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Improvement of feed efficiency in dairy cattle

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“Les hommes ont plus de timidité dans l'esprit que dans le cœur; et les esclaves volontaires font plus de tyrans que les tyrans ne font d'esclaves forcés”

Charles Pinot Duclos

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ABSTRACT

The improvement of feed efficiency in dairy cattle has traditionally been approached through an increase in production levels. However, the effectiveness of the energy partitioning underlying the dilution of maintenance phenomenon decreases with each successive increment in production relative to body size, and therefore will lose importance in the next years. The selection of more efficient animals (i.e. animals that consume less feed to achieve a fixed production) is limited by the cost and inherent difficulty in recording individual feed intake data, which prove pivotal to improve the accuracy of the estimated breeding values for feed efficiency traits. First aim of my project was to record and collect individual phenotypic data on the dry matter intake of dairy cattle under different dietary conditions and at different production stages. The collected data will become part of a larger collaborative dataset aimed at increasing the prediction power of genomic selection plans including traits related to the feed efficiency. Furthermore, following a multimodal approach essential for an effective improvement of the dairy enterprise efficiency, the effect of feed additives and alternative feed ingredients on the production, health status and efficiency of dairy cattle has been investigated.

In the first trial, the effect of camelina cake – a byproduct of camelina oil rich in proteins and unsaturated fatty acids – on milk fatty acid profile was investigated. Results from the study evidenced a higher concentration of n-3 PUFA ($p = 0.02$) and a lower n-6/n-3 ratio ($p = 0.01$) in the milk of cows fed camelina compared to control. The analysis of the ruminal content suggested a moderate effect of camelina on the ruminal environment and biochemistry.

In the second trial, the effect of *Saccharomyces cerevisiae* (live yeast) on the ruminal environment and its interaction with the production and health status of dairy cows

was investigated. Dietary treatment with *S. cerevisiae* did not affect the investigated ruminal parameters and had only mild effects on blood biochemistry. However, an effect on the ruminal pH was observed when cows were fed low-quality forages, with higher pH values in cows supplemented with *S. cerevisiae* compared to control ($p < 0.05$).

In the last trial, the effect of camelina on the ruminal microbial populations and the cumulus-oocyte complexes of growing dairy heifers was evaluated. The inclusion of camelina in the diet was associated with higher expression of all selected molecular markers of oocyte quality ($p < 0.05$). Moreover, feeding camelina affected the ruminal microbiota, as shown by the significant reduction in the alpha diversity of the treatment group ($p < 0.05$).

RIASSUNTO

In passato, il miglioramento dell'efficienza alimentare delle bovine da latte è stato tradizionalmente raggiunto grazie ad un miglioramento delle produzioni di latte, grazie al fenomeno della cosiddetta 'diluizione del mantenimento'. Tuttavia, l'efficacia nella ripartizione energetica verso la galattogenesi che questo effetto prevede è destinata a ridursi nel tempo, motivo per cui tenderà a perdere di importanza nei prossimi anni. La selezione di animali più efficienti, ossia animali che consumano meno alimento per un determinato livello produttivo, è limitata dalla effettiva complicatezza della raccolta di dati relativi al carattere di assunzione alimentare (DMI), essenziali al fine di incrementare l'accuratezza di stima del valore genetico relativo a tratti correlati all'efficienza alimentare. Obiettivo primario del progetto era quello di registrare e collezionare dati fenotipici individuali di assunzione alimentare in bovini da latte di diverse età, in diverse fasi produttive e sottoposti a diverse condizioni dietetiche. I dati raccolti entreranno a far parte di un più ampio dataset volto al miglioramento del potere predittivo dei protocolli di selezione genomica per l'efficienza alimentare, inseribili poi in piani di selezione nazionali. Inoltre, andando a mimare il potenziale approccio multimodale necessario per intervenire efficacemente sull'efficienza del settore bovino lattifero, sono stati valutati gli effetti su produzioni, stato di salute ed efficienza di alcuni additivi per mangimi ed ingredienti per razioni alternativi.

Nel primo lavoro sono stati valutati gli effetti di una dieta contenente panello di camelina (un sottoprodotto ricco in proteine ed acidi grassi polinsaturi) sulla produzione ed il profilo acidico del latte bovino. I risultati evidenziano una maggior concentrazione di PUFA omega-3 (p = 0.02) ed un minor rapporto omega-3/omega-6 (p = 0.01) negli animali alimentati con camelina rispetto al controllo, mentre la

contestuale valutazione di acidi grassi e dimetilacetali ruminali rivelano un ridotto effetto della camelina sul metabolismo del rumine.

Nella seconda prova, gli effetti del lievito vivo *Saccharomyces cerevisiae* sulla salute del rumine, e l'interazione coi parametri produttivi e di salute delle bovine in lattazione sono stati indagati. Il trattamento dietetico con lievito non ha sortito effetti significativi sul comparto ruminale delle bovine ed ha causato solo lievi variazioni nei parametri biochimici ematici. Tuttavia, un effetto significativo sul pH del contenuto ruminale è stato registrato modificando la razione con l'inserzione di foraggi di minor qualità, che ha determinato valori medi di pH superiori nelle bovine trattate con lievito ($p < 0.05$).

Nella terza e ultima prova, gli effetti della somministrazione di panello di camelina con la dieta sono stati valutati in relazione alle caratteristiche delle popolazioni batteriche ruminali e la qualità dei complessi cumulo ooforo-oocita (COCs) di manze da latte in crescita. La dieta con camelina si è rivelata statisticamente associata a livelli più elevati di espressione dei quattro marker molecolari di qualità dei COCs selezionati ($p < 0.05$). Inoltre, l'analisi metagenomica ha rivelato un effetto della camelina sul microbioma ruminale, in cui si è evidenziata una riduzione notevole della diversità alfa ($p < 0.05$).

Chapter 1|

General Introduction

BRIEF STATE OF THE ART

The last century has witnessed a huge increment in productive efficiency of dairy cattle, traditionally achieved through selection for improved milk production (Mccarthy et al., 2012). This is mainly due to the biological concept of the "dilution of maintenance", according to which as milk production increases, the proportion of energy (and feed) needed for maintenance decreases (VandeHaar & St-Pierre, 2006). Currently, high-producing Holstein cows produce at more than 4 times their maintenance requirement, fully exploiting the economic advantage offered by the dilution of maintenance (VandeHaar & St-Pierre, 2006). Further efforts to improve the production efficiency of dairies should aim at increasing the ability of cows to convert feed nutrients and energy into salable milk, i.e. improving the feed efficiency of dairy cattle.

Feed efficiency (FE) – defined as an animal's relative ability to convert feed-derived nutrients and energy into valuable productions - is a trait gathering increasing attention due to its implication in the economic sustainability of dairy industry. Accounting for more than 50% of total costs of milk production, feed represents the largest single expense of dairy production and, along with feed price volatility, may heavily impact farm profitability (Pomar et al., 2011; Bethard, 2013; Sommer & Eastridge, 2014; Vallimont et al., 2011). However, economic factors are not solely responsible for the serious concern about the current situation of the agri-food supply chain. In recent decades, the emerging concept of sustainable agriculture has rapidly imposed the safeguard of nature and environment, food safety and animal well-being as new central objectives for the dairy industry (Pretty & Bharucha, 2014). It is a fact that the animal production industry – and agriculture – are facing unprecedented challenges. The estimated growth of the global human population is leading to an increase in the demand for animal-derived energy and food products (OECD-FAO, 2012; Carolan, 2018). This means that dairy industries are likely to

compete for grain and feed resources with other farm animal industries, human consumption, and biofuel production. Should the trend be maintained, the double impact of population growth and the changing dietary patterns will require the global amount of crops to be doubled within thirty years from now (Tilman et al., 2011). Such a level of agricultural production will be difficult to achieve, as population growth will also lead to increased demand for building land (FAO, 2011). Moreover, further concerns are raised by the well-documented relationship between animal production system and greenhouse effect: farm animals are accountable for 14.5% of the total human induced greenhouse gas (GHG) emissions, 20% of which are pertaining to the dairy sector (Empel et al., 2016).

In the light of this greater awareness, the improvement of livestock efficiency – especially FE – represents one of the main challenges for the sustainability and profitability of the agri-food supply chain. Improving the FE of the herds is in fact a valid strategy to contain feed-related costs and reduce the extent of the agricultural land needed to support the animal production system (Herrero et al., 2013). Noteworthily, the benefits an improvement in livestock FE would have on the environment do not end here. Livestock contributes to a some extent to GHG emissions. Feed efficiency has been suggested to be the most important explanatory variable in farm greenhouse gas emissions (Thoma et al., 2013). Several scientific works have already reported that more efficient cattle are characterised by lower methane emissions (Waghorn & Hegarty, 2011), which may have implications for the mitigation of greenhouse effect (Negussie et al., 2017). Selection for FE has been estimated to reduce methane emission by 15% in a decade (de Haas et al., 2014).

In the past, genetic selection of dairy cattle has mainly been based on indexes of production and – more recently – reproduction-related traits, two traits with a well-documented negative phenotypic correlation (Berry et al., 2016). Feed efficiency (FE),

for its part, has been estimated to have an economic weight similar to that of longevity and greater than that of udder health, two traits included in dairy cattle breeding goals for more than a decade (Hietala et al., 2014; Miglior et al., 2005). Despite the huge benefits deriving from it, until recent years little attention has been paid to improving FE through direct selection on it. Overall, to make genetic progress a trait must be heritable, manageable and unexpensive to evaluate in large populations. The inclusion of FE in the breeding goals has in fact long been limited by the difficulty and cost it entails compared to other economically important traits, being based on the measurements of individual phenotypic traits (feed intake) of a very large number of animals. Nevertheless, recent advances in the field of the -omics science have made it possible to overcome this handicap, renewing interest in selecting of more efficient animals (Pryce et al., 2012; Finocchiaro et al., 2015). In the genomic approach to selection, phenotypic traits are recorded in a representative subset of a population and linked with genomic data to estimate breeding value (for those traits) of animals with known genomic – but not phenotypic – data. Currently, the number of FE records available at the national level is not adequate to create one phenotypic dataset that can be exploited for reliable inference and accurate prediction of genetic merits. For this reason, international collaborative efforts among research centers have already started, in order to combine datasets of populations from different countries (de Haas et al., 2012; Banos et al., 2012), yet with the potential limitation that a possible genotype per environment interaction could entail.

The adoption of measures of FE alternatives to ratio-based measures (e.g. gross feed efficiency), such as residual feed intake (RFI), represented a further step toward a feasible selection for FE. As already demonstrated for beef cattle, animals with lower RFI (i.e. more efficient) are believed to have lower methane enteric emissions (Hegarty et al., 2007; Basarab et al., 2013), endorsing the favorable correlation between FE and GHG emissions. RFI has been demonstrated to be an inheritable trait

(Lin et al., 2013; Tempelman et al., 2015), and since it is independent of the production level of the animals and considered indicative of intrinsic variations in their basic metabolic processes (Crews Jr, 2005, Kelly et al., 2011), it could represent a better selection tool for improving FE compared to gross feed efficiency (GFE). The adoption of RFI as an index of FE has been proposed as a strategy to minimize the negative genetic correlated response between GFE and energy balance (Spurlock et al., 2012; Hardie et al., 2017). Though some scientific publications support an unfavorable positive correlation between RFI and energy balance-related traits (Hurley et al., 2018), further investigations on the mutual effects exerted by the selection for different traits (e.g. RFI and resilience to production-related metabolic disorders) are essential to set up effective selection plans. Thus, the selection for dairy cows with improved FE represents a cornerstone on the road to a more sustainable agriculture, achievable through an integrated approach including: i) the introduction on farms of electronical and ICT-related technologies, in the frame of the Precision Livestock Farming (PLF) concept (Bánházi et al., 2009); ii) the search for new, environmentally-sound feedstuff; iii) the deepening of knowledge about feed ingredients and additives at our disposal.

METHODS TO MEASURE FEED INTAKE

As already mentioned above, the major limit for the introduction of FE as a genetic selection criterion is the collection of individual phenotypic feed intake data. To date, DMI data are difficult to collect, requiring long time observations and expensive tools, limiting their measurement to research stations equipped with specific technologies.

Automated feed monitoring systems

Automated feed monitoring systems using radio-frequency identification to track and record individual intakes as the animal visits the feed bunk are available for

confinement systems. However, these technologies are mainly used in small research facilities or feedlots due to their expensiveness. In dairy facilities, the introduction of automated feed bins is limited by their feeding capacity, unable to accommodate the high intakes of lactating cows.

