones. The authors of [13] reported that DON is also responsible for a reduction in ruminal activity, as found in this trial: the MTX diet reduced ruminal activity, as reported in Table 4.

Despite high DON contamination, cows who were fed the MMP diet had the longest rumination time. This difference may be due to the presence of the MMP, which, in this case, consists of antioxidants and gut modifiers in addition to bentonite and an immune modulator. Ingestion of mycotoxin-contaminated feed causes oxidative stress [37], and antioxidants in diets help reduce their negative impact [13,38]. Antioxidants, with their beneficial effects, could lead to an increase in the overall health status of cows, thereby promoting an increase in ruminal activity.

Another trial conducted by [31] observed a completely different trend in the rumination time: the minimum value was found for the mycotoxin-contaminated diet containing a mycotoxin degradation product, while the maximum one occurred in animals fed only the mycotoxin-contaminated diet. The differences could be due to the different composition of the mycotoxin mitigation product or the different dietary level of the mycotoxins. The mechanisms that cause differences in rumination time are not yet well understood and defined; therefore, further experiments will be needed to clarify these aspects.

3.3. Milk yield and its quality

The production and milk quality parameters show that there were no significant differences between the three diets tested in this trial. The milk production levels appear to be in line with what was observed by [33], while [32] reported that the administration of mycotoxin-contaminated diets did not affect milk production levels or its composition. The MTX diet led to lower milk yields and worsened quality parameters (except for the fat content, which was higher than that of the CTR diet). The authors of [32] also observed a numerical reduction in milk production in the mycotoxin-exposed group, while [31] noted significantly reduced milk production. These differences represent a serious economic loss at the farm level [31]. The MMP diet resulted in higher milk production levels than the CTR diet, with improved quality traits (except for protein and casein). Similar to what was observed in this trial, the authors of [39,40] observed that the production level increased, while [40] reported that the administration of zeolite as an MMP resulted in a significant reduction in milk fat and protein. In the trial conducted by [31], the diet containing an MMP resulted in higher milk production compared to the contaminated diet; however, the production value of the control animals was higher than all the other diets used in the trial. In contrast, the authors of

[24] observed that the use of MMP caused no changes in the milk yield or quality characteristics. Milk coagulation parameters have an essential role in the Italian dairy sector, as most of the milk produced is processed into high-quality products [41,42]. No differences were observed for most of the analyzed milk characteristics; the Rct_eq was the only significant result, and CFmax and CFp tended to be significant, with a tendency of an interaction effect W*T. Nevertheless, differences were observed that indicate a lower cheese aptitude of the MTX diet, which is in line with these results. The authors of [43] observed that mycotoxin intake negatively influenced milk cheesemaking, particularly the cheese curd firmness and whey volume. Although not significant, the rennet coagulation time was also influenced by the intake of mycotoxin-contaminated diets. The study conducted by [31] also showed that mycotoxins cause negative effects on milk coagulation, particularly in K20 and a30 parameters, whereas the intake of MMP restores the levels similar to those of the control diet. Additionally, in this study, the MMP diet accelerated the coagulation times. Further studies on the effects of mycotoxins on milk coagulation properties will be necessary to extend the current knowledge. Further studies should be performed to verify the effects of Fusarium mycotoxin on milk production with a larger number of cows to verify the numeric differences.

3.4. Digestibility traits, ruminal and fecal variables

The trial results show that the apparent CP digestibility was significantly lower in the MMP diet than in the other two diets. The reduction in NDF and starch apparent digestibilities were not significant; however, the reduction in the starch digestibility was more significant. The results obtained in the present trial differ with the bibliography: in particular, the authors of [31,44] observed that mycotoxin administration reduced the NDF digestibility, while the authors of [38,45] noted a decrease in CP digestibility following DON- and FB-contaminated diet administration. Further studies are needed to better understand these mechanisms and to identify why the apparent digestibility values were lower in the MMP diet than in the MTX diet.

3.5. Immuno-metabolic parameters

Regarding the immuno-metabolic parameters, the mycotoxin-contaminated diets caused minimal effects that were not statistically significant, partly in line with the trial conducted by [31]. Only one significant difference was observed in this trial, namely,

the BOHB parameter, which increased significantly in the MTX diet compared to the other two diets, differing from what is found in the literature. The authors of [46] observed that in cows fed a mycotoxin-contaminated diet with 60% concentrate, the BOHB value was lower than in the control diet. In this trial, differences could be observed for urea and globulin, and numerical decreases were noted for transaminases (GOT and GGT) and paraoxonase; however, the trend in transaminases differs from that observed by several authors [6,31,47], who found elevated transaminase levels. There is a potential positive impact of product supplementation that can be related to its impact on immunity. Indeed, the groups that received mycotoxins showed a slight increase of MPO, which suggests increased activity of neutrophils and/or monocytes, with a significant interaction W*T. Nevertheless, none of the analyzed immune indexes (i.e., acute phase proteins) or oxidative stress indicators (i.e., ROM) were altered in these cows; thus, all these aspects deserve deeper investigation in the future to be fully elucidated. The administration of the MMP diet did not cause significant differences in the immunological parameters; on the contrary, the blood parameters were very similar to those of the CTR group, supporting the effective reduction of mycotoxin absorption from the gut. Lastly, future trials should be carried out to produce more data to clarify some of the variations in the blood parameters in cows with a long mycotoxin exposure.

4. Conclusions

Dairy cows fed with a diet contaminated with Fusarium mycotoxins for an extended period (i.e., 2 months) were adversely impacted. The presence of DON, ZEN and FB in the dairy cow diets, even below the United States FDA and European Union guidelines, negatively impacted the milk yield and milk rennet coagulation properties. The MMP reduced the negative effects of the mycotoxin by increasing the ECM and milk quality (mainly protein, casein, lactose and clotting features). Cows fed a mycotoxin-contaminated diet (MTX) had a lower milk yield and feed efficiency than the CTR and MMP diets, even if these differences were only numerically different. Mycotoxin contamination modified the apparent digestibility of starch and protein and some milk coagulation properties, such as Rct_eq, CFmax and CFp parameters; however, the contamination did not severely modify the energy metabolism, liver functionality, innate immune system or oxidative stress.

These results provide better knowledge of the risks of mycotoxin exposure on the performance and quality of milk. Further analyses should be carried out to verify the effects of mycotoxin mitigation products on immune–metabolic responses and diet digestibility; a larger number of cows should be studied to verify the consistency of these results. This study shows that the use of additives for mycotoxin control can be a valuable strategy to prevent undesirable effects in animals and alleviate their adverse effects. The study of organic and inorganic additives has already been extensively studied, but research in this field is still necessary due to the new emerging technologies available on the market and new knowledge in the field of mycotoxins. Future scenarios in this area of research will be related to climate change and immunometabolic parameters of dairy cattle as marker of animal health. Moreover, future studies should be conducted to better investigate the effects of mycotoxin metabolites in biological fluids or a modified mycotoxin form in feeds when particular effects on animals cannot be fully explained with an analysis of regular mycotoxins.

5. Materials and Methods

The study was authorized by Italian Health regulations that belong to accommodation and care of animals used for experimental and other scientific purposes (authorization n. 138/2021-PR is-sued on February 2, 2021).

5.1. Experimental cows and diets

Thirty-six multiparous mid-lactation Holstein cows were involved in the experiment conducted at the CERZOO research and experimental center (San Bonico, Piacenza, Italy). CERZOO is an innovative agro-zootechnical research farm, and it is equipped with facilities designed to study applications to increase production efficiency, animal welfare and sustainability. In the barn, each cow is equipped with sensors to continuously monitor the state of health, weight, feeding behavior, various activities and the quantity and quality of production. The structures provide an automated system of ventilation, and the cows are constantly monitored for feed intake. In addition, in the veal farm, there are automation systems that allow individual control of the consumption of milk and feed. In the next paragraphs, we report the equipment with which CERZOO is provided, which was used in this trial.

The animals were raised in a free stall, provided with individual feeding stations and had free access to water. To ensure drinking water safety, an annual analysis for sodium, chloride, potassium, calcium, Salmonella spp. and Escherichia coli are performed in agreement with Italian regulations. The study was conducted during 2 periods (18 different cows for each period): spring (from March to May) and summer (from May to July). In each period, six animals/group were considered (12 cows for each experimental group total). The cows were grouped based on the days in milk (DIM), parity, milk yield, body condition score (2.75 0.35 parities and 101 37 days DIM at the onset of the first period or 2.99 0.34 parities and 145 61 days DIM at the onset of the second period) and randomly assigned to specific experimental groups. Each experimental period consisted of 9 days of adaptation, followed by 54 days of exposure. Each cow had free access to a feed bin of the RIC feeding system, which monitored their feed intake and behavior. A pedometer and a ruminometer were used to monitor the activity and rumination time, respectively. The daily milk production was

measured by an Afimilk system. The cows were milked twice daily at 4.00 a.m. and 4.00 p.m. The cows were weighed after each milking, and the data were averaged daily. The animals were randomly allocated, before the start of the study, to one of three experimental groups, with six dairy cows for each treatment in each experimental period. The treatments consisted of the following: (i) CTR diet, TMR with low contamination HMC (high-moisture corn) and beet pulp; (ii) MTX diet, TMR with highly contaminated HMC and beet pulp; and (iii) MMP diet, MTX diet supplemented with about 100 g/cow/day of MMP (i.e., TOXO® HP-R, Selko, Tilburg, The Netherlands). The number of parties for CTR, MTX and MMP were, respectively, 2.83 0.24, 2.95 0.35 and 2.88 0.4. The DIM for CTR, MTX and MMP were, respectively, 124 47, 131 32 and 128 50. The MY for DIM for CTR, MTX and MMP were, respectively, 39.65 4.5, 40.1 3.4 and 39.95 5.0. The BCS for DIM for CTR, MTX and MMP were, respectively, 3.00 0.1, 2.95 0.25 and 3.1 0.36. Each total mixed ration (TMR) used in this study had the same composition, and the cows were fed once a day at 08:00 h. Approximately 5% of expected orts were collected individually and weighed daily. The components (Table 1) were mixed in a mixer wagon (Rotomix 5000, Bravo srl, Cuneo, Italy) in this order: corn silage, barley silage, alfalfa hay, soybean meal (44%), dehulled sunflower meal (34%), salts, mineral–vitamin supplements and water. Low- or high-contaminated high-moisture corn (HMC), cracked cornmeal and beet pulp feed ingredients were added, respectively, to low- (i.e., CTR diet) or high- (i.e., MTX or MMP-diets) contaminated diets, and were mixed using Rotomix 5000 mixer wagon (Bravo, Cuneo, Italy), and then fed to the animals. The treatment consisted of 100 g/cow/day of mycotoxin mitigating product (i.e., TOXO® HP-R) directly added to the highly contaminated TMR, according to a procedure described by [18]. The whole TMR with low- or high-contaminated feed ingredients was provided to RIC based on the measurement of the dry matter intake (DMI) on the previous day. During the adaptation period, all the animals received the same CTR diet, which was similar in composition and contained low-contaminated feed ingredients. Representative dietary samples were collected each week and analyzed for nutrient composition and mycotoxins, as described below. Samples of individual ingredients were analyzed at the beginning and at the end of each experimental period, and their chemical compositions and mycotoxin contaminations were determined (Table 2).

5.2. Analysis of feeds, diets, and mycotoxins

TMR samples were collected weekly, whereas the feed samples were taken at the start and end of each exposure period. We collected TMR seven times for each experimental period for each cow group (in total, 14 TMR sampling for CTR, 14 for MTX and 14 for the MMP group). The chemical profile (i.e., TMR) and mycotoxin levels were evaluated (i.e., both TMR and low- and high-contaminated feed ingredients), as reported in [31]. Before the analysis, the samples were dried at 60 ◦C in a ventilated oven for 48 h and milled through a 1 mm screen using a laboratory mill (Thomas-Wiley, Arthur H. Thomas Co., Philadelphia, PA, USA), after which they were stored for subsequent analysis. The dry matter (DM) was determined by the gravimetric loss of free water after heating at 105 ◦C for 3 h (Association of Official Analytical Chemists [48], (method 945.15)). The DM concentration was calculated after drying the feed and TMR samples at 60 ◦C in a ventilated oven for 48 hr. The ash was considered gravimetric residue after incineration at 550 ◦C for 2 h [48] (method 942.05). An ether extract (EE) was obtained using the method proposed by [48], method 920.29, and the crude protein (CP; N 6.25) was determined by Kjeldahl's method [48] (method 984.13). The CP soluble fraction (expressed on a DM basis) was determined according to [49]. The neutral detergent (ND), acid detergent (AD) and lignin sulfuric acid (ADL) fiber fractions were sequentially determined using an AnkomII Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA), as described by [50]. The ND solution contained sodium sulfite and a heat-stable amylase (activity: 17,400 Liquefon U/mL, Ankom Technology Corporation, Fairport, NY, USA). The fiber fractions were corrected for residual ash (aND-Fom, ADFom). The starch content was determined by polarimetry (Polax 2 L, Atago®, Tokyo, Japan). The mycotoxins were analyzed in cornmeal and TMR, with the samples examined before extraction. Mycotoxin detection/evaluation followed the method of [51] for aflatoxins (AFs); [52] for FBs; [53] for ZEN, DON, T-2 and HT-2 mycotoxins; and [54] for OTA. In summary, the extraction of AFB1 was performed using a ratio of acetone:water (7:3 v/v), after which it was purified by immuno-affinity column (R-Biopharm Rhône LTD, Glasgow, Scotland, United Kingdom). High-performance liquid chromatography (HPLC) with a fluorescence detector (FLD) was used to screen AFB1, with a limit of detection (LOD) and limit of quantification (LOQ) of 0.05 and 0.15 μg/kg DM, respectively. The FBs

were extracted using a phosphate buffer and then purified using an immuno-affinity column (R-Biopharm Rhône LTD, Glasgow, Scotland, United Kingdom), after which quantification was completed by HPLC coupled with a mass spectrometer (HPLC–MS/MS) with an LOD and LOQ of 10 and 30 µg/kg DM, respectively. Extraction of the other mycotoxins, ZEN, DON, T-2 and HT-2 toxin, was achieved with acetonitrile:water (86:14 v/v). ZEN was purified using an immune-affinity column (R-Biopharm, Rhône LTD, Glasgow, Scotland, United Kingdom) and quantified using HPLC–FLD with LOD and LOQ values of 2 and 5 µg/kg DM, respectively. DON was purified using a Trilogy-puritox Trichothecenes column (R-Biopharm, Rhône LTD, Glasgow, Scotland, United Kingdom), followed by quantification using GC-MS, with LOD and LOQ values of 10 and 30 µg/kg DM, respectively. T-2 and HT-2 toxins were purified using a Trilogy-Puritox Trichothecenes column, followed by quantification by LC-MS/MS, with LOD and LOQ values of 0.5 and 1.5 µg/kg DM, respectively.

5.3. Rumination time, body weight, body condition score

The trial cows' daily rumination time was detected using an accelerometer collar (RuminAct, SCR Heatime, Netanya, Israel), and the average times are reported. The cows were weighed twice daily using an electronic scale (TDM, San Paolo, Brescia, Italy) and the averages are reported. The metabolic BW was calculated using this formula: BW0.75 [29]. The BCS was determined at the start and the end of the experimental periods using the 5-point scoring system [55].

5.4. Health status of cows

The cows' health status was monitored daily. Mastitis was diagnosed by visual evaluation (abnormal milk per quarter), routine milk conductivity level and somatic cell count (SCC) analysis, which was performed for suspicious cases. Diarrhea was diagnosed using the fecal score method (visual evaluation of consistency and color) [56]; diarrheic feces are those with a fecal score of 2 or less. A veterinarian visited all the animals, and no symptoms of disease were found before the start of the experimental periods. The husbandry was generally good, and the feces had normal consistency. Cows with severe diseases were not included in the trial. One animal fed the MMP diet was removed from the trial due to a health problem adjudged as non-conforming by the

veterinarian. This animal was comprehensively treated for its symptoms but was excluded from the trial due to reduced milk production and worsening of health. Other animals had some minor and transient health problems that did not cause their exclusion from the experiment. These events were recorded. Consequently, some data related to specific animals with minor health injuries were excluded from the statistical analysis due to reasons independent of the treatments and related to other management problems. The excluded data represented less than 2.0% of all the data.

5.5. Milk yield, composition and cheese making traits.

The individual milk yield was measured at each milking, twice per day. Each week of the experiment period, representative daily milk samples were taken. The milk samples were analyzed for fat, protein, casein, lactose and titratable acidity using near-infrared spectroscopy (NIRs) (MilkoScan FT 120, Foss Electric, Hillerød, Denmark). The daily production of fat, protein, casein and lactose was calculated according to [57]. Urea nitrogen was determined in skimmed milk using a spectrophotometric assay and a urea nitrogen kit (cat# 0018255440, Instrumentation Laboratory, Milano, Italy) in association with an autoanalyzer (ILAB-650, Instrumentation Laboratory, Lexington, MA, USA). The somatic cell count (SCC) was determined using an optical fluorometric method with an automated cell counter (Fossomatic 180, Foss Electric, Hillerød, Denmark). In addition, bulk milk samples were analyzed weekly for AFM1, as described previously. The analyzed levels of AFM1 were under the LOD in all the samples. The milk coagulation properties (MCP) were evaluated using two mechanical lactodynamographs (Formagraph, Foss Electric A/S, Hillerød, Denmark). The following traditional MCP traits were recorded: rennet coagulation time (RCT, min), the time taken for the start of the coagulation after rennet addition; curd firming time (k20, min), the time to reach a curd firmness of 20 mm; and curd firmness (a30, mm) at 30 min after rennet addition. The instrument recorded the width of coagulation every 15 s for 60 min, resulting in a total of 240 measurements. The collection of measurements was used in the modeling equation proposed by [30] as an estimation of the following curd firming and syneresis traits: RCTeq (min) is the RCT estimated with the curd firming equation; kCF (%min) is the curd firming rate constant; kSR (%min) is the curd

syneresis instant rate constant; CFmax (mm) is the maximum curd firmness reached within 45 min; and tmax is the time taken to reach CFmax.

The cheese yield and milk nutrient recovery traits were analyzed in duplicate using the 9-Milca method [58]. Briefly, the cheesemaking procedure was mimicked by adding 200 μL of a rennet solution (Hansen Standard 215 with 80 5% chymosin and 20 5% pepsin; Pacovis Amrein AG) diluted to 1.2% (w/v) with fresh distilled water in 9 mL of milk. The initial incubation step was 30 min at 35 ◦C, after which a stainless-steel spatula was used to make the first cut. Next, a curd-cooking phase of 30 min at 55 ◦C was performed, during which a second manual cut was made. Once the cooking phase was finished, the curd was separated from the whey for 30 min at a temperature near to 25 ◦C, by gently applying pressure to the curd to better drain the whey. Lastly, the whey composition (fat, protein, lactose and TS) was measured with an FT2 infrared spectrophotometer (Milkoscan FT2; Foss Electric A/S, Hillerød, Denmark). This procedure resulted in seven cheesemaking traits. The curd weight (CYCURD), curd dry matter (CYSOLIDS) and water retained in the curd (CYWATER) are expressed as a percentage of the total milk processed. The curd nutrient recoveries (REC), namely, RECPROTEIN, RECFAT, RECENERGY and RECSOLIDS (%), were calculated by measuring the difference in weight and composition between the milk and whey, according to [59]. For all the procedures, repeated measures were performed that were averaged prior to the statistical analysis.

5.6. Feces collection and nutrient digestibility

Fecal samples were collected from the rectum on days 0, 14, 28, 42 and 54 of the exposure periods. The feces were mixed, and subsamples were taken. The first subsample was dried at 60 ◦C, as described above. The ADL in this first subsample was used as an internal marker to calculate the digestion coefficients of the fecal samples.

5.7. Blood sampling and blood biochemistry

Blood samples were collected for chemical analysis before start of the trial and after 1, 4 and 8 weeks of the experimental period. The samples were collected in the morning (before feeding) by venipuncture of the jugular vein in 10 mL Li–heparin-treated tubes (Vacuette, containing 18 IU of Li–heparin/mL, Kremsmünster, Austria). After

collection, the samples were cooled immediately in an ice water bath. Part of the blood was used to calculate the packed cell volume (PCV) (Centrifugette 4203; ALC International Srl, Cologno Monzese, Italy). The other part was centrifuged (3500× g for 16 min at 4 ◦C) to collect the plasma, which was stored in aliquots at −20 ◦C until the analysis. The plasma metabolites were analyzed at 37 ◦C using an automated clinical analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA, USA), as described by [60]. Commercial kits from Instrumentation Laboratory SpA (Werfen, Italy) were used to measure the glucose, total cholesterol, urea, Ca, P, Mg, total protein, albumin, total bilirubin and creatinine. Kits from Wako (Chemicals GmbH, Neuss, Germany) were used to measure non-esterified fatty acids (NEFA), beta-hydroxybutyric acid (BHBA) and Zn. Electrolytes (Na, K and Cl) were measured using a potentiometer method (ion-selective electrode connected to ILAB 650). A kinetic analysis was used to determine the alkaline phosphatase (AP; EC 3.1.3.1), aspartate aminotransferase (AST; EC 2.6.1.1) and γ-glutamyltransferase (GGT; EC 2.3.2.2) activities, with kits from Instrumentation Laboratory SpA. The ceruloplasmin, haptoglobin, paraoxonase (PON) activity, myeloperoxidase (MPO) activity, reactive oxygen metabolites (ROMt) and ferric-reducing antioxidant power (FRAP) were measured as described by [61].

5.8. Statistical analysis

Data were collected daily (i.e., dry matter intake or DMI, milk yield or MY, rumination time or RT and body weight or BW), weekly (milk composition and fat- or energy-corrected milk) or at a lower frequency during several occasions during the study (blood samples at the start and weeks 1, 4, and 8). Before the statistical analysis, all the data were tested for normality. Variables that tested as non-normal distributions (such as SCC) were log 10-transformed before the statistical analysis. The data were analyzed using the MIXED procedure in SAS, and for all the data that was measured more than once in each cow, analyses with repeated measures were included [62] according to the following model:

$$Yijklm = \mu + Ti + Dj + (T \times D)\ ij + pk + cl + eijklm,$$

where Yijklm is the dependent variable, μ is the population mean, Ti is the fixed effect of the treatment, Dj is the fixed effect of the time (day or week) of measurement (repeated measurements), T Dij is the fixed effect of the treatment time (day or week) of mea- surement interaction, pm is the fixed effect of the period, cl is the random effect of a cow and eijklm is the residual error. The same model was separately run for both adaptation and exposure periods.

Five covariate model structures were used based on the finite sample corrected Akaike information criterion (AICC) and the Schwarz Bayesian criterion for the best fitting model. The five tested structures were compound symmetry, heterogeneous compound symmetry, unstructured, auto regressive (1) and anti-dependence [62,63]. A p value below 0.05 was considered significant, and a p value between 0.05 and 0.10 was considered to indicate a trend. In the tables, p values lower than 0.001 are reported as <0.001. If the main effects (i.e., T and W) or the first order interaction (T * W) were significant, a post-hoc multiple comparison T test was adopted to verify the differences among the least square means.

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Chapter 4

Effect of ZEN contamination and use of mycotoxin mitigation product on productive and reproductive performances in early lactating dairy cows.

It will be submitted to Journal of Dairy Science soon.