Many of the classic methods for measuring DMI require specific instrumentation that are unaffordable for the majority of the farms, besides having little applicability in intensive production systems. Automatic feeding systems measure individual FI allowing selective access to feed bins equipped with electronic scales; the recognition of the animal and subsequent access to the bin is ensured by the specific association between the feeder and the electronic transponder on the collar of the animal. Among these automatic feeding systems, the Calan gates and the Insentec RIC system are the most widely applied. The two systems are mainly superimposable, although the Calan system can be considered more suitable for pasture and outdoor environment. Both systems tend to limit the number of animals having access to the feed at one time and, despite they were proved to not affect total feed intake compared to traditional feeding systems, compensatory changes in feeding behavior were reported in cows fed by Calan gates (Ferris et al., 2006).

Mathematical models

Mathematical models to predict DMI have been proposed by several authors. The National Research Council (NRC, 2001) proposed a prediction equation to estimate DMI through the whole lactation period:

$$\text{DMI (kg/d)} = (0.372 \times \text{FCM} + 0.0968 \times \text{BW}^{0.75}) \times \{1 - e^{[-0.192 \times (\text{WOL} + 3.67)]}\},$$

where FCM is 4% fat corrected milk yield, $\text{BW}^{0.75}$ is kilograms of metabolic body weight and WOL is week of lactation. The NRC equation for predicted DMI has been included in larger predictive models aimed at estimating feed efficiency (Omodei

Zorini et al., 2020). As observed by some authors, the accuracy of the DMI prediction may increase when energy partitioning-related traits (milk energy output, body condition score, changes in BW) are included as covariates of dynamic equation models (Hayirli et al 2003; Ellis et al. 2006; de Souza et al 2019). A potential explanation for the role of BCS in voluntary DMI is the positive correlation between body fat percentage and circulating leptin, a hormone playing a key role in the appetite-satiety regulation (Block et al., 2003). The NRC model – and other models based on regression equation, e.g. the multiple regression model of the Agricultural Research Council (ARC 1980) and the Cornell Net Carbohydrate and Protein System (Fox et al 2004) – considers production data (BW, milk yield) as the sole explanatory factors of DMI, including outputs as model inputs. By consequence, the latter models predict the DMI required to sustain a fixed production without the possibility of being combined with other models to predict the effects of dietary changes on voluntary intake (Krizsan et al 2014; Friggens et al 1998). This may prove useful when diet composition is unknown, conversely representing a limit when assessing the effect of diet composition on DMI during the ration formulation process.

The INRA Fill Unit System is based on fixed "fill values". The fill values are fixed values of 'ingestibility' specific for each forage and elaborated by comparison with a reference pasture grass representing the "fill unit for cattle" (FUC). One FUC corresponds to 122.6 g/$BW^{0.75}$ (kg of metabolic body weight). In the Fill Unit System, each animal has a specific FI capacity defined by sex, age, LBW, production and parity level. The system proved reliable in predicting DMI when animals were fed low concentrate diets (less than 30-40% DM), losing in prediction power with increasing concentrate fraction inclusion (Jarrige et al., 1986).

More recently, Zom et al. (2012) proposed a model – mainly based on the classic INRA Fill Unit system – that accounts for several feed and animal characteristics and

may be combined with other models predicting the responses in cow performance to feeding strategies.

N-alkane technique

In grazing systems, the direct measurement of FI is inherently difficult, therefore requiring the use of indirect methods of measure. N-alkane are among the more commonly used indirect markers of DMI. N-alkanes are long-chain hydrocarbons (C25-C35), natural components of plant cuticular wax. The n-alkane technique indirectly estimates individual DMI by dividing the fecal dry matter output (FDMO) by the digestibility of the feed (Moore 1996). Fecal output is estimated (eFDMO) from an external marker while an internal marker (naturally occurring in vegetal feedstuffs) is used to estimate dry matter digestibility (eDMD). Since the odd-chain length n-alkanes are more widely represented than the even-chain length n-alkanes in vegetal species, the classical double n-alkane technique uses a dosed synthetic even-chain length n-alkane as external marker paired with a naturally occurring dietary odd-chain length n-alkane as internal marker (Mayes & Dove, 2006). The best estimates of DMI are achieved by using n-alkane pairs that differ by one carbon atom, because of their similar fecal recovery rates. For instance, the n-alkane pair C33/C32 has been shown to be one of the most accurate n-alkane pairs to use (Dillon, 1993). Various studies have documented the accuracy of the n-alkane technique for estimating DMI (Oliveira & Silva, 2007; Dillon, 1993; Dove et al., 2002). Dove and Mayes (1991) reviewed nine scientific articles evaluating the n-alkane technique in both cattle and sheep, reporting that the largest discrepancy between known and estimated herbage DMI, at a group level, was 2.6%. Moreover, the presence of unique patterns of concentrations of alkanes in different herbage species allows to estimate the diet composition in grazing animals (Dove, 1993). The marker technique has been criticized for its lengthy preparatory work (especially for external markers) and its practical inadequacies. As such, it had little or no application in large herds. On the

other hand, a meta-analysis conducted by Guinguina et al. (2019) showed high repeatability of the the estimated DMI calculated with the marker technique across lactation, despite a tendency to over-estimate DMI at high measured FI and underestimate it at low measured FI. The same authors also used the eFDMO to develop a predicting model for FE.

New technologies and Precision Livestock farming

Recently, the possibility of predicting DMI from mid-infrared (MIR) spectral data of milk has been documented (McParland et al 2014). Wallen et al. (2018) evaluated different prediction equations models for DMI using MIR spectrometry of milk, concluding that the best accuracy is reached applying partial least squares methods and including milk yield and NEI from concentrate into the model. Shetty et al. (2017) obtained similar encouraging results using Fourier-transformed infrared (FT-IR) spectroscopy to predict DMI and RFI in lactating cows, highlighting better accuracy during the early lactation period compared to across-, mid- and late-lactation estimations.

Shelley et al. (2016) explored the feasibility of measuring individual DMI by scanning feed volume, using 3-dimensional video cameras. By the application of a regression analysis system, the authors were able to evaluate the correlation between data about the density, volume and weight of the ration. The study demonstrated potential for on farm application of the technique, despite the challenges posed by the variability in density of the ration due to time and sorting by the animals.

MEASURES OF FEED EFFICIENCY

A plethora of different definitions of efficiency, gross feed efficiency, and net feed efficiency exists in growing and mature animals. The appropriate definitions of

efficiency – together with individual animal feed intake record – are therefore fundamental to achieving the necessary gains in efficiency.

Gross Feed Efficiency

The most common and well-known measure of FE is gross feed efficiency. The improvement of FE observed over the past 100 years has largely been due to increases in gross feed efficiency, achieved through the dilution of maintenance (VandeHaar and St-Pierre, 2006). Gross feed efficiency (GFE; also known as physical feed efficiency or feed conversion efficiency) is the ratio of total output divided by total input of an animal, thus representing the opposite measure of the feed conversion ratio; it is generally expressed as milk yield (more commonly corrected for fat-, energy- or fat and protein-content) per unit of dry matter intake (DMI).

The measure of GFE, based on a ratio of two simple traits, is relatively quick to calculate, and has the further advantage of being extremely easy to understand for dairy producers. However, the simplicity of GFE also leads to some limitations in its use. Firstly, GFE does not account for the homeorhetic adaptation mechanisms put in place by the cow across lactation stages (Martens, 2020). During the last weeks of gestation and the first weeks of lactation, cows commonly face a state of negative energy balance (NEB) (Herdt, 2000). The mobilization of body reserves (adipose tissue) is the main biological mechanism put in place to overcome the NEB and support the requirements of milk production (Contreras et al., 2017). Since GFE does not account for fat mobilization, animals losing more body condition during the first weeks of lactation may appear more efficient (Vallimont et al., 2011). Elevated losses in body condition are associated with poor reproductive performance and health status (Roche et al., 2009), potentially impairing the benefits of improved GFE. In a study conducted by Spurlock et al. (2012), the selection for GFE during mid-lactation (75-150 DIM) did not result in significant negative effects on health status. On the

contrary, particular attention should be paid in measuring GFE during early lactation, since NEB, lipid mobilization and the related metabolic disorders have the highest incidence during this period (LeBlanc et al., 2010; Spurlock et al 2012). For this reason, a general suggestion is to measure the GFE in mid-to-late lactation stage, with an assessment of 150 days or more to correct the effect of potential confounding factors.

Another limitation of the GFE, common to all ratio traits, is the intrinsic risk that one of the two traits comprised in the ratio proves disproportioned to the other, that would lead to uneven and unpredictable response to selection (Gunsett, 1984; Zetouni et al 2017). Optimal values of GFE have been suggested by authors. Hutjens et al. (2005) suggested values of 1.6-1.8 for multiparous cows between 150 and 225 DIM; values of 1.3-1.4 when over 200 DIM; and lower values for primiparous cows, characterized by higher energy partitioning to body growth. Lastly, the GFE is well known to be phenotypically and genetically correlated with measures of growth, production, and mature size (Crews Jr, 2005). Thus, the selection for the improvement of FCR and GFE would result in increased growth rate, mature size, and consequently increase maintenance requirements in an unbalanced breeding goal.

Residual Feed Intake

Residual Feed Intake (and its variation residual energy intake) is nowadays the most used measure of metabolic efficiency, suggesting individual differences in nutrient partitioning to milk production, body tissue maintenance and other uses (Herd & Arthur, 2009). The RFI has been proposed as a measure of FE alternative to the ratio-based indices (e.g. GFE) in dairy ruminants because of its ability to take into account the intrinsic changes in the metabolic and energy assets that take place over the course of the growth and lactation stages (Jasisnski et al., 2019; Connor et al., 2019). Because the regression model applied to calculate the RFI accounts for BW changes and other factors influencing the energy output through the lactation stages,

the RFI is less influenced by body reserve fluctuation than the GFE is and is considered independent of growth and milk production level (Potts et al., 2015; Koch et al., 1963). Despite this, some authors concluded that the stage of lactation may affect the RFI/REI of dairy ruminants (Mäntysaari et al., 2012). Since partial regression coefficients of the DMI on energy sinks vary throughout the lactation, so that early lactation is not closely related to later lactation stages, RFI values assessed during early and late lactation stages should be approached cautiously (Li et al., 2017). On the other hand, RFI values determined between 60 and 230 DIM revealed positively associated with the RFI across the entire lactation (Prendiville et al., 2011) and highly repeatable for a wide range of dietary starch levels (Potts et al., 2015).

An analysis conducted by Gonzalez-Recio et al. (2014) on 843 Australian Holstein heifers concluded that the inclusion of the heifer RFI in the multi-trait selection index currently in use in Australia (Australian Profit Ranking; APR) would increase overall farm profitability of 2.4%, while also diluting the potential effect of undesirable responses correlated to fertility and BCS.

The RFI express the difference between measured feed intake (DMI) of an animal and its expected DMI. The RFI is calculated as the residual of the regression of the observed phenotypic DMI on what are considered to be the main energy sinks of the animal, included in a predictive model to calculate its expected theoretical DMI. If for growing and meat-producing animals regressors of the equation are quite simple, for lactating cows – having multifunctional energy requirements for maintenance, growth, pregnancy, and lactation – the energy sinks of the regression model are represented by several measures of energy expenditure for lactation (e.g. milk energy output), maintenance (e.g. metabolic body weight) and body reserves accumulation (e.g. body weight change) (Manafiazar et al., 2013). Given this premise, the RFI should have less implications on the energy balance (and therefore indirectly on the

health status and reproductive performance) of dairy cattle compared to the GFE. Since the RFI equation accounts for variations in body tissues, cows that mobilize more adipose tissue to maintain production should not have an advantage in selection. In reality, the genetic correlation of the RFI trait was found to be positive for live body weight (Fan et al., 1996; Van Arendonk et al., 1991) and energy balance (Hurley et al., 2017), meaning that selecting for more efficient cows could result in larger animals more prone to negative energy balance. It has to be said that many of the studies investigating the RFI in dairy lactating cows were conducted during the first lactation only, with different experimental intervals and with observed DMI values collected weekly or monthly, from which the potential low predictive power (Zamani et al., 2008; Vallimont et al., 2011).

Random regression models including the effect of stage of lactation and parity level (Tempelman et al., 2015; Manafiazar 2013) should be refined in order to improve the predictive power of RFI, as already done for other measures of FE (Omodei Zorini et al., 2020).