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Abstract

Mycotoxins are secondary metabolites from different fungi genera, and they could be detected in several feed sources. *Fusarium* mycotoxins (DON, ZEN, and FBs) could cause gastrointestinal disorders, immune suppression, inflammation, hepatotoxicity, decrease milk performance and reproductive disorders. ZEN is one the most investigated *Fusarium* mycotoxin that can affect meanwhile productive and reproductive performances. ZEN and its metabolites had an estrogenic action and could modify the cow estrous cycle. In our preliminary trial we involved 30 Holstein dairy cows 21 multiparous and 9 primiparous) from 2nd to 8th weeks of lactations and fed with one of three experimental diets: control diet (CTR) with a low ZEN contamination, mycotoxin diet (MTX) with high ZEN contamination and finally MTX diet supplemented with 35 gr/cow/day of MMP (MMP). Though the experimental period we collected data of dry matter intake, rumination time, resting time, body weight, nutrient digestibility, milk production and quality, body condition score, immune-metabolic parameters, hormonal profile (anti-Mullerian AMH and progesterone), ovarian structure development and reproductive indexes. Primiparous cows seems to eat more dry matter in the MMP group, but others feeding traits dint' changed. ZEN contamination numerically decreased milk production but didn't affect quality. Only protein content decreased in primiparous. Milk urea increased in both parties' groups in MTX, with a MMP mitigation effect. ZEN contamination mainly modified some immune-metabolic parameters in multiparous cows like glucose, myeloperoxidase, and ALP. AMH didn't change among experimental group, but ZEN contamination tended to decrease the milk progesterone content. In addition, ZEN seemed to modify the ovarian activity recovery after calving. We noted that mycotoxin contamination decreased the number of corpus luteum in primiparous and increase the dimension of follicular cysts in multiparous. In our trial we detected many negative effects of ZEN contamination after calving and the inclusion of MMP seems to mitigate some of them. Moreover, these effects changed according to the number of lactations, suggesting a different susceptibility depending on cow's age. Further studies must be performed with large number of primiparous and multiparous cows to better understand the ZEN effect on productive and reproductive performance after calving.

Key words: *zearalenone, mycotoxins, milk production, reproduction, anti-Mullerian*

1. Introduction

Mycotoxin are secondary metabolites produced by many filamentous fungi belonging to the genera Fusarium, Aspergillus, and Penicillium spp that can cause toxic responses when ingested by humans and other vertebrates (Fink-Gremmels, 2008; Zinedine et al., 2007). Mycotoxins are generally very stable and can be find out in animal feeds and homegrown forage (Cheli et al., 2013; Gallo et al., 2015a; Kabak et al., 2006). Fusarium spp produce mycotoxins that are usually detected in several feeds because the fusarium molds are widespread and able to contaminate field crops in the temperate and warm climate zones ("Scientific Opinion on Risks for Animal and Public Health Related to the Presence of Nivalenol in Food and Feed," 2013). Previous studies have described the toxicological effects of fusarium toxins in farm animals. (Antonissen et al., 2014; Fink-Gremmels & Malekinejad, 2007; Pestka, 2007; Voss et al., 2007). In fusarium mycotoxins we found deoxynivalenol (DON) that can cause gastrointestinal disorders, immune suppression, and inflammation (Fink-Gremmels & Malekinejad, 2007; Pestka, 2007) and Fumonisins (FBs) that are cytotoxic, hepatotoxic, and nephrotoxic (Riley & Pestka, 2005). Another fusarium mycotoxin is Zearalenone (ZEN) with a resorcyclic acid lactone structure like estradiol, in particular the sex-hormone 17 β-estradiol (E2). That conformation allows ZEN to bind estrogenic receptors leading an estrogenic action (Riley & Pestka, 2005). ZEN oral bioavailability is different between the species (Catteuw et al., 2019) and in ruminant the adsorption is scarce due to the rumen microbiota efficient ZEN degradation in α-zearalenol (α-ZEL) and β -ZEL (Knutsen et al., 2017). α-ZEL is 60 times more potent than ZEN while the potency β -ZEL is 0,2x of that ZEN. The presence and ratio of ZEN, α-ZEL, β -ZEL changes in vitro and vivo conditions. Debevere et al reported a higher conversion of ZEN into α-ZEL than into β -ZEL in vitro (Debevere et al., 2020). Nevertheless, in vivo conditions β -ZEL is more produced than α-ZEL (Knutsen et al., 2017). Confirming this, Danicke found that in the bile of cows fed with a ZEN contaminated diets ZEN range from 12 to 20%, α-ZEL 6 to 13% and finally β-ZEL 68 to 76%, as the majority part of them. Different studies reported the interferences in the cow reproductive function when fed with ZEN contaminated diets (Metzler et al., 2010) including the disturbance of the estrous cycle, pathological changes of the reproductive tract of males and females, decreased fertility,

decreased neonatal survival in females and their offspring (Kuiper-Goodman et al., 1987; “Scientific Opinion on the Risks for Public Health Related to the Presence of Zearalenone in Food,” 2011). ZEN contamination at lower level (0,3 mg/kg) decreases the oocyte quality but embryo development wasn’t affected (Silva et al., 2021). Fushimi et al reported an increase in the population of ovarian antral follicles and increased synthesis of anti-Mullerian (AMH) hormone by follicular granulosa cell in cows exposed to ZEN at dose close to 1 mg/kg diet (Fushimi et al., 2015). Another study pointed out a time correlation between ZEN urinary value and AMH: Low ZEN in urinary tend to result in high AMH value in the next month (Widodo et al., 2022). In vitro Zen and its metabolites detected in bovine follicular fluids may be influenced by feeding conditions on farm but at the same time high concentration of ZEN may not have effects on bovine oocytes fertilization and development after IVF (Takagi et al., 2008a). The AMH hormone is produced by granulosa cells from growing follicles and regulates the recruitment of the quiescent ovarian follicles. The antral follicle count (AFC) is positively associated with plasma concentrations of AMH (J. L. H. Ireland et al., 2008; Rico et al., 2011) and it is suitable used as marker for ovarian reserve (Rico et al., 2011). Moreover, AMH in plasma varies little through the estrous cycle which facilities as marker at random stages of estrous cycle (J. L. H. Ireland et al., 2008; Rico et al., 2011). Different studies (J. J. Ireland et al., 2007; Rico et al., 2012; Singh et al., 2004) described a relationship between the AMH plasma concentration and the cow response to multiple ovulation treatments and the number of oocytes recovered by ovum pick up in dairy cows. Furthermore, Souza et al proposed the evaluation of AMH as a marker to identify cows with greater responses to super stimulation and thus improve efficiency of superovulation programs in dairy cows (Souza et al., 2015). In 2014 Ribeiro et al support a positive association between AMH and fertility in lactating dairy cows, because cows with low AMH concentrations had smaller pregnancy rates after first service and greater incidence of pregnancy loss between d 30 and 65 of gestation (Ribeiro et al., 2014).

In literature different studies evaluate the response of some mycotoxin deactivation product to minimize the negative effect of contaminated diets (citation). A previous study tested the effect of a MDP (Mycofix) on lactating dairy cows fed diets naturally contaminated by aflatoxins (Pietri et al 2009) or several fusarium toxins (Kiyothong et

al., 2012). The European Union has approved this product for the use in pig and poultry (Murugesan et al., 2015). This product is based on 3 principal properties: (1) its inorganic components (e.g., bentonite) adsorb polar, planar mycotoxins such as AF; (2) for mycotoxins unaffected by the inorganic components (e.g., trichothecenes, ZEA, FB), Eubacterium strain Biomin BBSH797 deactivates trichothecenes, a biological constituent deactivates ZEA, and a purified enzyme (fumonisin-esterase, FUMzyme 30,000 U/kg of Mycofix) biotransforms FB into nontoxic metabolites; and (3) its phycophytic substances (usually a blend of extracts from plants and algae) provide protective effects. FUMzyme can convert FB in their hydrolyzed forms (HFB) or partially hydrolyzed forms (pHFB) by acting an enzymatic degradation using fumonisin esterase FumD (D. Hartinger & Moll, 2011; Heinl et al., 2010). The aims of the study were to evaluate the effects of a diet contaminated by a regular concentration of ZEN as fusarium mycotoxin on feeding behavior, rumination activity, milk yield and quality, biochemical traits, reproductive performance, impact on the ovarian structure development in early lactation after calving and to determine the effect of a mycotoxin-deactivating product in preventing the negative effects of ZEN and its metabolites previous described.

2. Materials and methods

The trial was authorized by Italian Health regulations that pertain to the accommodation and care of animals used for experimental and other scientific purpose (n° 112/2022-PR).

2.1 Experimental cows and diets

Thirty Holstein dairy cows (70% multiparous and 30% primiparous) were involved in the study at CERZOO (San Bonico, Piacenza, Italy), experimental dairy farm of University Cattolica del Sacro Cuore di Piacenza. The trial period started from 7 days after calving until 56 days in milk (**DIM**), after an adaptation period from calving of 6 days. Cows were monitored at total for 49 days after calving (7 weeks), from the 2nd week of lactation to the 8th week. They were housed in a free stall barn with cubicles as resting area. Cows were milked twice daily (4.30 and 16.30 hr.), and fresh water was available at libitum through the entire period. Experimental total mixed ration (**TMR**) was daily distributed at libitum at 8.00 through feed bins of the RIC automatic feeding system (Hokofarm Group, Netherlands), with 5% expected orts. The TMR feeds were mixed in a self-propelled mixer wagon (Rotomix, Bravo, Cuneo, Italy) and then distributed. Dairy cows were allocated to the experimental groups according to number of party (7 multiparous and 3 primiparous for each experimental group), total milk production of the previous lactation for multiparous cows and body weight (**BW**) at calving. Cows were divided in three experimental groups: the control group (**CTR**), mycotoxin group (**MTX**) and treated group (**MMP**). Animals in CTR group were fed with TMR contaminated by a regular level of Fusarium mycotoxins (FB1 578,79 µg/kg DM, FB2 313,60 µg/kg DM, ZEA 55,42 µg/kg DM, DON 226,87 µg/kg DM), MTX group was fed with TMR contaminated by a higher level of Fusarium mycotoxins (FB1 613,49 µg/kg DM, FB2 338,06 µg/kg DM, ZEA 366,63 µg/kg DM, DON 1141,54 µg/kg DM) and MMP group received a MTX diet (FB1 559,56 µg/kg DM, FB2 282,82 µg/kg DM, ZEA 319,72 µg/kg DM, DON 1028,42 µg/kg DM) supplemented with 35 g/animal per day of a mycotoxin-deactivating product (Mycofix, BIOMIN Holding, GmbH, Tulln, Austria). Representative TMR samples were collected weekly and

analyzed for nutrient composition and mycotoxins. Diets compositions were reported in the table 1.

2.2 Analysis of feeds, diets and mycotoxins

The TMR from experimental groups were weekly sampled and each was dried at 60 C° in a ventilated oven for 48 h for DM determination, milled though a 1 mm screen using a laboratory mill (Thomas-Wiley, Arthur H. Thomas Co., Philadelphia, PA) and stored for other analysis. The TMR samples were analyzed with a near-infrared instrument by Foss (Hilloreroed, Denmark) NIR system 5000 spectrophotometers equipped with a monochromator and transport module, scanning over the wavelength range 400-2500nm, every 2 mm. TMR was characterized for crude protein (CP), NDF, ADF, ADL, Ash, ether extract (EE), starch, neutral detergent insoluble CP (NDICP) and acid detergent insoluble CP (ADICP). Energy evaluation of the diets were calculated according to NRC 2001.

Mycotoxin were weekly analyzed in experimental TMR. Measurement of mycotoxin followed the methods of Gallo et al. (2010) for AF the methods of Pietri and Bertuzzi (2012) for FB, the methods of Bertuzzi et al (2014) for ZEN, DON, T-2 and HT-2 toxin and methods of Rossi et al. (2006) for OTA. Mycotoxins were them measured using an HPLC instrument with a fluorescence detector (FLD). The limit of detection (LOD) and the limit of quantification (LOQ) were 0,05 and 0,15µg/kg, respectively. After extraction with a phosphate buffer and purification using an immuno-affinity column (R-Biopharm Rhone LTD), FB were quantified by HPLC coupled with a mass spectrometer.

The LOD and the LOQ were 10 and 30 µg/kg, respectively. The ZEN, DON, T-2, and HT-2 toxin were extracted with acetonitrile: water (86:14 vol/vol) and purified using an immuno-affinity column (ZEN) or a Trilogy-Puritox Trichothecenes column (DON, T-2 and HT-2 toxins) from R-Biopharm (Rhone LTD). Quantification was performed using HPLC-FLD (ZEN), GC-MS (DON), or LC-MS/MS (T-2 toxin and HT-2 toxin). The LOD and LOQ values were 2 and 5 µg/kg for ZEN; 10 and 30 µg/kg for DON; and 0.5 and 1.5 µg/kg for T-2 toxin and HT-2 toxin, respectively. OTA was extracted from samples using methanol-3% aqueous sodium hydrogen carbonate (50:50 vol/vol),

purified using an immunoaffinity column (R-Biopharm Rhone LTD), and quantified using HPLC-FLD. The LOD and LOQ for OTA were 0.2 and 0.6 μg/kg, respectively.

2.3 Feeding behavior, Rumination time, body weight and body condition score.

All cow in the study were fed through the feed bins of the RIC system which recoded the daily TMR consumption for each cow. The dry matter content of each experimental TMR was weekly analyzed and used to obtain the daily dry matter intake (**DMI**) for each cow. Moreover, rumination time (**RT**) and eating time (**ET**) of each cow were recorded using a neck sensor collar by an accelerometer (RuminAct, SCR Heatime, Netanya, Israel) and was described in results as daily average rumination time and daily average eating time. Each cow was weighted after milking twice a day by an electronic weigh (Afifarm, Israel). Body condition score (**BCS**) was visual performed by the same operator with a 1 to 5 scale according to (Edmonson et al., 1989) after the morning feed. Lying time was reported as daily total resting time and as mean resting time (**MRT**), measured by a leg pedometer (Afifarm, Israel).

2.4 *Health status of cows*

Health status of cows was daily monitored throughout the study. No animals with severe disease were included in the study. Furthermore, cows with severe disease in the adaptation period were excluded from the trial. Health problems of the cows were recorded but they didn't affect the milk production and animal behavior. Metritis and endometritis were diagnosed with ultrasound check by the farm vet. Mastitis was diagnosed by visual evaluation of abnormal milk, comatic cell count and on farm culture bacteria test were performed for suspicious cases. During the trial no signs of clinical mastitis was detected. Diarrhea was diagnosed by visual evaluation and color of feces using the feal score method (Ireland-Perry & Stallings, 1993), with diarrheic feces defined as those with a fecal score of 2 or less. Ruminal problems and lameness were examined by a veterinary practitioner.

2.5 Milk yield and quality

Milk yield was daily recorded by milk meter at the milking time (Afifarm, Israel). Weekly representative milk samples were taken during the experimental period for each cows enrolled. Milk samples were analyzed using infrared measurements (MilkoScan FT 120, Foss Eletric, Hillerod, Denmark) according to (Chessa et al., 2014) to determine the milk content of fat, protein, casein, lactose, urea. Additional weekly milk samples were taken for SCC determination using an optical fluorometric method with an automated cell counter (Fossomatic 180, Foss Eletric).

2.6 Blood sampling and blood biochemistry and AMH

Blood samples were weekly collected during the experimental period for chemical analysis from 2nd week of lactation to the 8th. Samples were taken in the morning (before feeding) by venipuncture of the jugular vein using a 10-mL lithium heparin tubes (Vacuette, containing 18 IU of Li-heparina/mL, Kremsmunster, Austria) and immediately cooled in an ice water bath. A small amount of blood was used to calculate packed cell volume (Centrifugette 4203; ALC International Srl, Cologno Monzese, Italy), the remaining blood was centrifuged (3,500 × g for 16 min at 4°C), and the resulting plasma was separated into aliquots and stored at −20°C until analysis. Plasma metabolites were analyzed at 37°C using an automated clinical analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA) as described by Calamari et al. (2016). Commercial kits from Instrumentation Laboratory SpA (Werfen, Italy) were used to measure glucose, total cholesterol, urea, Ca, P, Mg, total protein, albumin, total bilirubin, and creatinine. Kits from Wako (Chemicals GmbH, Neuss, Germany) were used to measure nonesterified fatty acids (NEFA), BHBA, and Zn. Electrolytes (Na, K, and Cl) were measured using a potentiometer method (ion-selective electrode connected to ILAB 650). Kinetic analysis was used to determine the activities of alkaline phosphatase (ALP; EC 3.1.3.1), aspartate aminotransferase (AST; EC 2.6.1.1), and γ-glutamyltransferase (GGT; EC 2.3.2.2), with kits from Instrumentation Laboratory SpA. Ceruloplasmin and haptoglobin were measured as described by Calamari et al. (2016), paraoxonase activity as described by Bionaz et al. (2007), myeloperoxidase activity as

described by Bradley et al. (1982), and ferric reducing antioxidant power as described by Benzie and Strain (1999). Blood analysis of AMH was performed every week during the study by a laboratory kit (Bovine AMH Elisa AL – 114; AnshLabs, Webster, US).

2.7 Feces collection and nutrient digestibility

Feces samples were individually collected from the rectum of each cow at the 3rd and 7th weeks after calving. Subsamples of feces were taken by sampling the cow for 3 consecutive days (twice a day: at 8.00 am and 3.00 pm the first day, 9.00 am and 4.00 pm the second day and 10.00 am and 5.00 pm at third day) at the 3rd and 7th lactation weeks. The subsamples were mixed thoroughly to obtain a representative sample of the sampled week. After that samples were dried at 60 C° in a forced draft oven for 48 h, ground with a knife mill (1-mm screen) and then DM, ash, NDF, ADF, ADL and CP were measured using an infrared measurement by Foss (Hilloreroed, Denmark). The ADL content was used as an internal marker to calculate apparent digestibility for starch, CP and NDF. The ADL faecal content was used as an internal marker to calculate digestion coefficients according to the following equations:

$$X\ digestibility = (1-([ADL]_{diet} \times [X]_{faeces})/([ADL]_{faeces} \times [X]_{diets})) \times 100\%$$

with: X digestibility = digestibility coefficient of specific nutrient X (starch, NDF or CP). The following suberscript indicated samples (with diet or faeces) in which the nutrient or internal marker were quantified, as described above.

2.8 Ultrasonography, ovarian activity, and milk progesterone.

Ovarian structures activity was examined by a veterinarian practitioner and measured by a transrectal ultrasonography (**US**, 7.5-MHz transrectal transducer, Aloka 210, Corometrics Medical Systems Inc., Wallingford, CT) every week from 2nd week of lactation to the 8th. At each US check the number and size of follicles (**FO**) (only follicles bigger than 0,5 mm was detected), corpus luteum (**CL**) and cystic structure (**CS**) were measured and recorded. Cystic structures were characterized as follicular structure that exceeded 25 mm in diameter in absences of CL (Garverick, 1997). Milk progesterone (P4) measured over time is widely known as a practical parameter to

understand the cow's reproduction status (Friggens & Chagunda, 2005). P4 trend was monitored from the 3rd to 8th week of lactation during the experimental period. Milk samples for P4 determination were collected twice a week with an equal day interval between sampling and immediately frozen (at -18C°) at the farm. At the end of the trail all samples were send to laboratory for analysis. P4 concentration of milk samples was measured with competitive ELISA, using a commercial milk P4 kit (Ridgeway, Gloucester, UK) and expressed as pg/ml. Besides the US check for ovarian structures detection and measurements, we recorded at the same time the cleanliness of the uterine lumen system and the degree of the uterine involution after calving. This quality check was recorded with a uterine wellness score (WU) for the statistical analysis as other studies….. Our score was based on 4 grades of wellness: 1 (corrected uterine involution development with absence of material and liquid in the uterine lumen), 2 (corrected uterine involution development with low uninfected liquid and no material in the uterine lumen), 3 (slowed uterine involution development with low infected liquid and no material in the uterine lumen) and 4 (slowed uterine involution development with infected liquid and material in the uterine lumen).

2.9 Reproduction performance

All cows at the end of the experimental period started a Double Ovsynch protocol (Souza et al., 2008) for the first artificial insemination (**AI**) and all cows were inseminated between 83 and 89 d after calving. First pregnancy diagnosis was performed by ultrasonography between 32 and 38 d after each AI (Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004 a,b; Chebel et al., 2006; Juchem, 2007). The same pregnancy diagnosis method was performed after each following AI. Second pregnancy diagnosis was executed with the previous method between 80 and 87 d after AI to detect any embryo losses by US (Fricke, 2002). All cows enrolled in the experiment detected as pregnant at the first pregnancy diagnosis were still pregnant at the second pregnancy check. Consequently, no embryo losses were detected during the experimental trial. Throughout the paper, the first pregnancy diagnosis was designed as d 35. Pregnancy at double ovsynch (**P/TAI**) was calculated as the proportion of inseminated cow pregnant at the first pregnancy check. Day Open (**DO**) was calculated

as the proportion of time between calving and the pregnancy date and conception rate (**CR**) was calculated as 1 divided the number of AI for pregnancy. Heat detection was recorded by a pedometer heat detection system (Afifarm, Israel). Days interval between calving and first heat detection (**1HD**) was recorded. Cows were classified as anovular when no CL was detected in the experimental period as recommended by Bartolome et al., (2000). We considered cycling cows when at least 1 CL was detected by US check. In addition, we used data from heat detection and P4 milk to determine cyclin cows. Time at first ovulation after calving was detected combining the data of presence/absence of ovarian structures at weekly US, heat detection and milk P4. Day at the first ovulation (**1OV**) was calculated as time interval between calving and the day that occur the first ovulation previously detected.

3.0 Statistical analysis

Variables with non-normal distributions (such as SCC) were log 10-transformed before the statistical analysis. Data were collected daily or weekly. Data that was measured more than once in each cow during each experimental period was tested for normality and analyzed as repeated measurements using the MIXED procedure in SAS (SAS Inst. Inc., Cary, NC, release 9.3, 2002-2010) according to the model:

$$Yijklm = \mu + Ti + Dj + (T \times D)\ ij + pk + cl + eijklm;$$

where: Yijklm is the dependent variable, μ is the population mean, Ti is the fixed effect of treatment, Dj is the fixed effect of time (day or week) of measurement (repeated measurements), T x Dij is the fixed effect of treatment x time (day or week) of measurement interaction, pm is the fixed effect of order or parity, cl is the random effect of a cow, and eijklm is the residual error. The same model was separately run for both adaptation and intoxication periods.

Five covariate model structures were used based on the finite-sample corrected Akaike information criterion (AICC) and the Schwarz Bayesian criterion for the best fitting model. The 5 tested structures were compound symmetry, heteroge-neous compound symmetry, unstructured, auto-regressive (1), and ante-dependence.

Data that was measured once in each cow was tested for normality and analyzed using the MIXED procedure in SAS (SAS Inst. Inc., Cary, NC, release 9.3, 2002-2010) according to the model:

$$Yijkl = \mu + Ti + pj + ck + eijkl;$$

where: Yijklm is the dependent variable, μ is the population mean, Ti is the fixed effect of treatment, pm is the fixed effect of period, cl is the random effect of a cow, and eijklm is the residual error. The same model was separately run for both adaptation and intoxication periods.

A P-value below 0.05 was considered significant, and a P-value between 0.05 and 0.10 was considered to indicate a trend.