Economic FE

Because dairy sustainability also requires dairy profitability, evaluation of the economic efficiency is prudent. Despite the positive relation between FI and cow performance, milk production is not necessarily related to profitability. Fluctuations in both milk and feed prices may heavily affect farm profitability, reducing the effectiveness of production-oriented management plans. Measures of economic FE may prove useful to direct and support profitable financial decision-making at an enterprise level.

Ration cost efficiency (RCE) is the simplest measure of economic FE. It is calculated as the ratio of the fiscal value of sold milk divided by the fiscal cost of

consumed dry matter (Robinson and Erasmus 2010). Unfortunately, the RCE does not account for BW changes, growth, health, longevity, DIM, feed refusal and shrink. Moreover, Robinson and Erasmus (2010) observed that at equal level of RCE, higher levels of DMI and milk yield are more profitable than lower ones, raising questions on the reliability of the RCE as a measure of dairy efficiency.

Feed cost per hundredweight (45.4 kg) is calculated as the accumulated feed cost for lactating cows divided by the hundredweights of milk shipped. As for PFE, it does not consider the heifer enterprise, neither the variability in milk composition (Bethard, 2013). St-Pierre and Gamocic (2000) advised against minimizing feed cost per hundredweight of milk, instead supporting a system of accurate nutrient value estimation based on market prices of many feed ingredients and optimization of feed resources and production.

Income over feed cost (IOFC) is defined as the economic margin of net farm income over feed costs. IOFC is generally calculated on both a herd- and cow-per-day basis, despite the latter poses higher risks of biased results as it does not account for non-producing enterprise (heifers and dry cows), neither for non-market output (e.g. milk from ill or treated cows). In order to calculate the net farm income, daily milk yield, current milk price and the cost of each ingredient of the diet must be known. IOFC may be helpful for short-term feeding and management decisions, although it proves a less reliable tool for long-term herd performance assessment, as it depends on fluctuations in milk and feed prices (Bethard 2013). IOFC may or may not include costs of feed shrink and refusal, cow health, dry cow and heifer management. A survey conducted from 2009 to 2012 on 95 Pennsylvania dairy herds determined a mean IOFC value of 7.71$ (Buza et al., 2014). As for other measures, higher milk yield (through proper nutrition plans) positively affected IOFC in a larger proportion compared to reduced feed costs (Buza et al., 2014). Nutritional and management

strategies may be effective in improving IOFC: feeding multiple diets to lactating cows clustered by nutritional and production status has been reported to improve IOFC (Kalantari et al., 2016); in farm application of complex optimizing algorithm for cow grouping may furtherly improve the economic FE (Wu et al 2019). The IOFC measure has recently been improved: Money Corrected Milk (Bethard, 2013) more accurately reflects herd performance over time, although it still does not include costs of feed shrink and refusal, cow health, dry cow and heifers management.

BIOLOGICAL FACTORS AFFECTING FEED EFFICIENCY

Given the tight connections between energetic metabolism and FE, the biological bases of the latter may be better understood when considering the sources of energy loss along the process of conversion of nutrients into the final products (milk and body tissue). Gross energy (GE) – defined as the gross calorific potential of feedstuff – provides little information on the energy available to the animal, as a significant part of the former is not retained for digestion and is lost in the feces. Despite the energy that remains after the fecal losses – defined digestible energy (DE) – is potentially usable by animals, a part of it is still lost with urine and gaseous products of fermentations. The remaining metabolizable energy (ME) comprises another 30% of losses due to the heat production associated with feed consumption (heat increment), leaving only 70% of ME available as net energy. Net energy (NE) is therefore the portion of feed-derived energy that dairy ruminants can effectively use for maintenance (NEM), growth (NEG) and lactation (NEL).

All energy lost during the flow from GE ingested with feed to NE is not available for milk production. Containing energy losses has the potential for improving FE of dairy animals by reducing the difference between GE intake and NEL. The identification of the biological processes and parameters involved in the energetic

metabolism may give new insight and direct the decision-making process aimed at obtaining more efficient dairy cows.

Compared to the wealth of knowledge relating to laboratory and monogastric farm animals, knowledge on the biological basis of FE in ruminants are limited. Despite the huge interest in identifying the relative contribution of each parameter to FE in dairy cattle, it proves difficult to discern accurately, as metabolic experiments shall take into account the complex homeorhetic adaptation of the energetic metabolism enacted by lactating cows. For this reason, most of data reported below are inferred from studies conducted on beef cattle. The RFI is considered independent of the animal production level, representing – at least theoretically – a measure of efficiency of the intrinsic metabolic processes. Therefore, most of the investigation on the metabolic and biological bases of FE were conducted using the RFI as an index of FE.

Feed intake and dry matter digestibility

The contribution of FI to variation in FE mainly refers to the biological variation in DM digestibility and heat production that associates with higher intakes. The average heat increment deriving from feed consumption in ruminant species is around 9% of the GE (NRC, 2001). Greater DMI values have been associated with greater heat production in dairy cattle, while more efficient cows characterized by lower intakes proved less susceptible to heat stress (DiGiacomo et al 2014; Arndt et al., 2015). The greater ME utilization capacity of feed efficient cows may be the key to explain the results of studies suggesting that animals selected for low RFI have lower energy requirements for maintenance (Herd e Bishop 2000; Castro Bulle et al., 2007; Gomes et al., 2012). The latter hypothesis – while fascinating – is still debated, since long-term and expensive trials are required to precisely measure maintenance requirements of ruminants.

Another proposed mechanism behind the effect of FI on RFI is the variation in ruminal retention time between cows that differ in FI level (Nkrumah et al., 2006). As reported, high intake levels increase the rate of passage of feed through the rumen, considerably reducing the digestibility of fiber and organic matter (Robinson et al., 1985; Shaver et al., 1986). The effect of digestibility on the RFI is discussed below.

Dry matter digestibility – expressed as the ratio of consumed energy divided by fecal energy loss – has been correlated with the RFI of beef cattle, accounting for 14% to 19% of the variation in RFI (Richardson et al., 1996; Richardson and Herd, 2004). Studies conducted on dairy cattle produced more mixed results, with a contribution to the variations in FE ranging from 0 to 31% (Fischer et al., 2018; Potts et al., 2017). Moreover, doubts have been posed on the reliability of the results, since confounding factors (physical activity, feeding behavior, diet composition, rumen and environmental temperature) may influence digestibility values (Fischer et al., 2018). As already mentioned, higher FI is followed by an increase in GE intake, however associated with an overall lower efficiency in feed utilization due to increased rate of passage (Tyrell and Moe, 1975). A certain degree of genetic variability in digestive capacity has been detected for equal intake level. Beecher et al. (2014) found that Jersey dairy cattle had higher digestive efficiency compared to Holstein-Friesian, a feature associated with an overall greater gastrointestinal tract weight and – interestingly enough – with both higher GFE and DMI capacity. Individual DM digestive capacity detected by near-infrared (NIR) spectroscopy of feces was used to assess the heritability of the trait, ranging from 0.13 to 0.51 in dairy cattle (Mehtiö et al. 2019). In countertendency, other authors did not detect any correlation between digestibility and FE (Cruz et al 2010; Gomes et al 2013; Fitzsimons et al 2014). Caution should be posed in taking digestibility as one of the major determinants of RFI and FE because of the inherent difficulty in its measurement.

Metabolism and body composition

Since maintenance requirements have been cited as a factor affecting the efficiency of dairy cows it is logical to think that pathological states can lead to a reduction in FE. The activation of the immune system consequent to inflammatory and infectious insults has a mandatory price represented by the energy and nutrients needed for its augmented functionality (Elsasser et al., 2008). Despite the energy requirements of a fully activated immune system are not well established in ruminants, evidence from human medicine indicates that a severe inflammatory response can increase the energy requirements for maintenance of 30-40% (Frankenfield & Ashcraft, 2011).

Excluding pathophysiological fluctuations in maintenance requirements, specific metabolic pathways have been identified as a factor affecting FE. Mitochondrial proton leak and oxidative phosphorylation uncoupling, a process responsible for significant energy dissipation through thermogenesis (Busiello et al., 2015), has been noted to be related to the RFI in beef steers (Fernandez et al., 2019) and pigs (Lonergan, 2015). Other authors however failed to find differences in proton leak kinetics in beef cattle diverging by RFI (Acetoze et al., 2015; Fonseca et al., 2015). Consistently, Nkrumah et al. (2006) reported 21% reduction in mitochondrial respiration rate in low-RFI steers.

Variation in body tissue composition may influence FE of animals. The standard efficiency for fat deposition is 70-95%, while for protein deposition is 40-50%. Contrary to what expected, results from a study conducted on slaughtered beef steers showed that the progeny of low-RFI parents had less body fat and more body protein than the progeny of high-RFI parents (Richardson et al., 2001). A subsequent study from the same research group, conducted on live animals, showed positive correlation of RFI with serum leptin and urea (markers of lipid metabolism), and

negative correlation with serum creatinine (marker of muscle metabolism) (Richardson et al., 2004). Results from these studies should be read cautiously, as difference in body fat to protein ratio explains only 5% of variation in FI (Herd & Arthur, 2009).

Methane emissions

Ruminal methane is a by-product of the NAD+ reduction to NADH, a cofactor essential for the regulation of the ruminal pH and the synthesis of volatile fatty acids (van Lingen et al., 2016). Methanogenesis is an energetically inefficient process, estimated to waste 2 to 12% of the ingested GE (Qin et al., 2012). It is well established that DMI has a strong positive correlation with methane production (Kriss et al., 1930; Hristov et al., 2018), so much so that DMI has been successfully used as an indirect trait to predict methane emission (Charmley et al., 2016; Niu et al., 2018). Nonetheless, the diet composition can affect methane production independently of the DMI. Animals fed high-fiber diets rich in cellulose have higher methane emission compared to animals fed high-concentrate (Fahey e Berger, 1988). The low methanogenic potential of starch is likely due to its highest degradation to propionate compared to fiber, as microbial propionate production is a competitive pathway with hydrogen uptake (Ungerfeld, 2015). Moreover, other dietary factors such as concentrate composition (Lovett et al., 2006) and forage quality (Molano & Clarck, 2008) may be used to modulate methane emission of feed efficient dairy cattle.

In theory, improved FE decreases daily methane emissions by reducing the DMI for a fixed production level (Waghorn and Hegarty, 2011). Results from experimental trials seems to be inconsistent on the relation between RFI and methane emissions. Lower total methane production (expressed as g/d and g/kg of MBW) have been reported in beef (Fitzsimons et al., 2013) and dairy cattle (Waghorn & Hegarty, 2011) selected for improved FE. The two studies did not find a reduction in methane

production per kilogram of DMI (methane yield), suggesting that the mitigation of methane production was to be imputed to the lower DMI rather than an improved ruminal metabolism. This is in contrast with what observed by Nkrumah et al. (2006) and Dini et al. (2018) in beef cattle, for which low-RFI subjects produced less methane even when DMI was used as covariate of the equation. On the contrary, other authors surprisingly found that methane yield (but not daily methane production) was higher for low- than high-RFI dairy cows. Olijhoek et al. (2018) evaluated the effect of DMI, dietary concentrate level and digestive capacity on methane production of Holstein-Friesian and Jersey dairy cows using respiratory chambers. The authors found that when considering RFI as a continuous variable, more efficient animals had lower DMI but higher methane yield (methane per kg of DMI) and – in Holstein-Friesians – higher DM digestibility. Higher levels of dietary concentrate were able to slightly mitigate methane emissions, with a more pronounced effect on Jersey cows, that were characterized by overall higher methane production. In a twin study, the metagenomic analysis of the ruminal populations concluded that the RFI does not affect the composition of the methanogenic microbiota (Noel et al., 2019), as confirmed by other publications (Bowen et al., 2020). Further studies are needed to more precisely assess the interactions between FE, DMI and methane production.