3. RESULTS

3.1 Diets and mycotoxin contamination

Table 1 shows the chemical composition of the experimental TMR and mycotoxin contamination levels. About the chemical composition the CTR, MTX, and MMP diet were similar in fiber fraction NDF, ADF and ADL and Ash content. The MTX diet was slightly higher in protein content (14,95%) than MMP (14,74%) and CTR diet (14,58%). The average value for starch (25,74% for CTR, 25,61% for MTX, 25,45% for MMP) and sugar content are very similar between experimental diets. For this reason, we can assume that the three groups were fed with iso-energetic diets during the proof. Regarding regulated mycotoxin contaminations in the TMR, we can remark a difference in mycotoxin contamination specifically between the CTR group and MTX-MMP groups. The average values of ZEN and DON are 366,63 and 1141,54 µg/kg DM in MTX and 319,72 and 1028,42 µg/kg DM in MMP groups, respectively. As expected, the lowest ZEN and DON contamination we found in the CTR diet with an average of 55,42 µg/kg DM and 226,87 µg/kg DM, respectively. ZEA and DON contamination in the MTX and MMP groups were 6- and 5-times greater than CTR groups to properly achieve the aims of the study previously described. Anyway, the mycotoxin contamination was under European commission limit levels (0,5 mg/kg for ZEN and 5 mg/kg for DON) (*COMMISSION RECOMMENDATION on the Presence of Deoxynivalenol, Zearalenone, Ochratoxin A, T-2 and HT-2 and Fumonisins in Products Intended for Animal Feeding (Text with EEA Relevance) At the Request of the Commission the European Food Safety Authority (EFSA) Adopted Opinions on the Myco-Toxins Deoxynivalenol*, 2006). Otherwise, the FB1 and FB2 contamination among diets did not differ much each other because the FB1 levels were approximately around 500/600 µg/kg DM and 300 µg/kg DM for FB2. Therefore, there wasn't a significant difference in FB1 and FB2 mycotoxin contamination among experimental groups.

Table 1. Chemical composition, digestibility, and energy evaluations of experimental total mixed ration (TMR) diets fed to lactating dairy cows in the trial.

Items	Treatment		
	CTR	MTX	MMP
Ingredients (% DM)			
Corn meal	16,24	16,24	16,24
Barley meal	4,06	4,06	4,06
Soybean, solvent meal 44%	14,77	14,77	14,77
Alfalfa hay	11,33	11,33	11,33
Ryegrass hay	3.23	3.23	3.23
Mineral-vitamin supplement2	1,89	1,89	1,89
Fat (palm oil)	0,76	0,76	0,76
Corn silage	34,61	34,61	34,61
Sorghum silage	12,58	12,58	12,58
Placebo	0,34	0,15	
Mycotoxin Add		0,19	0,19
MMP product			0,15
Chemical composition (% DM)			
DM (% as fed)	47,13	49,21	49,28
CP	14,57	14,94	14,71
soluble CP	5,75	5,66	5,69
ash	5,74	5,69	5,78
aNDFom	32,63	32,09	32,24
ADFom	17,49	17,22	17,30
ADL	2,39	2,34	2,25
NDFD 24h			
EE	3,19	3,07	3,12
Starch	25,73	25,60	25,44
Sugar	4,58	4,73	4,71
Mycotoxin contamination4 in TMR (µg/kg DM)			
FB1 in TMR	578,79	613,49	559,56
FB2 in TMR	313,60	338,06	282,88
ZEA in TMR	55,42	366,63	319,72

3.2 Feeding behavior, Rumination time, body weight and body condition score.

Table 2 reported the DMI, BW, RT, BCS and resting time and mean resting analyzed for each week during the trial. The three groups had no significant differences in daily DMI for multiparous cows even though a numerically difference in CTR group with higher DMI (26,23 kg/DM/cow/day) compared with MTX (22,85 kg/DM/cow/day) and MMP group (23,41 kg/DM/cow/day). We observed that primiparous cows tended to eat more DM (19,17 kg/DM/cow/day; P<0,10) in early lactation in the MMP group when compared with primiparous fed with CTR (17,39 kg/DM/cow/day) and MTX (17,50 kg/DM/cow/day) diets. The treatment effect was significant at 5^{th}, 7^{th} and 8^{th} lactation weeks (P<0,05) with higher amount of feed ingested after first calving in MMP group. BW didn't differ among groups with an average of 550 kg for primiparous and 640 kg for multiparous cows. Similarly, the BCS was comparable among groups with not statistically differences. Rumination time have an opposite trend among primiparous and multiparous cow. After first calving cows spent more time daily to ruminate (548,56 min/cow/day) when fed with MMP diet. Oppositely multiparous cow had the highest RT when fed with CTR diets. Unfortunately, not statistically difference we found in the rumination behavior due to the grater variability among each cow. Cows had lower numerically RT in MMP (551,96 min/day/cow) than MTX (569, 26 min/day/cow) and CTR (598,67 min/day/cow). The daily average RT for the entire experimental period was numerically higher respectively in CTR group, then MTX and finally in MMP with not statistically difference. Only primiparous spent more time for each resting time (P<0,05) at 7^{th} and 8^{th} lactation weeks when fed with MXT diet (44,11 min/cow/day and 43,06 min/cow/day) compared with CTR (34,64 min/cow/day and 35,5 min/cow/day) and MMP (28,04 min/cow/day and 31,56 min/cow/day).

3.3 Feces collection and nutrient digestibility

About feces analysis the result is reported in tab. n° 2 with the previous described coefficient of apparent digestibility for NDF, starch and protein. We didn't identified any statistically differences between experimental groups for data showed in table n°6. Apparent digestibility for NDF, starch, and protein appeared comparable among different groups. Similar comparison was detected for the nutrient profile of feces collected during the trial at 3rd and 7th weeks of lactation.

Table 2 (FEEDING BEHAVIOUR) Least squares means and associated SEM for feeding intake, rumination time, body weight, resting time and apparent digestibility of Holstein cows fed control diets with low mycotoxin contamination level (CTR), diet with high mycotoxin contamination level (MTX), or MTX diet added with about 35 g/cow/day of MMP product (MMP-) during the experimental period.

Items		Parties		Treatment			Sem	P of the model				
		Primiparous	Multiparous	CTR	MTX	MMP		Party	Treatment (T)	Day (D)	D * P	D*T
DMI	Primip+Multip	18,02	24,16	21,81	20,18	21,29	0,533	<0,05	0,424	<0,05	0,170	0,881
kg/d	Primiparous			17,39	17,50	19,17	0,498		0,064	<0,05		0,600
	Multiparous			26,23	22,85	23,41	0,948		0,185	<0,05		0,947
RT	Primip+Multip	525,36	520,29	538,08	501,69	528,72	296,91	0,865	0,583	<0,05	0,678	0,545
min/d	Primiparous			526,20	501,33	548,56	313,45		0,412	<0,05		0,491
	Multiparous			549,96	502,05	508,88	507,23		0,628	<0,05		0,551
BW	Primip+Multip	549,45	643,23	592,09	582,63	615,31	12,662	<0,05	0,267	<0,05	0,996	0,932
kg	Primiparous			544,43	535,73	568,20	15,530		0,448	<0,05		0,999
	Multiparous			639,75	627,53	662,42	20,073		0,486	<0,05		0,752
Resting time	Primip+Multip	534,85	611,74	598,67	569,26	551,96	315,42	<0,05	0,393	<0,05	0,870	0,998
min/d	Primiparous			551,04	539,84	513,67	450,72		0,537	<0,05		0,988
	Multiparous			646,03	598,68	590,24	445,48		0,493	0,151		0,928
Mean resting	Primip+Multip	34,20	60,33	43,83	50,15	47,82	4,798	<0,05	0,487	<0,05	0,159	0,556
min	Primiparous			35,82	37,20	29,59	4,149		0,395	<0,05		0,520
	Multiparous			51,83	63,10	66,05	8,389		0,147	<0,05		0,515
NDF digestibility	Primip+Multip	72,80	72,07	71,05	73,12	73,11	4,952	0,524	0,251	0,133	0,856	0,603
%	Primiparous			71,87	73,37	73,26	4,597		0,700	0,769		0,726
	Multiparous			70,37	72,98	72,98	5,33		0,322	0,070		0,368
Starch digestibility	Primip+Multip	98,51	98,29	98,37	98,63	98,20	0,080	0,317	0,259	0,986	0,700	<0,05
%	Primiparous			98,50	98,62	98,41	0,177		0,744	0,485		0,745
	Multiparous			98,27	98,65	98,00	0,105		0,264	0,425		<0,05
Protein digestibility	Primip+Multip	85,34	84,28	84,32	85,09	85,01	1,976	0,276	0,771	0,284	0,960	0,084
%	Primiparous			85,02	85,47	85,53	4,738		0,926	0,997		0,892
	Multiparous			83,78	84,73	84,449	1,934		0,839	0,098		<0,05

DMI = dry matter intake; RT = rumination time; BW = body weigh

3.4 Milk yield and quality

Measurement of the milk yield and milk composition are shown in table n°3. Milk yield has a different numerically trend between experimental groups according to the number of lactations. Primiparous in MMP group produced 2 kg milk more (37,29 kg/day) than cows in CTR and MTX (35,31 kg/day and 35,13 kg/day). Otherwise, multiparous milk yield was higher in CTR and MMP groups than in the MTX groups (+6,3 and +2,5 kg/day, respectively). Same numerically variations were observed for FCM and ECM daily production as showed in tab n°3. Feed efficiency was very comparable between groups and nearly 2 kg of milk for each kg oof DMI in any experimental group. Regarding milk quality, mycotoxin contamination hadn't showed any differences in fat and casein content. A different trend was observed according to the number of lactations. After first calving ZEN contamination tended ($P<0,10$) to decrease the milk protein content from higher percentage in CTR and MMP groups (3,13%) to MTX (2,99%). Together an opposite trend was observed for the lactose content: after first calving cow fed with MMP diet (4,68%) produced less milk lactose ($P<0,05$) than other with diets (CTR 4,86% and MTX 4,81%). However, these differences weren't' replicated for multiparous cows. An interest result was the milk urea content that was significantly different between experimental groups ($P<0,05$). Regardless the number of parties, a high ZEN contamination led to an increase of milk urea (29,31) compared to diet with a normal ZEN contamination (26,35) or when the MMP was added into the diet (26,11%). About the SCC statistically variations weren't remarked.

Table 3 (MILK PRODUCTION AND QUALITY) Least squares means and associated SEM for feeding behavior, body weight, milk yields, feed efficiency and milk parameters of Holstein cows fed control diets with low mycotoxin contamination level (CTR), diet with high mycotoxin contamination level (MTX), or MTX diet added with about 35 g/cow/day of MMP product (MMP-) during the experimental period.

Items		Parties		Treatment			Sem	P of the model				
		Primiparous	Multiparous	CTR	MTX	MMP		Party (P)	Treatment (T)	Day (D)	D * P	T*D
MY	Primip+Multip	35,91	45,56	42,11	38,88	41,21	0,339	<0,05	0,440	<0,05	0,421	0,283
kg/d	Primiparous			35,31	35,13	37,29	0,303		0,721	<0,05		0,980
	Multiparous			48,91	42,63	45,14	0,624		0,237	<0,05		0,795
FCM	Primip+Multip	34,90	43,24	40,60	37,05	39,56	0,797	<0,05	0,465	<0,05	0,289	0,971
kg/d	Primiparous			33,75	34,11	36,83	0,756		0,506	<0,05		0,338
	Multiparous			47,45	39,99	42,28	1,278		0,214	<0,05		0,271
ECM	Primip+Multip	34,55	42,66	40,33	36,48	39,00	0,672	<0,05	0,402	<0,05	0,299	0,922
kg/d	Primiparous			33,69	33,50	36,45	0,573		0,497	<0,05		0,869
	Multiparous			46,97	39,47	41,54	1,155		0,194	<0,05		0,441
Feed efficiency	Primip+Multip	2,06	1,93	2,02	2,01	1,95	0,007	0,200	0,822	<0,05	0,910	0,810
$dmnl^2$	Primiparous			2,11	2,07	1,99	0,012		0,697	<0,05		0,741
	Multiparous			1,94	1,94	1,92	0,010		0,987	<0,05		0,784
Fat	Primip+Multip	3,87	3,75	3,75	3,77	3,90	0,042	0,444	0,708	<0,05	0,636	0,613
wt/wt	Primiparous			3,70	3,89	4,04	0,063		0,461	<0,05		0,115
	Multiparous			3,78	3,67	3,78	0,055		0,879	<0,05		0,819
Fat	Primip+Multip	1,36	1,68	1,56	1,43	1,57	0,010	<0,05	0,495	<0,05	0,176	0,553
kg/d	Primiparous			1,31	1,33	1,48	0,003		0,408	<0,05		<0,05
	Multiparous			1,82	1,51	1,64	0,004		0,311	<0,05		<0,05
Protein	Primip+Multip	3,12	3,08	3,13	3,03	3,13	0,002	0,598	0,399	<0,05	0,332	0,125
wt/wt	Primiparous			3,13	2,99	3,21	0,002		0,068	<0,05		0,064
	Multiparous			3,13	3,06	3,04	0,003		0,732	<0,05		0,591
Protein	Primip+Multip											
kg/d	Primiparous											
	Multiparous											
Lactose	Primip+Multip	4,78	4,70	4,79	4,73	4,71	0,002	<0,05	0,160	<0,05	<0,05	0,536
wt/wt	Primiparous			4,86	4,81	4,68	0,004		<0,05	<0,05		0,073
	Multiparous			4,73	4,65	4,73	0,003		0,366	<0,05		0,916
Urea	Primip+Multip	26,19	28,33	26,35	29,31	26,11	3,846	0,071	0,050	<0,05	0,510	0,557
mg/100ml	Primiparous			24,93	29,12	24,23	9,967		0,107	0,066		0,602

	Multiparous			27,81	29,43	27,79	3,296		0,546	<0,05		0,367
SCC	Primip+Multip	287,36	152,69	68,21	258,57	333,30	35048	0,308	0,200	0,413	<0,05	0,415
log_{10} (cells/ml x 1,000)	Primiparous			83,18	138,70	615,63	82259		0,134	0,228		0,353
	Multiparous			53,23	363,57	41,26	35752		0,135	0,672		0,758
Casein	Primip+Multip	2,38	2,36	2,40	2,31	2,39	0,002	0,772	0,405	<0,05	0,388	0,462
wt/wt	Primiparous			2,39	2,28	2,45	0,001		0,124	<0,05		0,128
	Multiparous			2,42	2,34	2,32	0,003		0,611	<0,05		0,867

MY = milk yield; FCM = fat corrected milk; ECM = energy corrected milk; SCC = cell comatic count,

Table 4 (BLOOD PARAMETERS AND AMH HORMONE) Least squares means and associated SEM for blood and parameters and plasma AMH of Holstein cows fed control diets with low mycotoxin contamination level (CTR), diet with high mycotoxin contamination level (MTX), or MTX diet added with about 35 g/cow/day of MMP product (MMP-) during the experimental period.

Items		Parties		Treatment			Sem	P of the model				
		Primiparous	Multiparous	CTR	MTX	MMP		Party (P)	Treatment (T)	Day (D)	D * P	T*D
Packed cell volume	Primip+Multip	0,31	0,29	0,30	0,30	0,30	0,00005	<0,05	0,443	0,786	0,658	0,543
vol/vol	Primiparous			0,31	0,31	0,31	0,00007		0,888	0,230		0,097
	Multiparous			0,29	0,30	0,29	0,00006		0,327	0,815		0,089
Glucose	Primip+Multip	4,24	3,83	4,11	3,97	4,03	0,017	<0,05	0,305	0,170	0,303	0,820
mmol/L	Primiparous			4,24	4,23	4,26	0,010		0,987	<0,05		0,769
	Multiparous			3,98	3,70	3,81	0,034		<0,05	0,888		0,833
Cholesterol	Primip+Multip	3,68	4,02	4,00	4,06	3,49	0,019	0,193	0,153	<0,05	0,129	0,384
mmol/L	Primiparous			3,58	4,25	3,21	0,013		0,100	<0,05		<0,05
	Multiparous			4,43	3,86	3,78	0,037		0,226	<0,05		0,832
Urea	Primip+Multip	5,52	6,48	5,98	6,35	5,67	0,114	<0,05	0,159	<0,05	0,994	0,896
mmol/L	Primiparous			5,49	5,90	5,18	0,146		0,495	<0,05		0,555
	Multiparous			6,46	6,81	6,16	0,169		0,311	0,427		0,925
Total protein	Primip+Multip	77,61	81,20	79,18	80,59	78,45	1,095	<0,05	0,478	<0,05	0,569	0,717
g/L	Primiparous			77,28	79,78	75,75	1,874		0,257	<0,05		0,511
	Multiparous			81,07	81,39	81,14	1,511		0,990	<0,05		0,557
Albumin	Primip+Multip	36,90	37,07	37,08	37,04	36,84	0,189	0,754	0,933	<0,05	0,767	0,274
g/L	Primiparous			36,83	37,23	36,64	0,367		0,864	<0,05		0,892

	Multiparous			37,33	36,84	37,05	0,247		0,830	<0,05		0,090
Globulin	Primip+Multip	40,70	44,13	42,10	43,55	41,60	0,892	<0,05	0,578	<0,05	0,740	0,893
g/L	Primiparous			40,45	42,55	39,11	1,585		0,364	<0,05		0,420
	Multiparous			43,74	44,55	44,10	1,119		0,955	<0,05		0,262
GOT	Primip+Multip	91,90	91,50	95,08	90,14	89,87	42,495	0,936	0,618	<0,05	0,090	0,326
U/L	Primiparous			87,84	92,69	95,16	132,84		0,787	<0,05		0,677
	Multiparous			102,42	87,59	84,59	26,119		<0,05	<0,05		0,356
GGT	Primip+Multip	17,64	24,65	19,86	25,01	18,56	1,637	0,070	0,352	0,704	0,655	0,552
U/L	Primiparous			16,417	19,301	17,19	0,582		0,363	0,112		<0,05
	Multiparous			23,30	30,72	19,92	2,904		0,335	0,264		0,055
Bilirubin	Primip+Multip	2,87	2,10	2,18	2,63	2,64	0,303	<0,05	0,530	<0,05	0,908	0,130
µmol/L	Primiparous			2,68	2,99	2,93	0,894		0,924	<0,05		0,736
	Multiparous			1,69	2,26	2,35	0,199		0,381	<0,05		<0,05
ALP	Primip+Multip	73,02	38,79	52,84	54,59	60,29	25,228	<0,05	0,749	<0,05	0,100	0,888
U/L	Primiparous			59,41	70,73	88,92	102,79		0,485	0,197		0,784
	Multiparous			46,26	38,44	31,65	4,246		<0,05	<0,05		0,870
Haptoglobine	Primip+Multip	0,24	0,12	0,16	0,20	0,18	0,005	<0,05	0,775	<0,05	0,547	0,940
g/L	Primiparous			0,24	0,27	0,21	0,013		0,856	<0,05		0,950
	Multiparous			0,08	0,13	0,15	0,005		0,360	<0,05		0,537
NEFA	Primip+Multip	0,54	0,38	0,39	0,48	0,51	0,004	<0,05	0,142	<0,05	0,731	0,108
mmol/L	Primiparous			0,47	0,59	0,57	0,009		0,418	<0,05		0,727
	Multiparous			0,31	0,37	0,45	0,006		0,222	<0,05		0,097
BOHB	Primip+Multip	0,57	0,68	0,54	0,67	0,67	0,011	0,182	0,362	<0,05	0,711	0,892
mmol/L	Primiparous			0,52	0,62	0,57	0,006		0,428	<0,05		0,554
	Multiparous			0,56	0,71	0,78	0,022		0,386	0,183		0,812
Trigliceridi	Primip+Multip	0,14	0,11	0,12	0,13	0,12	0,00003	<0,05	0,832	0,699	0,954	0,865
mmol/L	Primiparous			0,13	0,14	0,14	0,00006		0,905	0,462		0,816
	Multiparous			0,10	0,11	0,11	0,00003		0,941	0,898		0,931
Creatinine	Primip+Multip	85,37	82,95	82,23	84,60	85,65	1,693	0,183	0,293	<0,05	0,349	0,106
µmol/L	Primiparous			85,31	84,80	86,00	3,245		0,935	<0,05		0,936
	Multiparous			79,16	84,39	85,29	2,217		0,078	<0,05		<0,05
SHp	Primip+Multip	303,49	309,78	299,14	308,90	311,85	103,18	0,570	0,618	<0,05	0,378	0,630
µmol/L	Primiparous			301,24	311,43	297,79	226,36		0,518	0,192		0,229
	Multiparous			297,18	306,38	325,91	111,59		0,353	0,321		0,738

Myeloperoxidase	Primip+Multip	451,35	406,22	446,14	411,57	428,65	420,28	<0,05	0,119	0,090	0,180	0,452
U/L	Primiparous			456,87	451,67	445,51	1308,66		0,924	0,118		0,201
	Multiparous			435,40	371,47	411,79	187,96		<0,05	0,729		0,083
ROMt	Primip+Multip	14,81	12,95	13,32	14,08	14,24	0,307	<0,05	0,665	<0,05	0,165	0,555
mgH_2O_2/100ml	Primiparous			14,11	16,10	14,21	0,593		0,485	<0,05		0,837
	Multiparous			12,53	12,06	14,266	0,398		0,195	<0,05		0,332
FRAP	Primip+Multip	139,25	138,19	140,60	139,53	136,02	24,746	0,708	0,383	0,225	0,476	0,648
µmol/L	Primiparous			142,91	140,61	134,22	38,460		0,157	0,312		0,805
	Multiparous			138,18	138,44	137,82	37,979		0,991	0,771		0,611
Paraoxonase	Primip+Multip	107,97	91,37	99,63	100,35	99,05	13,694	<0,05	0,984	<0,05	<0,05	0,113
U/L	Primiparous			102,44	120,27	101,21	30,334		0,160	<0,05		0,265
	Multiparous			96,83	80,43	96,88	15,038		0,120	<0,05		0,633
AMH	Primip+Multip	0,155	0,225	0,199	0,178	0,193	0,0002	0,106	0,923	0,647	0,149	0,950
pg/ml	Primiparous			0,222	0,102	0,141	0,0003		0,258	0,490		0,993
	Multiparous			0,176	0,254	0,245	0,0003		0,478	0,142		0,509

GOT = aspartate ammino transferase; GGT = γ-Glutamyl transferase; ALP = alkaline phosphatase; NEFA = non esterified fatty acids; BOHB = β-hydroxybutyrate; SHp = thiolic groups; ROMt = total ractive oxygen metabolite; FRAP = ferric ion reducing anti-oxygen power; AMH = anti-mullerian hormone,

3.4 Blood biochemistry and AMH

Most differences in immune-metabolic profile were recorded mainly for multiparous cows and not for primiparous. Measurements of blood variables (tab n°4) indicated that creatinine tended (P<0,10) to be greater in MMP (85,29) and MTX (84,39) groups compared to CTR (79,16) in multiparous cows. Blood glucose level was higher (P<0,05) in CTR (3,98) group than in MMP (3,81) and MTX (3,70). Similar significant comparison was observed for myeloperoxidase blood content (P<0,05): multiparous cows fed with CTR diets showed the greater MPO (435,4) than MMP (411,79) and MTX (371,47). However, the lowest level of ALP (P<0,05) resulted in MMP group (31,65) compared to others mycotoxin contaminated groups (46,26 and 38,44 respectively for CTR and MTX). Blood biochemistry results indicated a significant difference (P<0,05) of aspartate amino transferase (GOT) with greater value in CTR (102,42) compared to MTX (87,59) and MMP (84,59), still referring to multiparous cows. Measurements of the AMH in blood denoted no difference among experimental groups. However, we observed different numerically AMH concentration after first calving and in multiparous cows. Considering the mycotoxin contamination in diets (MTX and MMP group compared to CTR), we noted higher numerically AMH levels in multiparous when fed with higher ZEN contamination (0,254 and 0,245) compared to lower ZEN contamination (0,176). An opposite trend was observed after first calving, with higher AMH level in CTR group (0,222). Unfortunately, not statistically difference we found in AMH content due to the grater variability among each individual cow. No differences between groups for other biomarkers were highlighted.