Rumen microbial populations

Evidence suggests a strong link between host genetics and the rumen microbiome (Hernandez-Sanabria et al., 2013; Roehe et al., 2016). A linkage between the rumen microbiota and host variations in FE has been first proposed in beef cattle by Guan et al. (2008). From then on, many studies investigated the effect of selecting for RFI on rumen volatile fatty acids and microbiota composition, albeit with inconclusive results. Rius et al. (2012) reported similar rumen microbial composition in dairy cows divergently selected for RFI, while detecting greater digestive capacity in more efficient cows. It has to be mentioned that the RFI and the microbiota of animals

selected in the study were not investigated contextually. Carberry observed a higher abundance of *Prevotella* spp. in high-RFI beef cattle, though the effect was strongly influenced by the diet (Carberry et., 2011). Jami et al. (2013) did not find any correlation between rumen bacterial community composition and RFI of dairy cattle. Results from the trial have to be read cautiously since conducted on a small sample of 15 lactating cows. Myer et al. (2015) identified significant variations in the rumen microbioma of beef steers diverging for GFE. A recent study conducted on 20 dairy cows in early lactation stage detected significantly greater abundance of *Succinivibrio dextrinosolvens* (a propionate-producing bacterium) and lower abundance of *Butyrivibrio pseudoclasticus* (a butyrate-producing fibrolytic bacterium) and *Streptococcus bovis* (a lactate-producer heavily involved in the pathogenesis of subacute ruminal acidosis) in low-RFI cows compared to high-RFI (Elolymi et al., 2018). Unexpectedly, the analysis of the enzymatic activity conducted in the same study revealed lower activity of cellulase and protease in highly efficient cows compared to less efficient ones. Given the concurrent improvement in DM digestibility detected in the more efficient cows, authors speculated that the minor enzymatic activity of highly efficient cows may be due to the lower DMI and slower rate of passage, that would allow a longer lasting microbial degradation of the organic matter. In contrast are the results of a study conducted by Li & Guan (2019) on beef cattle, in which the RFI values were positively associated with the abundance of ruminal *Lachnospiracee* spp., a family of butyrate-producing bacteria. Since a ruminal VFA ratio against propionate is positively associated with methane emission and energy retention (Russell, 1998), this may explain the effect of the ruminal microbial composition on the FE of beef cattle. The study also evidenced that the ruminal microbiota of more efficient steers was overall less diverse (lower alpha diversity), a finding confirmed by studies conducted on dairy cattle (Shabat, 2016).

Suboptimal conditions of the ruminal environment – and in particular subacute rumen acidosis (SARA) – can negatively affect the FE of ruminant species by reduced fiber digestibility. The cellulosolitic bacterial community is particularly sensitive to pH drops, and its reduction during SARA can lead to a reduction in fiber digestibility of about 20-25% (Krajcarski-Hunt et al., 2002). In contrast are the results from Potts et al. (2017), that observed no explanatory effect of the rumen digestive ability on variations of the FE (expressed as RFI) when cows were fed high-starch (potentially acidogenic) diets. Instead, authors found that fiber digestibility account for 9 to 31% of the variation in RFI when cows were fed low-starch diets. This could be due to the reduced energy density of low-starch diet, driving higher DMI in order to sustain the energy requirements of cows and – by consequence – increased passage rate responsible for low DM digestibility.

NUTRITION, MANAGEMENT AND FEED EFFICIENCY

It is well established that a correct nutrition of livestock represents a cornerstone for animal production. Diets formulated to meet the nutritional requirements of animals for specific production periods and levels are paramount to maintain healthy, productive and efficient cows and heifers (McGrath et al., 2018; Sordillo et al., 2016; Eastridge, 2006).

Given the strict relation between nutrition and physiology, differing the nutritional factors affecting the FE from the biological ones is often difficult and – in most of cases – it represents a merely scholastic distinction.

The importance of correct dietary plans should be particularly stressed for dairy cows in late gestation and early lactation (transition period), during which 50 to 75% of the incidence of several metabolic and infectious diseases is reported (LeBlanc 2010). As already mentioned, the activation of the immune surveillance in sick

animals can cause a significant increase in the energy requirements for maintenance (Elsasser et al., 2008). Since many of the predisposing factors associated with disease are approachable through nutrition, particular attention must be paid in formulating diets for transition and early cows.

The energy content of the ration is likely one of the main factors affecting the FE of dairy cows (Casper and Mertens, 2007). In growing dairy heifers, feeding a low-energy diet in an energy restriction dietary regime resulted in reduced FE compared to high-energy diet fed ad libitum, despite associated with better average daily gain values (Williams et al., 2019). A large body of literature can be found on the effect of diets differing for energy density on the performance and health status of transition dairy cows. Despite some contrasting opinions (Winkelman et al., 2008), general observations converge on the conclusion that restricting the energy intake prior to parturition in order to not exceed the requirements – by reducing the energy density of the diet or restricting the access of animals to the ration – may have beneficial effects on the future milk production, energy balance and susceptibility to disease (Janovick et al., 2011; Agenäs et al., 2003; Guo et al., 2007), all factors potentially affecting the FE of lactating animals. Regardless of the effects on the immune and metabolic status of transition cows, a dietary strategy based on energy restriction may also pose some problems regarding the efficiency of farm animals. Low-to-moderate-energy diets proved effective in stimulating the DMI of dairy cows (Dann et al. 2006). Higher DMI is desirable in transition cows, as the reduction of the voluntary intake is considered one of the main responsible for the development of the production-related metabolic disorders (Hayrli et al 2003; Allen et al., 2020). However, an increased DMI also unavoidably pose the risk of impairing the GFE of animals, as well as reducing the rate of ruminal passage (Tyrrell and Moe 1975).

Optimal nutrition of dairy cattle results even more essential in late pregnancy and early lactation, during which the highest incidence of disease is recorded (LeBlanc et al., 2010). As already mentioned, an impairment of the health status causes a relevant increase in the energy requirements for maintenance (Elsasser et al., 2008), that together with the impairment of the production performance and the costs related to veterinary services may heavily impact the economic efficiency of the enterprise. In this sense, the improvement of FE on a herd level can also be achieved by optimizing the health status of the animals through a nutritional approach (Miglior et al., 2017)

Dietary changes in dairy herd management should aim to reduce the energy losses by improving feed utilization. In order to optimize the feed utilization and minimize the fecal losses of ruminants it is essential to maximize starch and fiber digestibility (Oba e Allen 2000; Kendall et al 2009). The inclusion in the diet of high-quality forages proved to be effective in improving fiber digestibility and production performance of dairy cattle (Ding et al., 2015; Allen et al., 1996). Recently, Pino et al. (2018) investigated the effect of diets including high-quality (corn silage; NDF 39.8%) versus low-quality (rye grass; NDF 48%) forages on GFE of beef heifers, evidencing that high-quality forages may have an ameliorative effect on beef cattle efficiency. Adequate levels of physically effective NDF (peNDF) are necessary for a correct rumen motility and rate of passage, exerting a protective effect against SARA (Kroger et al., 2019). However, the positive effects of increased peNDF on health and rumen status should always be weighed against the potential reduction in ration energy content and digestibility, leading to a reduction of FE (Yang e Beauchemin, 2006).

The inclusion in the diet of starch sources with different degradation rates allows to increase the digestibility of the diet while containing the risks associated with high-starch diets. Fast rates of starch degradation in the rumen may result in excessive accumulation of short chain fatty acids and a net reduction in ruminal pH,

representing one of the main risk factors for SARA (Penner et al., 2009). As already reported, cows affected by SARA display a significant impairment in productivity, organic matter digestibility and – ultimately – FE (Oetzel 2017). Chemical and physical features of different grains affect their content in rumen undegradable starch – with the highest levels found in maize and sorghum, followed at a distance by wheat, rye and barley (Benninghoff et al., 2015). Knowing the rumen undegradable starch content of the grains included in the ration makes possible to modulate the degradability of dietary starch through the diet formulation. Regardless of the type of grains included in the ration, grain particle size is another important factor affecting the ruminal degradability, with fine grounded grains more readily fermentable compared to coarse grounded ones. Rémond et al. (2004) compared in vivo ruminal degradation of fine grounded, medium grounded and coarse rolled corn, finding a 20% reduction in rumen degradability between fine grounded and rolled corn. Another work compared the effects of different processing techniques on rumen degradability of several starch sources, finding a linear decrease in degradability from fine grounded to rolled barley, wheat, rye and pea meal; and a quadratic decrease for corn, sorghum, oat and faba meal (Gallo et al., 2018). The processing treatment applied to the grains can also affect the proportion of starch reaching the hindgut. For example, steam-flaking increased by 50% the ruminal degradation of corn and sorghum compared to dry-rolling (Theurer et al., 1999). Ensiling high moisture corn (Hoffman et al 2011) and extrusion of dried distillers (Claassen et al 2016) are other nutritional strategies able to improve starch degradability.

Despite less investigated, dietary composition may affect FE of cattle by other factors, such as protein content (Giallongo et al., 2016) and roughage-to-concentrate ratio of the diet (Phuong et al., 2013).

From a study conducted on beef cattle it has been hypothesized that feeding patterns and behavior may contribute to 2% of the genetic variation in RFI (Basarab et al., 2013). Evidence from beef (Basarab et al., 2013; Williams et al., 2011) and dairy cattle (Connor et al., 2013) suggests that animals with lower RFI (i.e. more efficient) consume the ration at a slower rate, less often (lower number of meals per day) and spend overall less time feeding per day. These findings seem to be confirmed by a study evaluating a 2 years feeding trial conducted on 842 dairy cows, in which genomic tools were used to assess the genetic correlation between RFI and traits related to the feeding behavior (Lin et al., 2013). Results from the study showed that feeding behavior traits were moderately heritable and that the feeding duration trait (rate of feeding) had moderate genetic correlation with RFI (0.27). A slow rate of feeding may positively affect FE by its effect on the rate of passage of ingesta through the rumen and – indirectly – by improving the fiber and DM digestibility (Aikman & Beever, 2008). Less time spent feeding each day means a reduced standing time. Standing time associated with feeding has been quantified in an average of 4.4 hours per day for dairy cows raised in housing systems and twice that under grazing conditions (Cook et al., 2008), proving a substantial energy expenditure. Moreover, longer feeding time causes reduction in time spent resting and ruminating, with a potential negative impact on energy metabolism (Soriani et al., 2013). Feeding behavior is a complex trait influenced by the characteristics of the diet (e.g. palatability, roughage to concentrate ratio, particle size), patho-physiological state of the animal and environmental stressors (Allen et al., 2020). In confined raising systems, longer feeding time increases the risk of interspecific competition, which in turn may furtherly affect feeding pattern and efficiency of animals (DeVries & Keyserlingk, 2009; Proudfoot et al., 2009). Moreover, recent transcriptomic analysis conducted on various cattle breeds identified breed-specific hepatic metabolic pathways that would explain the impact of liver functionality on FE (Salleh et al., 2017). If confirmed, these findings could corroborate the hypothesis of the hepatic

oxidation fuel, proposed by Allen et al. (2020), as a control mechanism behind FI and feeding behavior. Another behavior to be avoided is feed sorting, as it represents a predisposing factor for SARA. Homogeneous mix of the total mixed ration – that can be monitored using a Penn State Particle Size Separator – is essential for reducing the chance of feed sorting. Moreover, Leonardi et al. (2005) documented that adding water to dry diets improved the DMI and reduced the feed sorting behavior of dairy cows, as humidity allow the particles to better bind together.

Purchasing by-product feedstuff may reduce feed expenses and improve economic feed efficiency (Buza et al 2014; Karlsson et al., 2018), even though attention should be addressed to its high variability in quality and nutrient characteristics compared to standard products (Bradford and Mullins 2012). The cost of feed shrink and spoilage should be assessed (Kertz 1998). Likewise, selecting alternative feedstuff with low production costs may increase the economic feed efficiency.

Ultimately, a variety of feed additives commonly used in dairy cattle nutrition can be supplemented with the indirect aim of improving the efficiency of the animals. Feed additives like *Saccharomyces cerevisiae* and its fermentation products are used to stabilize the ruminal environment and improve organic matter digestibility (Thrune et al., 2009; Chaucheyras-Durand et al., 2016). Antilipolytic agents (e.g. niacine) may reduce the detrimental effects on health caused by excessive lipid mobilization (Chen et al., 2019). Essential aminoacid like rumen-protected methionine proved capable of improve milk production and immune response (Batistel et al., 2017).

GENETICS OF FEED EFFICIENCY AND GENOMIC SELECTION

Since both GFE and RFI traits have been evaluated to be moderately heritable – with values of 0.14 to 0.37 (Spurlock et al., 2012) and 0.01 to 0.38 (Rolfe et al 2011; Vallimont et al., 2011) respectively, FE may represent a relevant trait to consider in

selecting cattle for breeding goals. The improvement of feed utilization of dairy cattle through genetic selection may represent a feasible solution to increase both the environmental and economic sustainability of dairy industry. Understanding the genetic basis of FE is therefore imperative such that selective strategies can be optimized.