3.5 Ovarian activity and milk progesterone

Weekly US during the experimental period led us to monitor the development of different ovarian structures after calving between groups. From 2nd to 8th lactation weeks the number and dimension of FO, CL and CS were reported in tab n°6. Average number of CL by US was significant different after calving between experimental groups (P<0,05). High ZEN contamination in MTX diets decrease significantly the

mean number of CL weekly detected (0,25) in comparison with diet with a regular ZEN contamination (0,64 CTR). The value of CL weekly detected in MMP group was intermediate to others. The contamination effect wasn't so considerable in multiparous cows, but numerically there still be a decrease of weekly CL detected with higher ZEN contamination. During the trial the average dimension of CL was close to 2 mm in all experimental group. CL detected on ovarian surface of multiparous were bigger in MTX group (2,41 cm), followed by CTR (2,05 cm) and MMP (2,00 cm). Concerning follicular waves after calving the average number of FO was quite comparable among groups. We observed at US check a mean of 2,3 follicular structure of more than 0,5 mm each week. However, the number of parties, cows in MTX group showed more FO on ovarian surface (2,53) than cows in CTR (2,37) and MMP (2,10) group at weekly US check. About the mean dimension of FO, we noted that the multiparous MMP FO ($P<0,10$) tended to be greater (1,35 mm) than FO in MTX (1,23 mm) and CTR (1,15 mm). In addition, US check indicated same difference in the development of CS after calving. During the trial CS presence on ovarian surface was greater in MXT group (0,22) in comparison with CTR (0,06) and MMP (0,15). As reported in tab n°6 the greater CS incidence resulted in multiparous cows with an increase in CS incidence (0,37 and 0,24 for MTX and MMP groups respectively) when diet was contaminated by higher level of ZEN. Unfortunately, due to the low number of cows involved that difference wasn't statistically. Concerning primiparous cows, no CS was identified in MMP group and at least only 1 CS in CTR and 2 CS in MTX group. A significant variation appeared in the CS dimension of each structure weekly measured by US ($P<0,05$), especially in multiparous cows ($P<0,05$). CS were greater in cows fed with MTX diet (3,55 mm) than when they were fed with MMP (3,38 mm) or CTR (3,33 mm) diets. The mycotoxin contamination in diets affected the average P4 measured from 3rd to 8th weeks of lactation in both primiparous and multiparous cows. ZEN contamination tended ($P<0,10$) to decrease the mean P4 milk content. These results were showed in tab n° 6 where the P4 higher level was measured in CTR group (103,9 ng/ml), followed by MMP (55,7 ng/ml) and finally the MTX group (35,41 ng/ml). Moreover, data indicated a rapid increase in the first 4 weeks of lactation of the average milk P4 when cows were fed with a regular ZEN contamination.

3.6 Reproduction performance

About reproduction performance DO, CR and number of inseminations for pregnancy were comparable among group, as reported in tab n°5, probably due to few numbers of cows enrolled in the study. The results of conception rate at the first AI after the experimental period was described in the same tab. We perceived same modifications in the recovery of the ovarian activity after calving. Through the trial we identified a total of 9 anovular cows: 4 in MTX group (3 primiparous and 1 multiparous), 3 in MMP (2 primiparous and 1 multiparous) and 2 in CTR group (all of them multiparous). Other cows were considered cyclic because of the presence at least of one CL during the experimental period. We noted a tendency (P<0,10) of main incidence of anovular cows after first calving in MTX (75%) and MMP (50%) compared to CTR group, where no one anovular cow was detected. The 1OV interval cows was numerically greater in MTX (29,50 d) than in CTR and MMP treatment (24 d and 25,14 d respectively). Moreover, heat detection data revealed a different heat behavior among cows considering the number of parties (P<0,10). All cycling CTR multiparous cows showed at least one heat after calving with the shorter 1HD of 21 d. 60% of multiparous in MTX group showed the first heat on an average of 46 d as 1HD. On the other hand, the 50% of multiparous cows in MMP group had an 1HD interval shorter (38,50 d) than cows in MTX. All cycling primiparous in MTX and MMP groups showed a heat after calving but then cows in MTX had an earlier 1HD (45 d) in comparison with MMP cows (27 d) after calving. However, only the 25% of CTR primiparous showed a heat after calving with a 1HD of 38 d. The index of wellness of the uterine system (WU) wasn’t affected by ZEN contamination and the uterine involution after calving was similar between experimental groups.

Table 5 REPORDUCTIVE INDEX Least squares means and associated SEM for reproductive traits of Holstein cows fed control diets with low mycotoxin contamination level (CTR), diet with high mycotoxin contamination level (MTX), or MTX diet added with about 35 g/cow/day of MMP product (MMP-) during the experimental period.

Items	Parties		Treatment			Sem	P of the model	
			CTR	MTX	MMP		Party	Treatment (T)
DO	Primip+Multip	d	105,12	105,00	110,90	42,71431		0,9443
	Primiparous	d	111,75	108,00	102,75	41,21421		0,9533
	Multiparous	d	98,50	102,00	116,33	48,43959		0,8262
P/TAI	Primip+Multip	%	50,00	62,50	50,00	52,64731		0,8564
	Primiparous	%	25,00	75,00	50,00	52,70463		0,4402
	Multiparous	%	75,00	50,00	50,00	54,35573		0,7451
CR	Primip+Multip	%	0,76	0,75	0,70	0,337966		0,9285
	Primiparous	%	0,71	0,82	0,71	0,354371		0,8925
	Multiparous	%	0,82	0,69	0,70	0,362284		0,8610
ANOVULAR	Primip+Multip	%	0,20	0,40	0,30	0,475317		0,6469
	Primiparous	%	0,00	0,75	0,50	0,440959		0,099
	Multiparous	%	0,33	0,17	0,17	0,447214		0,7613
HD	Primip+Multip	%	0,50	0,40	0,40	0,519972		0,8845
	Primiparous	%	0,25	0,25	0,50	0,527046		0,7479
	Multiparous	%	0,67	0,50	0,33	0,527046		0,5615
1HD	Primip+Multip	d	24,40	45,75	32,75	11,91931		0,0673
	Multiparous	d	38,00	45,00	27,00	12,72792		0,6390
	Multiparous	d	21,00	46,00	38,50	12,20997		0,0846
1OV	Primip+Multip	d	24	29,5	25,14	9,12		0.527
	Multiparous	d	24,5	33	26	7,01		0.597
	Multiparous	d	23,5	28,8	24,8	10.80		0.743

DO = day open; P/TAI = conception rate at first AI; CR = conception rate; ANOVULAR = % of anovular cows; HD = % of cows with at least 1 heat detection after calving; 1HD = day interval from calving to first heat detection; 1OV = days interval from calving to first ovulation

Table 6 OVARIAN STRUCTURE AND P4 Least squares means and associated SEM for number (N°) and dimension (DIM) of ovarian structure (CL, FO and CS) weekly detected by US and milk progesterone (P4) of Holstein cows fed control diets with low mycotoxin contamination level (CTR), diet with high mycotoxin contamination level (MTX), or MTX diet added with about 35 g/cow/day of MMP product (MMP-) during the experimental period

Items		Parties		Treatment			Sem	P of the model				
		Primiparous	Multiparous	CTR	MTX	MMP		Party	Treatment (T)	Day (D)	D*P	D*T
WU	Primip+Multip	1,27	1,52	1,34	1,42	1,43	0,046	0,096	0,878	<0,001	0,063	0,491
	Primiparous			1,14	1,25	1,43	0,151		0,184	<0,05		<0,05
	Multiparous			1,54	1,60	1,43	0,196		0,818	0,007		0,300
N° CL n	Primip+Multip	0,35	0,5	0,63a	0,25b	0,41	0,003	0,208	<0,05	<0,001	0,5353	0,627
	Primiparous			0,64a	0,09b	0,32	0,003		<0,05	<0,001		<0,05
	Multiparous			0,62	0,39	0,49	0,005		0,515	<0,001		0,867
DIM CL cm	Primip+Multip	2,12	2,09	2,05	2,41	2	0,088	0,995	0,903	<0,05	0,963	0,307
	Primiparous			2,16	2,65	1,02	0,152		0,722	0,181		0,539
	Multiparous			1,98	2,37	2,04	0,140		0,938	0,066		0,438
N° FO n	Primip+Multip	2,33	2,32	2,37	2,53	2,10	0,039	0,950	0,434	<0,05	0,684	0,483
	Primiparous			2,21	2,60	2,21	0,058		0,508	0,005		0,772
	Multiparous			2,52	2,45	1,99	0,054		0,466	<0,05		0,660
DIM FO mm	Primip+Multip	1,32	1,25	1,24	1,28	1,33	0,018	0,260	0,514	0,658	0,676	0,980
	Primiparous			1,32	1,29	1,33	0,032		0,962	0,129		0,319
	Multiparous			1,18	1,23	1,35	0,022		0,097	0,800		0,844

N° CS n	Primip+Multip	0,04	0,24	0,05	0,22	0,15	0,005	<0,05	0,409	<0,05	0,514	0,262
	Primiparous			0,04	0,08	0	0,002		0,510	0,169		0,654
	Multiparous			0,07	0,36	0,29	0,009		0,298	<0,05		0,293
DIM CS mm	Primip+Multip	3,12	3,45	3,25	3,5	3,38	0,357	0,960	<0,05	0,365	0,555	0,224
	Primiparous			3	3,25		0.512		0,06	0,351		0,368
	Multiparous			3,33	3,54	3,38	0,415		<0,05	0,373		0,328
P4 pg/ml	Primip+Multip	63,80	68,39	103,19a	35,41b	55,70	762,12	0,845	0,064	<0,001	0,635	0,580
	Primiparous			116,85	25,94	58,04	1521,05		<0,05	<0,05		0,301
	Multiparous			98,22	51,54	54,54	834,29		0,449	<0,05		0,329