Genetic evaluation of feed efficiency

Historically, because of the expense and difficulty of measuring individual cow intakes, selection for FE has been conducted by indirect selection on correlated linear-type traits, such as production parameters and BW. However, the low phenotypic correlation between these linear traits and RFI makes poor indicator traits of the former (Veerkamp et al., 1995; Coleman, 2010). Moreover, given the high genetic correlation between milk yield and GFE (Vallimont et al., 2011), it is reasonable to deduce that the additional ameliorative contribution of the inclusion of GFE trait into breeding plan would be negligible. Dry matter intake is a linear trait. Despite DMI has been estimated to be moderately-to-highly correlated with RFI (0.35 to 0.61) (Lin et al., 2013; Manafiazar et al., 2016), suggesting that if we selected to reduce RFI then DMI would also be reduced, the intrinsic difficulty in measuring individual DMI limits its application as an indicator trait. Encouraging preliminary results were obtained by McParland et al. (2014), that found a phenotypic correlation of 0.65 between RFI and milk fat content measured by mid-infrared (MIR) spectroscopy.

Genomic selection offers the possibility to capture additive genetic variation in FE, in addition to what is predicted by production and BW traits. Moreover, a genomic approach to selection allows to select animals directly for the marker genotype, bypassing the need for DMI measurement (Meuwissen et al., 2001). Compared to other species, genome-wide association studies (GWAS) characterizing the gene expression and gene regulatory mechanisms related to FE are quite recent in

dairy cattle (Berry & Crowley, 2013). To date, many of the GWAS conducted in dairy cattle are based on relatively small datasets (Pryce et al., 2012; Yao et al., 2013). Fortunately, larger GWAS conducted on beef cattle proves useful, as some of the genomic regions found to be related to FE are shared by dairy cows (Sherman et al., 2010; Lu et al., 2013; Yao et al., 2013).

In GWAS, the relatively recent sequencing of the bovine genome, coupled with the availability of high-density genomic information via single nucleotide polymorphisms (SNPs) made possible to identify quantitative trait loci (QTL), that can in turn be exploited to identify the heritable genomic regions correlated with FE and its relation with other traits. In a population of beef cattle, Saatchi et al. (2014) identified 10 significant 1-megabase SNP windows located on 8 autosomes for RFI; the larger windows, located on chromosome 15, was estimated to explain 2.4% of the genetic variance of RFI. A GWAS exploiting 625.000 SNPs conducted on growing heifers identified a significant correlation between regions of the genome in RFI and energy metabolism control (Pryce et al., 2012). In Holstein-Friesian cows estimated for RFI, a GWAS aimed at evaluating the gene copy number variations (CNVs) - segments of DNA that exhibit individual variations in number of copies and can influence the gene expression of the subject (Bickhart et al., 2012) – identified a major overlapping of genes involved in inflammation and immune response with RFI-related regions in more efficient cows; while specific CNVs regulating tissue and skeletal growth were more associated with RFI-related regions in less efficient cows (Hou et al., 2012).

Current difficulties in genomic selection for FE in dairy cattle

Genomic selection proves particularly useful for phenotypic traits difficult to measure in farm. The evaluation of the genetic effect of each marker (SNP) across the genome by comparison with a reference population (animals with known phenotype

and genotype), and the subsequent comparison with the genotype of the subject under evaluation allow to predict its breeding value (genomic breeding value; gBV) without phenotypic recording. Whether the accuracy of gBV for FE were high enough, animals could be selected for this trait with breeding based on DNA information alone. However, in order to increase the accuracy of gBV phenotypical data on a very large reference populations of genotyped individuals are needed. Moreover, phenotypes need to be re-assessed cyclically in order to maintain the accuracy of prediction. With genome-wide association studies (GWAS) now more affordable, the identification of quantitative trait loci (QTL) harboring the highest genetic variance for a specific trait is made possible. As already mentioned, GWAS for FE-related traits of dairy cattle have recently been performed, though on small datasets with still limited prediction power (Pryce et al., 2012; Yao et al., 2013). For this reason, international collaboration efforts are underway to create a larger reference dataset of genotyped and phenotypically tested subjects, under different conditions in terms of dietary and stabulation management (Difford, 2018). Currently, the accuracies of gEBV for RFI and DMI are promising, suggesting that genetic gains could be made through genomic selection (Pryce et al., 2014).

Genomic studies on dairy cattle are also useful for identifying unintentional, often undesirable correlated response to selection. For example, an aspect raising concern about the selection for FE is its potential antagonism with fertility (Shaffer et al., 2011; Vallimont et al., 2013). Gonzalez-Recio et al. (2014) explored the interactions between selection for FE and reproductive traits, founding that genomic correlations between calving interval and DMI or RFI were 0.26 and -0.13, respectively. This means that correlated response of fertility to RFI is undesirable, as cows with low RFI (i.e. more efficient heifers) have longer calving intervals as cows (i.e. are less fertile). Nonetheless, the unfavorable correlation between RFI and calving interval was still

smaller than the unfavorable correlation between calving interval and milk yield (0.41; Haile-Mariam et al., 2013).

Another concern, especially for dairy cattle, is that the selection for more efficient animals characterized by lower intake levels may result in thinner cows with inadequate body reserves to face the negative energy balance typically experienced in early lactation. Despite BCS and LBW traits being accounted for the calculation of RFI, in growing dairy heifers the genetic correlation between RFI and BCS was 0.71 (Gonzalez-Recio et al., 2014), endorsing the hypothesis of a correlated response between the two traits. This pose some questions on the adequacy of selecting for RFI, as negative energy balance has been demonstrated to result in lower fertility (de Vries & Veerkamp, 2000; Pryce et al., 2004).

Finally, temporal changes in FE may affect the accuracy of its genomic estimations. With the variation in biological asset and energy demands across the lifetime and production stages of dairy cow, FE and its genetic basis vary accordingly (Spurlock & VandeHaar, 2013). Estimated genomic correlation between dairy heifers and lactating cows is 0.67 (Pryce et al 2014).

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Chapter 2|

Camelina Cake in Dairy Cow Diets: Effects on Production and Milk Composition

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ABSTRACT

Increasing the nutritional quality of dairy product fat is a rising consumers claim prompted by the beneficial effects of supplemental long chain n-3 fatty acids and lower n-6/n-3 ratio on human health. Camelina is a sustainable crop and a good source of n-3 fatty acids. The goal of the trial was to evaluate the effects of the dietary inclusion of camelina cake on production and milk composition of lactating dairy cows. Twenty-two primiparous and multiparous Holstein Friesian dairy cows housed at the Experimental Station (Centro Zootecnico Didattico Sperimentale) of the University of Milan in Lodi were divided in two homogeneous groups for milk production, parity and DIM. Diets consisted of a TMR corn silage based, supplemented with 800g/h/d of camelina cake (CAME) or an isonitrogenous and isoenergetic soybean-based premix (CTR). Performances were recorded weekly. Milk composition was assessed weekly on fresh samples. Milk samples for milk fatty acid compositional analysis were collected on day 0, 21 and 42 of the trial. Data were analyzed by MIXED procedure by SAS. No differences were detected for milk production, feed intake, live body weight and body condition score. CAME group had a reduced milk fat percentage ($p < 0.05$) compared to CTR but not a reduced production of fat (kg). Saturated milk fatty acids were also reduced in CAME compared with CTR. Linolenic acid and rumen biohydrogenation intermediates were increased in CAME whereas stearic acid decreased. Camelina cake could be useful to improve nutrition value of milk, but its impact on rumen metabolism and possible interferences on biohydrogenation steps need further attention.

INTRODUCTION

The exponential growing in human population and the consequent increased global demand for dictates the need for a reforming of animal production industry, guided by enterprise choices vowed to the economical as well as environmental sustainability. Improving the FE of livestock allows to raise more productive herds

while containing economic expenses and crops grown for animal consumption. Mindful selection of adequate dietary strategies comprising non human-edible feed ingredients (by-products) and more sustainable vegetal cultures may represent a viable solution to be applied in conjunction with genomic selection for breeding plans.

Camelina sativa (also known as false flax and gold-of-pleasure) is an oilseed plant belonging to the family of *Brassicaceae*. In the last decade camelina oil attracted the attention of the scientific community as a potential alternative energy source to fossil fuels (Shonnard et al., 2010; Hoseini et al., 2018), due to its renewable nature and the low emissions of GHG that characterize its combustion (Petre et al., 2013). Previous studies highlighted camelina requires few water for its growth, it is poorly affected by plant density, and that high plant density camelina crops can effectively suppress weed growth (Kim et al., 2015; Gesch & Cermak, 2011). Camelina seeds and oil are naturally rich in protein, alpha-linolenic acid (one of the main n-3 PUFA) and tocopherols, and relatively low in erucic acid (Abramovic & Abram, 2005, Putnam et al., 1993), making them particularly suitable for animal feeding (Bullerwell et al 2016; Brandao et al., 2018; Lawrence et al., 2016). Despite the expected partial loss of nutrients due to processing, camelina meal and cake still represent products of high nutritional profile (Peiretti et al., 2007), to the extent of being recommended as a valid alternative for fish oil (Hixson & Parrish, 2014). Despite the high content in UFA pose a risk for the oxidative stability of camelina oil, the antiradical activity of camelina meal extracts was found comparable to that of vitamin C in a study by Mierina et al. (2017), with a higher oxidative stability than other widely used oilseed crops (e.g. rapeseed, flaxseed). The high stability of camelina by-products, in spite of their high content in UFA, may be justified by its content in tocopherols and polyphenols (Belayneh et al., 2015). These data suggest the potential of camelina as a feed supplement for animal nutrition, capable of enriching the acidic profile of animal

products (Cieslak et al., 2013; Rokka et al., 2002) while containing agricultural land extension, pesticides usage and water consumption.

Current public health policies recommend a decrease in the dietary consumption of SFA and an increase in PUFA, specifically n-3 fatty acids (FA), to lower the incidence of cardiovascular and metabolic diseases (Kris-Etherton & Krauss, 2020). At the same time, increasing the nutritional value of dairy products is a rising consumers claim, moved by the growing interest in functional foods. Dietary manipulation of milk fatty acid (FA) composition represents an interesting approach to improve the biological and nutraceutical properties of milk and dairy products, with beneficial effects on both human and animal health.

The high content in ALA makes camelina products good candidates for an economic and sustainable feedstuff, alternative to the dietary source of PUFA traditionally used in animal nutrition (e.g. marine-derived oil and linseed). Despite dietary lipid supplements have largely proved effective in modulating the milk FA composition (Boekaert et al., 2008; Toral et al., 2010), the transfer efficiency of dietary PUFAs into milk remains low in ruminants (Cattaneo et al., 2006) due to their biohydrogenation by the rumen microbial population. During the biohydrogenation process, a large fraction of dietary unsaturates – ranging from 60% to 90% (Mattos & Palmquist, 1977) – undergo hydrolysis and several consecutive isomerization reactions that lead to their saturation. By consequence, the main FAs that reach the duodenum (and subsequently the mammary gland) are stearic acid (C18:0) and several intermediates of reaction, mostly *trans*-isomers of oleic and linoleic acids (Buccioni et al., 2012). While some of the intermediates of the biohydrogenation process proved beneficial for human health – in particular some conjugate linoleic acid (CLA) (Pariza et al., 2001) – others are considered among the main causal factors of the 'milk fat depression' phenomenon, a condition characterized by a significant

reduction in expected milk fat content (up to 50%) with little or no change in lactose or protein (Bauman and Griinari, 2000). The production of these trans- intermediates is favored by two main factors, namely i) the reduction of the ruminal pH below the physiological range of values (i.e. subacute ruminal acidosis); ii) the excessive supplementation of PUFA (Dewanckele et al., 2020). Therefore, the development of new technological treatments and the exploration of new dietary sources of PUFA is pivotal to produce more health dairy products without interfering with production levels.

MATERIALS AND METHODS

The trial was performed at the Animal Production Research and Teaching Centre of the Polo Veterinario (CZDS), Università degli Studi di Milano (Lodi, Italy). The trial lasted for a period of 42 days (half of October 2018-end of November 2018). Animals used in this study were treated in accordance with the European Commission recommendation 2007/526/EC and 2010/63/UE on revised guidelines for the accommodation and care of animals used for experimentation and other scientific purposes. All the experimental procedures included in the trial were reviewed and approved by the Animal Care and Use Committee of the University of Milan.