4. Discussion

The levels of mycotoxin contamination in the diets during the trial are typical mycotoxin levels found in the feed of dairy cows in Europe. ZEN as fusarium mycotoxin is quite common in complete animal feed and feedstuffs (Gruber-Dorninger et al., 2019). ZEN contamination may primarily derive from maize silage as reported by Reisinger et al., 2019. Many surveys recorded the presence of ZEN from corn silage and corn grain (Weaver et al., 2021). Despite this presence in animal feed, Gallo et al., (2015) summarized that most of ZEN was detected at medium and lower levels, which are lower than the maximum recommended levels in the complete feed. Our trail contamination in experimental diets were under the recommended level of 500 µg/kg DM for feedstuffs by European Commission. As expected, the ZEN contamination was similar between MTX and MMP groups, 5 and 6 times more than CTR group. Same differences were observed for DON. Despite that outcomes, FB1 and FB2 diets content were equal between experimental groups. Cows that received different mycotoxin contaminated diets had similar DMI after calving until the 8th lactation week. This result was similarly reported by other authors (McKay et al., 2019a). However primiparous cows tended to eat more TMR after calving in MMP group as opposed trend to the multiparous feeding behavior in our trial. The MMP treatment probably had an apparent effect on DMI after first calving. ZEN contamination decreased numerically the daily RT, especially in multiparous cows where the MMP product probably may not be able to mitigate effects of mycotoxin contamination. As for the DMI we noted a different feeding behavior in primiparous with highest RT in MMP (548,56 min/day/cow). This increase could be related to the increase of DMI in MMP primiparous group previously described. Apparent digestibility of NDF, starch and protein weren't affected either by Fusarium mycotoxin contamination or MMP presence/absence in the diets. Data about NDF digestibility isn't' in agreement with other studies where fusarium mycotoxin decreased degradation of crude fiber with higher (Hildebrand et al., 2012) or similar (Gallo et al., 2020a) DON contamination levels to our study. Moreover, the presence of MMP product didn't modify the nutrient digestibility, in contrast with Kiyothong et al., (2012). Milk yield, FCM and ECM weren't statistically different between experimental

groups, even if we observed a numerically decrease in milk yield when cows were fed with MTX diet, as stated by Gallo et al., (2020). Our results suggest a lower numerically decrease in milk production when the MMP product was added in a mycotoxin contaminated diet, in accordance with other papers that pointed out an improvement in milk yield upon addition of MMP products (*396-JMES-1529-Zouagui*, n.d.; Jovaišiene et al., 2016). Milk quality didn't differ among treatment in multiparous cows as reported by other studies (McKay et al., 2019a). Although the primiparous showed different data: mycotoxin contamination seems to slightly decrease the protein content when cows were fed with MTX diet (2,99%). MMP products probably mitigate this negative effect due to the similar protein content between MMP and CTR groups (3,13%). The lowest lactose content in MMP groups is an odd result with no explanation to the best of our knowledge. ZEN contamination increases the milk urea content and the MMP product seems to mitigate this effect. Opposing to the data of Gallo et al., (2020) this milk urea increase was probably due to a different protein efficiency in the rumen, where ZEN could probably modify the rumen microbiota (T. Hartinger et al., 2022). These milk performance data led to us to assert that fusarium mycotoxin didn't' significantly affect milk yield as reported by other studies with different contamination level of fusarium mycotoxin contamination in diets (Korosteleva et al., 2007; McKay et al., 2019b; Winkler et al., 2014). However, the numeric difference noted in milk production among groups can represent an important variation in farm gate income. The trial variations in blood parameters of the cow's metabolic status among experimental groups were mostly limited, nevertheless some of them in multiparous groups were statistically significant. About energy metabolism we found that blood glucose was numerically higher in CTR group, followed by MMP and MTX groups. This trend could be related with the different milk yield among groups. Liver transaminases (GOT and GGT) had a different data result with each other. We found a decrease in GOT level when cows were fed with higher contaminated diets (MTX and MMP) regardless the presence of MMP product. Anyway, the lower GOT level in contaminated diets contrasted the transaminases trend noted by several authors (Faixová et al., 2010; Gallo et al., 2015b, 2020a) who instead higher transaminase levels were founded. We noted a different variation for GOT parameters referring to primiparous or multiparous. Data of GGT agree with other studies by Gallo et al., (2015b, 2020b) even if in other stages of lactations. The

numerically increase of GGT and blood urea could be probably related to an increase of liver detoxification activity due to the mycotoxin's contamination, partially recovered by MMP product. However, our results agree to another study of fusarium mycotoxin contaminated diets for an extended period (Catellani et al, 2023, under submission) with similar DON and lower ZEN contamination levels. This GOT trend among groups was additionally observed for ALP and MPO in disagreement to other previous studies (Gallo et al., 2015a, 2020, Catellani 2023, under submission). Fusarium mycotoxins seems to decrease the MPO blood content in MTX groups in contrast to what Gallo et al., (2020b) detected. We could assert that probably in multiparous cows at early stage of lactation ZEN contamination could mitigate the leucocyte response. The MMP supplementation appears to partially recover this negative immune effect. Our data indicated that trial mycotoxin contamination levels and administration of MMP diets didn't' significantly modify the immune-metabolic status of cows after calving, especially for primiparous cows where not statistically differences were detected. One of the aims of our study was to evaluate the possible impact of ZEN contamination on the development of ovarian activity and structure after calving. The measurement of plasma AMH was performed because previous study compared the serum AMH with fertility performance (Ribeiro et al., 2014), AFC (J. L. H. Ireland et al., 2008), ovarian reserve (?), estrus cycle phases (J. J. Ireland et al., 2007; Rico et al., 2012; Singh et al., 2004), superovulation (Rico et al., 2012; Souza et al., 2015) and ZEN diet contamination (Fushimi et al., 2015; Widodo et al., 2022). We found not significantly differences of AMH plasma concentration between experimental groups, probably due to the high individual cow variability and the small number of cows involved. Even though the numerically different variation in serum AMH between primiparous and multiparous (lowest AMH concentration in MTX for primiparous and CTR for multiparous), no party effects was detected. Our results suggest that the ZEN contamination didn't affect the AMH serum content in contrast with other studies (Fushimi et al., 2015; Widodo et al., 2022), in a different stage of lactation, ZEN contamination levels and cow breed. The number of FO weekly detected wasn't affected by treatments. Although, we noted that ZEN contamination affected the follicle genesis in multiparous cow with greater FO in MMP and MTX group. Regarding the presence of CL and their dimension we detected a ZEN contamination effect:

mycotoxin contamination had decreased the presence of CL on ovarian surface in comparison to diet with regular contamination level. This trend was particularly significant in primiparous cows. This data was quite interesting because in accordance with the P4 levels detected and previously described. P4 is produced primarily by the CL. So major presence of CL on surface could explain the higher level of P4 in control group. In addition, average CS dimension was higher when cows where fed by MTX diet. Two last results are quite correlated because we considered, as stated by Gaverick et al 1997, a CS as follicular structure that exceeded 25 mm in diameter in absences of CL. Therefore, we see an increase in mean FO and CS diameter when cows where fed with ZEN contaminated diets as reported by Mamhoud et al 2013. However, the MMP products seems to mitigate the previous described effect of ZEN on the CS dimension, with similar CS diameter of cow in CTR group, but not on FO. The incidence of CS on ovarian surface was numerically higher when cows where fed with MTX diets and followed by the MMP groups, but unfortunately not statistically significant. This numerically trend was in accordance with (Takagi et al., 2008b) who noted a higher ZEN detection in CS than normal FO, hinted that ZEN can increase the likelihood of cystic ovaries problems. Measurements of milk P4 was suitable for defining different reproductive traits but itself as reproductive performance parameters (). In our trial we noted a tendency of ZEN contamination to decrease by about 64% the mean milk P4 in MTX and by 50% in MMP group. This effect could be linked with the ZEN metabolites α-ZEL and β -ZEL. As described by Olsen, (1989), in one of two major biotransformation pathway ZEN is hydroxylated in α-ZEL and β -ZEL, catalyzed by 3α and 3β hydroxysteroid dehydrogenase (HSDs). This can cause the accumulation of active components, interfering with the biochemical pathway of various steroid hormone, including E2, testosterone and P4, where HSDs play a pivotal role. Consequently, ZEA is also a substrate for 3α-HSD and 3β-HSD present in many steroidogenic tissues, such as liver, kidney, testis, prostate, hypothalamus, pituitary, ovary, intestine (Olsen, 1989). 3β-HSD catalyzes the conversion of pregnenolone into P4. Regarding the primiparous group we observed a drastically decrease ($P<0,05$) in milk P4 with MTX diet (by 80%). At the same time the addition of MMP products seems to slightly mitigate this numerically negative effect with a less severe P4 reduction. However, this effect wasn't observed in multiparous cows. Reproductive

index as CR, DO and number of inseminations for pregnancy were similar between groups and no mycotoxin contamination or MMP product effect were detected on this reproduction index. This result was probably due to double ovsynch protocol after experimental period for first TAI, which modify the natural follicular waves of cows and cycle estrus (Souza et al., 2008). For the same reason no difference wasn't noted at P/TAI. Regarding the recovery of ovarian activity after calving of the cycling cows, we noted that cows fed with ZEN contaminated diets recovered the cycling status mean 5/6 days later than cows fed with CTR diet. Enhancing this result, time to first estrus detection behavior agrees with this trend: cows in MTX groups showed the first heat after calving later than cows in CTR group. This data agrees with the P4 result above described, with less P4 concentration after 3rd lactation week in MTX group. We probably assert that ZEN contamination after calving could delay the recovery of cyclic ovarian activity with numerically greater 1OV - 1HD interval and less milk P4 concentration in early lactation. To the best of our knowledge no other studies investigate the ZEN contamination effect on the recovery of cyclic ovarian activity on dairy cows with the study mycotoxin contamination levels.

5. Conclusion

Dairy cows fed with fusarium mycotoxin diet experienced adverse effects. The presence of ZEN contamination, even when below the guidelines of United States and European Union, negatively affected the production and reproduction performance of milking dairy cows. Our results suggest that ZEN contamination in early lactation after calving decrease milk yield, modify milk quality and some immune metabolic parameters. In relation to reproduction performance, our preliminary trial data indicated ZEN contamination had a negative impact on recovery of ovarian cyclic activity after parturition, normal follicle development and follicular cysts occurrence. Results suggested different performance output according to parities, between primiparous and multiparous. The MMP products mitigated some negative mycotoxin effects on milk production and quality (especially urea), the recovery of ovarian cyclic activity and development of ovarian structures, as follicular cysts dimension. We noted that many traits changed according to number of parties, probably identifying a different susceptibility to ZEN contamination depending on cows' age. On the best of our knowledge, our results led us to believe that negative effects of ZEN contamination after calving on reproductive e productive performance during the entire lactation will be better investigated with other studies. Further studies with larger number of cows of several parities and with different ZEN contamination level should be performed to better understand this preliminary results.

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Conclusions

Nowadays, the objective of dairy cattle breeding is to produce high-quantity and high-quality milk at low cost and, at the same time, to ensure animal welfare and to guarantee a low environmental impact. To achieve these goals, dairy breeders can manage different aspects of the dairy breeding. Two important aspects of the dairy process are: i) the genetic selection and ii) nutritional-safety traits of diets provided to animals. The aim of thesis was to evaluate the effects of genetics and anti-nutritional substances on performance, metabolism, feed efficiency and milk quality of lactating dairy cows. In the first two chapters, we studied the effect of two dairy breeds, Brown Swiss and Holstein, on nutritional and physiological adaptation during the transition period, being this an important period of productive life of livestock. In a commercial farm trial (Chapter 1), we observed the Brown Swiss had a different adaptation than Holstein and Simmenthal when they are breed in the same environmental conditions and feed with the same diets. After these results and to better investigated these differences between Brown Swiss and Holstein, we carried out an experimental farm trial in the facility of our university (Chapter 2). Our study reported that Brown Swiss cows had a different feeding behavior, milk quality performance, nitrogen metabolism, rumen fermentation and use of body reserve than Holstein cows. In particular, results from this study suggested that a specific Brown Swiss management and nutrition should be considered in Brown Swiss farms to achieve the best milk performance and high standard of welfare conditions. This also suggests that farms that host dairy cows with different breeds should adopt specific strategies to guarantee the nutritional requirements, especially in the first two lactations.

In the second part of this thesis, we considered the effect of anti-nutritional factors, especially mycotoxins as DON and ZEN, on the performance of lactating dairy cows. We found that Fusarium-produced mycotoxins had a negative impact on the dairy productive and reproductive performances, even when the contamination level was under the European guidelines for feed used in animal diets. However, the use of some commercial mycotoxin deactivating products resulted able in mitigating many of these negative effects on milk production and reproduction performance.

In conclusion, these results highlight useful information for dairy farmers and strategies to maximize the dairy performance of their herds. First, farmers that like to maintain different breeds in the same group should manage and feed cows according to the breed requirements. Secondly, farmer should mitigate the negative effects of the mycotoxin contamination feeding some mycotoxin deactivating products. In this thesis, we had investigated some of the genetic and anti-nutritional effects on milk performance, but in the future, many other aspects should be studied in more details: the breed specific requirements and management and mycotoxin contamination effects on metabolism and reproduction.