A total of 22 healthy Holstein-Friesian dairy cows of the same age (17 ± 2.5 months) deriving from the same commercial herd were housed in the free-stall facility of the CZDS. All animals enrolled have previously had been genotyped by the Italian Holstein-Friestian and Jersey Breeder Association (ANAFIJ). Cows were allocated at random into two experimental groups (n = 11) stratified for days in milk (DIM), parity, and previous production performance. Groups were fed a basal total mixed ration for lactating cows with the addition of a soybean-based premix (CTR) or 800 g/heifer camelina cake (CAME) (Table 1).

The diets were formulated to be isoenergetic and isonitrogenous and to meet the nutritional requirements of lactating cows (Table 2). The diets were prepared and mixed daily directly on the farm in order to obtain individual rations that ensured the uniformity of the mixture and the correct amount of premix per subject. Ration was delivered twice per day in order to assure the animals *ad libitum* access to the feed.

Diet samples and analysis

On d 0, 21, 42 representative samples of the rations were collected to determine the nutrient profile of the diets. Multiple grabs of the TMR in front of the cows was composited in a final sample (500 g), vacuum-packed and stored at -20 °C before analysis.

Feed samples were dried in a 60 °C forced air oven for at least 48 hours and analyzed for DM (procedure 4.1.06; AOAC, 2000). Samples were ground to 1 mm particle size in a Wiley mill and used for chemical analysis on analytical composition.

With the same time schedule, the main forages included in the diet were sampled. Silage samples were collected by mixing and compositing 10 sub-samples collected coring the fresh front of the silage trench on evenly distributed heights. Final representative samples (500 g) were delivered to Rock River Laboratory Europe (Heiddorf, Germany) for the evaluation of NDF digestibility at 24h or 30h, 120h, 240h (%) and kd $CHOB_3$ (fiber fraction).

Milk samples and analysis

Starting from d 0 and on a weekly basis on a weekly basis (d 0, 7, 14, 21, 28, 35, 42), individual milk samples from two consecutive milkings (morning and evening) were collected, preserved with sodium azyde and clustered in individual 100 mL composite samples representative of the 24-hour milk production. Composite samples were refrigerated at 4 °C until analysis of chemical composition and somatic

cell count (SCC). Fat, protein, lactose, urea, casein, total saturated-, total unsaturated-, monounsaturated- and polyunsaturated fatty acids (SFA, UFA, MUFA and PUFA respectively) content was assessed by Fourier transform infrared (FT-IR) spectroscopy. Somatic cell count (SCC) was performed using an automatic cell reader (Bactoscan 8000, FOSS Electronic).

On d 0, 21, 42, additional individual milk aliquots were collected and directly sent to laboratories for the evaluation of the FA composition by gas chromatography.

Rumen fluid samples and analysis

Rumen content samples were collected on d 0, 21, 42 using an oro-ruminal probe (Ruminator, Profs-Products). The metallic probe was inserted through the oral cavity and moved forward until reaching the caudal ventral blind sac of the rumen. A suction pump was connected and used to collect the fluid into the sterilized glass case that completed the set. After removing the first 100 mL of fluid in order to avoid saliva contamination, samples were transferred into sterile tubes ((Falcon™ 50mL Conical Centrifuge Tube) in triplicates and freezed at -80 °C until being analyzed.

Rumen fluid samples were thawed and prepared via transesterification (FAME) (Kramer et al., 1997). Fatty acid methyl esters (FAME) and DMA were identified and analyzed by gas chromatography, using a GC 8000 TOP gas chromatograph (ThermoFisher Scientific Inc., Milan, Italy) equipped with a flame-ionization detector (GC-FID) and a WCOT CP-Select capillary column (100 m; 0.25 mm i.d.; 0.25 μm film thickness; Chrompack, Middelburg, the Nederlands).

Measurements and recorded parameters

Daily recording of milking data was not possible due to a malfunction of the milking parlor, thus data from two consecutive milkings were recorded manually on a weekly basis (d 0, 7, 14, 21, 28, 35, 42).

Individual daily FI values were gathered processing the raw data recorded in real time by the software connected to the automatic feeders (Insentec RIC System). The RIC system was used to collect data for feeding behaviour evaluation, in terms of number of accesses at the bin per day, average intake per access, time spent at the bin per access, time spent at the bin per day.

Starting from day 0 and on a weekly basis (d 0, 7, 14, 21, 28, 35, 42) individual LBW was measured – before the ration was delivered –with an electronic scale located outside the facility. Individual BCS were determined as the mean of the evaluation sites on a 1 (very thin) to 5 (very fat) point scale with 0.25-point resolution.

At the same times, fecal consistency was evaluated on fresh feces collected from the rectal ampulla by means of a 1 (liquid) to 5 (dry and segmented) point scale with a 1-point resolution.

Environmental parameters (moisture and temperature) were recorded *in continuum* using two HOBO change-of-state data logger (Onset Computer Corp., Bourne, MA) with a sampling time of 48 detections/24 hours period.

All animals were subjected to a daily veterinarian clinical examination in order to check health status. Individual morbidity rate was recorded in terms of clinical signs, diagnosis, clinical assays, therapeutic treatments adopted, illness duration (days), and health recovery. Moreover, all given medications including vaccines, de-wormers and metaphylactic treatments, specifying administered dosages, were recorded. Mortality rate was also recorded by day and cause.

Calculations and statistical analysis

Milk to feed ratio (M:F) was calculated as individual energy-corrected milk divided by individual DMI. Energy-corrected milk (ECM) was calculated according to the NRC (2001), following the formula:

$$ECM(kg) = 0.327 \times MY(kg) + 12.95 \times FC(kg) + 7.2 \times PC(kg),$$

where MY was the daily milk yield, FC was the milk fat content, PC was the milk protein content, LC was the milk lactose content.

Fat corrected milk (FCM) was expressed as 3.5% fat-corrected, according to the equation reported by Parekh et al. (1986):

$$FCM(3.5\%; kg) = 0.35 \times MY(kg) + 18.57 \times FC(kg).$$

Residual feed intake (RFI) per cow was estimated applying a model of regression of DMI on the main energy sinks of lactating cows. The effect of diet was also included in the equation as follow:

$$DMI_i = \beta_0 + \beta_1(MilkE_i) + \beta_2(MBW_i) + \beta_3(\Delta BodyE_i) + Diet + \varepsilon_i,$$

where DMI_i was the observed individual DMI, $MilkE_i$ was the individual milk energy output, MBW_i was the mid-test individual metabolic body weight, ΔbodyE was the estimated change in body energy. RFI was defined as the error term of the model (ε_i).

Milk energy output (MilkE) was calculated by following the equation reported by the NRC (2001):

$$MilkE(Mcal/d) = 9.29 \times FC_{(kg)} + 5.63 \times PC_{(kg)} + 3.95 \times LC_{(kg)},$$

where FC, PC and LC were expressed as the daily mean value for the whole trial period.

Estimated change in body energy (ΔbodyE) was obtained by modifying the equation of the NRC (2001):

$$\Delta bodyE = (2.88 + 1.036 \times BCS) \times ADG,$$

Where BCS is the average individual BCS during the whole trial period, and ADG is the individual average daily gain.

Statistical analysis of calculated parameters and sample data was performed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC v.9.4 2015). Cow was the experimental unit for all the accounted parameters. The model included diet, time and diet nested with time as the main effects. Significance level was considered for $p < 0.05$ and $p < 0.01$.

RESULTS

Overall milk production levels were similar for CAME and CTR groups. A significant time per group effect was found for milk fat percentage ($p = 0.03$) at d 42, but not in total milk fat production expressed in kg/d. CAME diet was associated with a 9.04% reduction in milk fat percentage at d 42 compared to CTR diet (4.27% and 4.72% respectively). No effect of camelina inclusion was found on DMI and energy use of cows. Animals from the two experimental groups displayed similar mean values of LBW and BCS.

Calculation of the RFI resulted in the following equation:

$$DMI = 3.271 - 0.033MilkE + 0.073MBW + 0.012\Delta BodyE + 0.184Diet + RFI$$

No difference was found for FE, expressed as both RFI and GFE (Table 3).

Camelina was capable to alter the FA composition of milk, with significant difference detectable at d 42. Milk from CAME had an overall increase in n-3 PUFA content (0.88 vs 0.70; $p = 0.02$) and a lower n-6/n-3 ratio (3.86 vs 4.84; $p = 0.01$). Milk from CAME group had increased levels of total *trans*-18:1 isomers compared to CTR (4.34 vs 3.34; $p = 0.01$) and a tendency toward higher α-linoleic acid (0.58 vs 0.48; $p = 0.06$). CAME milk had increased *cis*-9, *trans*-11 CLA (4.34 vs 3.34; $p = 0.03$) (Table 4).

Analysis did not detect significant difference in rumen DMA content, with only a tendency toward significance for dimethoxy dodecane (DMA C12:0; $p = 0.07$) and DMA C18:1 *cis*-11 ($p = 0.06$) (Table 5).

Relative to the ruminal FA composition, camelina cake supplementation was associated with increased levels of several *trans*- intermediates – in particular *trans*-18:1 isomers – and significantly higher levels of C20:0 and C20-derived MUFA, that were also found in higher concentration in CAME milk (Table 6).

For more details and significancy levels, see the tables.

DISCUSSIONS

In the present study, the inclusion of camelina cake in the diet of dairy cows did not affected overall DMI and FE of animals. As a general observation, however, it must be underlined that investigations on FE of small populations with large standard deviations for lactation stage and days in milk may result in highly circumstantial results.

The main aim of this study was to investigate the effect of dietary camelina cake on the ruminal and milk FA profile, with particular regard on the ruminal

polyunsaturated fatty acids (PUFAs) biohydrogenation process. The inclusion of camelina cake (CAME group) resulted in substantial changes in milk FA composition, characterized by lower content in SFA and n-6 PUFA, lower n-6 to n-3 ratio, and greater levels of unsaturated FA (both MUFA and PUFA), n-3 PUFA and C18:1 *trans*-isomer. Similar modifications had previously been reported when supplementing camelina oil (Bayat et al., 2015). Despite the potential benefits deriving from human consumption of PUFA and CLA enriched milk, camelina cake also caused a significant decrease in percentual milk fat content, that could negatively affect milk market price and its attitude to dairy processing. Results from the rumen content analysis may help explain the mechanism behind these effects.

Milk MUFAs derive from two main route: i) the desaturation of C18:0 by the mammary Δ9 desaturase enzyme; ii) the *cis*- isomers of C18:1 that escape the rumen (Shingfield et al., 2013). Given the similar concentrations of stearic acid (C18:0) observed in the ruminal content of CAME and CTR cows, and the higher concentration of C18:1 *cis*- isomers in CAME – though significant only for C18:1-*cis*9 (255.38 vs 199.95 g/100g; $p = 0.02$) – increased MUFA content in milk is more likely due to an improvement in the latter mechanism.

Several *trans*- and CLA- reaction intermediates resulting from the incomplete ruminal biohydrogenation of the unsaturated FAs are supposed to inhibit the mammary synthesis of FAs, displaying anti-lipogenic effects of variable magnitudes. Among the biohydrogenation intermediates, CLA-*trans*10-*cis*12 received particular attention in dairy cows for its alleged role in the pathogenesis of the milk fat depression (Chillard & Ferlay, 2004; Baumgard et al., 2008). In our study, CLA-*trans*10-*cis*12 was not found in detectable concentrations in the ruminal fluid of cows included in the CAME as well as the CTR group. The metabolic pathway leading to CLA-*trans*10-*cis*12 synthesis has been investigated in literature, with a recent research

suggesting its production being imputed to *Cutibactrium acnes* (formerly *Propionibacterium acnes)* (Dewanckele et al., 2020). Since the ruminal microbial composition was not investigated in this study, the reasons for the undetectable levels of CLA-*trans*10-*cis*12 in cows involved in our trials remains unclear. Nonetheless, the inability of camelina cake to increase the ruminal concentration of CLA-*trans*10-*cis*12 seems to suggest that there may be more than one factor driving milk fat depression in dairy cows. A hypothesis in this regard takes into account the Δ9-desaturase-mediated conversion of milk C18:0 (positively related to milk fat content in goats) to *cis*-9 C18:1, in order to reduce the milk fat fluidity and facilitate milk secretion (Toral et al., 2014; Loor et al., 2005). This theory seems to find confirmation in the study conducted by Toral et al. (2015), who recorded a significant decrease in milk C18:0 and C18:1-*cis*9 content and an increase in C18:1-*cis*9/C18:0 ratio in cows supplemented with fish oil. In contrast, CAME supplementation in our study was not associated with an increase in C18:1-*cis*9, that was detectable in (not significantly) lower concentration in CAME milk.

Stearic acid (C18:0) was the most abundant FA in the rumen of both groups. The absence of depressing effect on ruminal C18:0 production in CAME group suggests that supplementation with camelina cake did not exert toxic effect on the C18:0 producing bacteria, that is one of the main hypotheses suggested as explanation for the incomplete reduction of C18 unsaturates associated with milk fat depression (Maia et al., 2010). Another hypothesis imputes the condition to lower ruminal pH, negatively affecting dietary lipid degradation (Kalscheur et al., 1997). Unfortunately, in our study the rumen pH was not measured, thus association between the latter and rumen C18:0 content was not investigated.

Consistently with what reported in literature, in our study the *trans*- 18:1 FA were the major intermediates produced during the biohydrogenation process, and a

significant increase of their concentration was detected in CAME compared to CTR. Despite no effect of diet was detected on the concentration of C18:0 (the final product of the ruminal biohydrogenation process), the highest levels of C18:1 isomeres is therefore in line with an alleged modification of the biohydrogenation pathways.

Ruminal content of C18:1-*trans*10 was higher in CAME compared to CTR. Despite the metabolic pathway of C18:1-*trans*10 is still not completely clear, its association with starch content of the diet and its antilipogenic effect have previously been documented in dairy cows (Griinari & Bauman, 1999; Shingfield et al., 2010). The biological effect of C18:1-*trans*10 may be taken as explanatory for the lower milk fat content recorded in cows fed camelina cake in the present study.

The inclusion of camelina in the diet was not effective in increasing the ruminal concentration of C18:1-*trans*11, a *trans*- isomer derived by the biohydrogenation of alpha-linolenic (C18:3-n-3) and linoleic acid (C18:2-n-6) (Harfoot & Hazlewood, 1997). Increasing the C18:1-*trans*-11 rumen outflow is desirable as it acts as a substrate for mammary Δ9 desaturase enzyme, catalizing the formation of the *cis*-9,*trans*-11 CLA (Griinari & Bauman, 1999), which has been shown to have potential positive effect on human health status (Pariza et al., 2001). Although camelina is reported to be rich in C18:3-n-3, no increase in ruminal C18:1-*trans*11 was observed in CAME group. It is interesting to note that despite this, milk from cows fed camelina cake had higher concentrations of both C18:3-n-3 and CLA-*cis*9-*trans*11, enriching the nutritional profile of milk.

Contrary to what observed for the FA composition, the ruminal DMA composition of CAME did not differ from that of CTR. Dimethyl acetals are derived from the plasmalogen lipids of bacterial membranes, and their composition is similar to FA profile, showing odd, even, saturated and unsaturated chains from C12 to C18.

The variation of DMA profile is linked to the ability of bacteria to incorporate surrounding FA in their cellular membrane so as to modify its fluidity, and can be seen as a marker of resilience to environmental changes (Goldfine, 2010; Kaneda, 1991). In our study, the DMA composition of CAME did not differ from that of CTR. However, higher – even if not significantly – concentrations of DMA 18:0, 18:1-*trans*11, 18:1-*cis*9 and 18:1-*cis*11 were measured in the rumen of CAME group, with a tendency toward significance for the latter DMA 18:1-*cis*11 ($p = 0.06$). It can be argued that camelina cake included in the diet was not sufficient to trigger a relevant modification in the FA composition of the membranes of ruminal bacteria, though mild signs of adaptation could be presumed.

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TABLES AND FIGURES

Table 1. Chemical composition of the camelina cake

(% DM)	
Crude protein	38.41
Ether extract	8.11
NDF	20.24
Ash	6.07
Calcium	0.51
Phosphorus	0.73
ME(kcal/kg)	2230

Table 2. Feed ingredients and nutrient composition of the experimental diets

	Dietary treatment	
Ingredients (% as fed)	**CTR**	**CAME**
Corn silage	60.09	59.90
Corn grain ground	12.52	12.48
Grass silage	15.02	14.97
Soybean meal	9.92	8.98
Soybean flaked	0.78	0.00
Camelina cake	0.00	2.00
Salt	0.18	0.17
Mineral-vitamin premix	0.13	0.12
Sodium bicarbonate	0.75	0.75
Calcium carbonate	0.63	0.62
Total	100.00	100.00
Calculated composition (% DM)		
Moisture	48.85	48.71
Crude protein	16.51	16.40
Ether Extract	3.43	3.41
NDF	34.09	34.52
NFC	39.87	39.88
$NEL_{(mCal/kg)}$	1.70	1.68
Calcium	0.75	0.76
Phosphorus	0.37	0.37

Table 3. Average productive performance and FE of experimental cows

	CTR	CAME	SEM	*p-value*
DMI (kg)	19.47	20.16	0.5	0.34
BCS	2.98	3.12	0.1	0.34
LBW (kg)	565.53	584.51	15.91	0.41
Milk yield (kg)	25.15	26.23	1.25	0.55
Milk fat (%)	4.72	4.27	0.14	0.03
Milk fat (kg)	1.17	1.11	0.05	0.34
Milk protein (%)	3.53	3.5	0.09	0.85
Milk protein (kg)	0.88	0.91	0.03	0.52
Milk lactose (%)	4.94	4.9	0.03	0.32
Milk casein (%)	2.75	2.74	0.07	0.90
MUN (mmol/L)	27.04	26	1.06	0.49
SCC (x 1000)	124.00	105	48.35	0.79
ECM (kg)	29.69	29.45	1.14	0.88
FCM (kg)	29.84	29.28	1.2	0.75
GFE	1.54	1.49	0.08	0.63
RFI(kg)	0.09	-0.09	0.25	0.67

Table 4. Milk fatty acid composition (g/100g) at d 42

	CTR	CAME	SEM	*p*-value
C14	10.52	11.48	0.32	0.04
C14-1c9	0.96	1.18	0.12	0.21
C16iso	0.23	0.22	0.02	0.71
C16	31.14	30.19	0.94	0.48
C16-1t9	0.07	0.08	0.00	0.09
C16-1c7	0.23	0.23	0.01	0.66
C16-1c9	1.65	1.85	0.12	0.24
C18	8.17	7.92	0.44	0.44
C18-1t6-8	0.30	0.34	0.01	0.04
C18-1t9	0.26	0.33	0.02	0.01
C18-1t10	0.50	0.72	0.05	0.00
C18-1t11	1.20	1.44	0.10	0.10
C18-1t12	0.51	0.71	0.04	0.00
C18-1c9	22.17	21.02	1.03	0.43
C18-1t15	0.24	0.35	0.02	0.00
C18-1c11	0.55	0.58	0.03	0.49
C18-1c12	0.41	0.53	0.04	0.02
C18-1c13	0.06	0.08	0.01	0.03

C18-1t16	0.31	0.43	0.03	0.00
C18-2t9t12	0.39	0.54	0.03	0.01
C18-2t11c15	0.07	0.10	0.01	0.06
C18-2cc	2.50	2.30	0.09	0.12
C20	0.16	0.22	0.01	0.00
C18-3n6	0.03	0.03	0.00	0.98
C20-1c9	0.14	0.22	0.01	0.00
C18-3n3	0.48	0.58	0.03	0.06
CLA9-11ct	0.68	0.89	0.06	0.03
C21	0.04	0.04	0.00	0.65
C20-2n6	0.04	0.05	0.00	0.25
CALA	0.03	0.03	0.00	0.84
C22	0.05	0.05	0.00	0.36
C20-3n6	0.12	0.11	0.01	0.87
C20-4n6	0.18	0.16	0.01	0.19
C23	0.03	0.03	0.00	0.83
C20-5n3	0.03	0.03	0.00	0.16
C24	0.04	0.04	0.01	0.72
C22-4n6	0.04	0.04	0.00	0.55
C22-5n3	0.07	0.06	0.00	0.26
SFA	65.31	64.42	1.11	0.57
UFA	34.69	35.58	1.11	0.57
PUFA	4.69	5.01	0.19	0.22
MUFA	30.00	30.56	1.01	0.69
PUFA n6	3.31	3.24	0.10	0.65
PUFA n3	0.70	0.88	0.05	0.02
n6/n3	4.84	3.86	0.27	0.01
trans 18-1 tot	3.34	4.34	0.25	0.01

Table 5. Profile of identified dimethyl acethals (DMA) in rumen fluid at d 42

	CTR	**CAME**	**SEM**	***p*-value**
DMA_C12	2.31	2.98	0.26	0.07
DMA_C13	2.66	2.72	0.17	0.81
DMA_C14	13.25	15.44	1.18	0.19
DMA_C14iso	9.58	9.79	0.91	0.87
DMAC15ante	19.53	21.51	1.24	0.26
DMA_C15	10.85	11.97	0.90	0.38
DMA_C16	69.28	74.28	5.87	0.54
DMA_C15iso	8.84	9.24	0.75	0.71
DMA_C18	6.17	6.66	0.84	0.68

DMA_C18-1t11	3.15	3.44	0.32	0.53
DMA_C18-1c9	15.61	18.02	1.97	0.39
DMA_C18-1c11	8.42	10.78	0.86	0.06
DMA_C18-1c12	4.97	4.90	0.57	0.93

Table 6. Fatty acid profile of rumen fluid at d 42

	CTR	**CAME**	**SEM**	***p*-value**
C10	2.17	2.14	0.63	0.97
C12	19.09	16.33	4.10	0.63
C13iso	3.74	3.90	0.50	0.82
C13	2.56	2.52	0.30	0.92
C14iso	9.02	7.54	0.88	0.24
C14ante	71.42	46.86	9.08	0.06
C13-1c12	4.18	5.67	0.65	0.11
C14	34.20	40.89	4.82	0.33
C15iso	24.89	24.12	1.72	0.75
C14-1t9	178.30	141.61	12.22	0.04
C15ante	56.85	58.33	2.81	0.71
C14-1c9	4.47	5.07	0.89	0.63
C15	47.26	47.13	3.26	0.98
C16iso	18.40	16.42	2.24	0.53
C16ante	16.94	20.36	2.22	0.28
C16	650.99	661.44	48.24	0.88
C17iso	12.70	11.49	1.22	0.48
C16-1c7	1.77	2.38	0.23	0.07
C16-1c9	3.93	4.07	0.43	0.81
C17ante	21.68	19.98	2.15	0.57
C17	22.73	23.17	1.82	0.86
C18iso	1.46	1.31	0.27	0.69
C18	1828.67	1862.77	203.63	0.90
C18-1t5	3.10	3.50	0.47	0.54
C18-1t6-8	26.24	31.41	3.12	0.24
C18-1t9	17.59	23.82	2.20	0.03
C18-1t10	41.82	64.93	5.39	0.01
C18-1t11	200.83	227.94	27.64	0.49
C18-1t12	41.82	62.75	5.57	0.01
C18-1c9	199.95	255.38	15.68	0.02
C18-1t15	32.03	54.04	5.77	0.01
C18-1c11	23.89	31.55	3.07	0.09
C18-1c12	32.41	42.85	4.08	0.08

C18-1c13	1.95	2.85	0.38	0.10
C18-1t16	35.92	58.73	5.65	0.01
C18-2t9t12	5.74	9.04	2.90	0.42
C18-2t11c15	7.79	9.32	1.68	0.52
C18-2cc	173.34	178.27	20.68	0.86
C20	31.45	55.04	5.38	0.00
C18-3n6	2.14	1.81	0.21	0.27
C20-1c11	3.89	10.61	1.18	0.00
C18-3n3	44.51	53.34	3.81	0.11
CLA9-11ct	3.03	2.78	1.04	0.86
C21	2.51	1.98	0.29	0.21
C18-4n3	13.00	14.29	1.65	0.58
CALA	4.09	3.60	0.46	0.45
C22	19.26	20.67	1.85	0.59
C23	9.14	8.61	0.84	0.65
C24	23.93	23.45	2.54	0.89

Chapter 3 |

Effects of the supplementation of *Saccharomyces cerevisiae* on milk performance, rumen pH and metabolic indicators of lactating dairy cows

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ABSTRACT

Subacute rumen acidosis (SARA) is a highly prevalent though underdiagnosed technopathy characterized by multiple moderate dropping of ruminal pH. Modifications in the ruminal environment can impair cow's digestive ability, with negative consequences on milk performance, health status and feed efficiency. *Saccharomyces cerevisiae* is a yeast species reported to stabilize ruminal pH values and improve fibrolytic activity of the microbial populations, sustaining the high milk productions of dairy cows. The aim of this study was to investigate the effects of supplementing a commercial strain of *S. cerevisiae* on the ruminal environment, productive performance and blood parameters of dairy cows in early-to-mid lactation. Thirty-six primiparous and multiparous Holstein-Friesian dairy cows housed at the Experimental Station (Centro Zootecnico Didattico Sperimentale) of the University of Milan in Lodi were divided in two groups homogeneous for milk production, parity and DIM. Diets consisted of a TMR for high-producing lactating cows, supplemented with 0.13% *S. cerevisiae* on wet basis (YEAST) or 0.13% sodium bicarbonate on wet basis (CTR). Performances were recorded weekly. Six cows per group received an indwelling wireless pH-meter for continuous measurement of ruminal pH. Blood samples were collected on d 0, 30, 60, 90 for blood gas and biochemical analysis, together with non-invasive biomarkers for pH measurement, and ruminal fluid, on which the analysis of VFA and microbiota will be performed. Ultrasonography of the ruminal mucosa was also performed. Data were analyzed by MIXED procedure by SAS. No difference was detected for productive performance, DMI and body condition. YEAST group had lower hematic concentration of total proteins and globulins compared to CTR ($p < 0.05$). No effect of *S. cerevisiae* was detected on ruminal pH, though a significant effect of *S. cerevisiae* per silage quality was observed ($p < 0.05$), with higher pH in YEAST compared to CTR when fed low-quality forage. Results seems to endorse an alleged ameliorative effect of *S. cerevisiae*, manifesting more intensely under harsh ruminal conditions.

INTRODUCTION

Saccharomyces cerevisiae is a microbial feed additive composed of highly concentrated viable yeast cells. It has been designed to improve the performance of a functioning ruminant by modulating rumen function and the activities of its microflora.

A consistent body of literature supports that supplementing live yeast has beneficial effects in ruminants, going from the improvement of growth performance (Leismeister et al., 2004), to the development of functional forestomach (Turney et al., 2017), to an improvement of milk production and feed efficiency (De Ondarza et al., 2010).

The effects and modes of action of *Saccharomyces cerevisiae* CNCM I-1077 on the ruminal environment have been extensively studied over the last 15 years. The main effects of live yeast include i) the stabilization of ruminal pH, which is also demonstrated to be causally linked to the interactions with lactate-metabolizing bacteria (Marden et al., 2008); ii) the maintenance of the anaerobic rumen environment (Newbold et al., 1996), favoring growth and enzymatic activity of the cellulosolytic microbial populations; and iii) the improvement of rumen maturity, which is demonstrated by *S. cerevisiae* CNCM I-1077's action in favoring microbial establishment in pre-ruminants (Chaucheyras-Durand et al., 2008).

Knowing that the high-energy diets fed to modern cows to increase milk performance may pose a risk of excessive ruminal fermentations, rumen management tools such as the diet physical structure (limited sorting, optimized chewing) and feed additives are part of the currently available solutions. Though subacute rumen acidosis (SARA) may represent a latent metabolic issue with well-recognized negative economic and health consequences, its detection in farm remains subtle.

Besides rumen pH, non-invasive parameters have recently been assessed to predict the occurrence of this disease, by investigating several biological matrices: milk, saliva, urine and faeces.

Supplementing *S. cerevisiae* to lactating cows may result in a more stable ruminal environment, favoring an improvement of organic matter ruminal degradation, in turn leading to higher milk production and feed efficiency (De Ondarza et al, 2010).

MATERIALS AND METHODS

The trial was performed at the Animal Production Research and Teaching Centre of the Polo Veterinario (CZDS), Università degli Studi di Milano (Lodi, Italy). The trial started at the end of January 2019 and lasted for a period of 90 days. Due to the lack of animals meeting the requirements of uniformity in terms of production performance, parity and days in milk, two batches of cows were used in the trial. A first batch (n = 26) started the trial in January 2019; a second one (n = 10) started in March 2019. All cows remained under evaluation for a period of 90 days, prolonging the trial until June 2019. Animals used in the study were treated in accordance with the European Commission recommendation 2007/526/EC and 2010/63/UE on revised guidelines for the accommodation and care of animals used for experimentation and other scientific purposes. All the experimental procedures included in the trial were reviewed and approved by the Animal Care and Use Committee of the University of Milan.

A total of 36 healthy Holstein-Friesian dairy cows between 30 and 150 days in milk, deriving from the same commercial herd, were housed in the free-stall facility of the CZDS. All animals enrolled have previously had been genotyped by the Italian Holstein-Friestian and Jersey Breeder Association (ANAFIJ). Cows were allocated at random into two experimental groups (n = 18) stratified for days in milk (DIM),

parity, and previous production performance. Groups received the same basal diet, in form of total mixed ration (TMR). Basal diet formulation was as much as possible constant across the trial period, with a major change in April 2019 due to the exhaustion of silage under usage (Tables 1 and 2). Basal diet was supplemented with two different premixes: the control group (CTR) diet was added 0.13% on wet basis of sodium bicarbonate; the treatment group (YEAST) diet was added 0.13% on wet basis of live yeast premix (minimum of 10^109 CFU/head/d). Diets were formulated to be isonitrogenous and isoenergetic, and to meet the nutritional requirements for early-mid lactation high-performance cow, as reported by the NRC (2001).

Before the start of the trial, cows were subjected to a seven days period of adaptation to the Insentec RIC feeding system.

Diet samples

On d 0, 30, 60, 90 representative samples of the rations were collected to determine the nutrient profile of the diets. Multiple grabs of the TMR in front of the cows was composited in a final sample (500 g), vacuum-packed and stored at -20 °C before analysis.

Feed samples were dried in a 60 °C forced air oven for at least 48 hours and analyzed for DM (procedure 4.1.06; AOAC, 2000). Samples were ground to 1 mm particle size in a Wiley mill and used for chemical analysis on analytical composition.

With the same time schedule, the main forages included in the diet were sampled. Silage samples were collected by mixing and compositing 10 sub-samples collected coring the fresh front of the silage trench on evenly distributed heights. Final representative samples (500 g) were delivered to Rock River Laboratory Europe (Heiddorf, Germany) for the evaluation of NDF digestibility at 24h or 30h, 120h, 240h (%) and kd $CHOB_3$ (fiber fraction).

Simultaneously, samples of Saccharomyces cerevisiae premix were collected and sent to an external laboratory for the quantification of colony forming unit (CFU/g).

Milk samples and analysis

Individual milk samples from two consecutive milkings (morning and evening) were collected every 15 days (d 0, 15, 30, 45, 60, 75, 90) preserved with sodium azyde and clustered in individual 100 mL composite samples representative of the 24-hour milk production. Composite samples were refrigerated at 4 °C until analysis of chemical composition and somatic cell count (SCC). Fat, protein, lactose, urea, casein, total saturated-, total unsaturated-, monounsaturated- and polyunsaturated fatty acids (SFA, UFA, MUFA and PUFA respectively) content was assessed by Fourier transform infrared (FTIR) spectroscopy. Somatic cell count (SCC) was performed using an automatic cell reader (Bactoscan 8000, FOSS Electronic).

On d 0, 30, 60, 90 additional individual milk aliquots were collected and freezed at -80 °C until analysis of the FA composition by gas chromatography.

Blood sampling and analysis

At the start of the trial and at the beginning of each month (d 0, 30, 60, 90) two aliquots of individual blood samples were collected from the coccygeal vein into evacuated collection tubes containing serum separator gel (BD Vacutainer® SST), K_2EDTA (BD Vacutainer® EDTA) or lithium heparin (BD Vacutainer® heparin) and placed in a refrigerated bag. Blood samples were collected before the morning meal, closing the feed gates and negating access to the bins from 3 hours before sampling.

The first aliquot of blood was readily used as such (whole blood) for blood gas and electrolyte analysis using a portable haemogasanalyser (OPTI CCA-TS).

The second aliquot of blood was centrifuged, with harvested sera and plasma were transferred into 1 mL microvials and stored at -20 °C, until quantification of glucose, β-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), urea, total cholesterol, triglycerides, aspartate transaminase (AST), γ-glutamyl transferase (GGT), bilirubin, creatin-kinase (CK), total protein, albumin, globulins, calcium, phosphorus and magnesium via liquid gas chromatography.

Rumen fluid sampling and analysis

Rumen content samples were collected on d 0, 30, 60, 90 using an oro-ruminal probe (Ruminator, Profs-Products). The metallic probe was inserted through the oral cavity and moved forward until reaching the caudal ventral blind sac of the rumen. A suction pump was connected and used to collect the fluid into the sterilized glass case that completed the set. After removing the first 100 mL of fluid in order to avoid saliva contamination, samples were transferred into sterile tubes (Falcon™ 50mL Conical Centrifuge Tube) in triplicates and freezed at -80 °C until being analyzed.

Rumen fluid samples were thawed and prepared via transesterification (FAME) (Kramer et al., 1997). Fatty acid methyl esters (FAME) were identified and analyzed by gas chromatography, using a GC 8000 TOP gas chromatograph (ThermoFisher Scientific Inc., Milan, Italy) equipped with a flame-ionization detector (GC-FID) and a WCOT CP-Select capillary column (100 m; 0.25 mm i.d.; 0.25 μm film thickness; Chrompack, Middelburg, the Netherlands).

Non-invasive matrices

On d 0, 28, 56 individual samples were collected from a series of non-invasive biological matrices: urine, feces, saliva and cerumen.

Urine samples were collected by spontaneous micturition and transferred into 15 ml sterile collection tubes. Sterile rectal swabs were used and stored in transport

medium pending metagenomic analysis, while faeces aseptically collected from the rectal ampulla were stored in sterile tubes (Falcon™ 50mL Conical Centrifuge Tube). All fecal samples were readily freezed at -80 °C. Disposable spatulas were used to collect cerumen samples for lipidomic analysis and stored at -20 °C. Saliva samples for urea dosage were collected by making each heifer chew a saliva sampling sponge. Sponges were centrifuged and harvested saliva was transferred into 1 mL microvials stored at -20 °C.

All samples were collected in duplicates, with one aliquot per sample type used straight for pH measurement, using a digital pH meter with combination electrode (HI2002-02 edge, Hanna Instruments).

Measurements and recorded parameters

Daily recording of milking data was not possible due to a malfunction of the milking parlor, thus data from two consecutive milkings were recorded manually on a weekly basis (d 0, 7, 14, 21, 28, 35, 42).

Individual daily FI values were gathered processing the raw data recorded in real time by the software connected to the automatic feeders (Insentec RIC System). The RIC system was used to collect data for feeding behaviour evaluation, in terms of number of accesses at the bin per day, average intake per access, time spent at the bin per access, time spent at the bin per day. Data on feeding behaviour are currently under evaluation.

Starting from d 0 and on a weekly basis individual LBW was measured – before the ration was delivered – with an electronic scale located outside the facility. Individual BCS were determined as the mean of the evaluation sites on a 1 (very thin) to 5 (very fat) point scale with 0.25-point resolution.

Before the start of the trial, a sub-group of both CTR and CAME groups (n = 6/group) – selected and stratified as previously reported – received a ruminal sensor bolus for monitoring daily rumen pH variations *in continuum*. Boluses were placed in the reticolo-rumen of each animal via oesophagus, using a bolus gun. Data were recorded using the indwelling data transmitting system associated with boluses (smaXtec Animal Care GmbH, Grax, Austria). Parameters evaluated were total time spent below the physiological ranges, number of times below the thresholds values, minimum and the maximum daily pH values.

On days 0, 30, 60, 90 changes in rumen mucosa thickness per animal was evaluated via transabdominal ultrasonographic examination. After clipping and cleansing animals with alcohol, the ultrasound probe (linear probe with 5 MHz frequency) was positioned on the examination window placed on the intercept of the lines from the third lumbar vertebra and the costal bone cartilage (Mirmazhari-Anwar et al., 2013). Rumen mucosa thickness was calculated as the mean of three measurements obtained using the distance measurement function of the ultrasonographic scan.

Fecal consistency was evaluated by means of a 1 (liquid) to 5 (dry and segmented) point scale with a 1-point resolution.

Environmental parameters (moisture and temperature) were monitored using two HOBO change-of-state data logger (Onset Computer Corp., Bourne, MA) with a sampling time of 48 detections/24 hours period.

All animals were subjected to a daily veterinarian clinical examination in order to check health status. Individual morbidity rate was recorded in terms of clinical signs, diagnosis, clinical assays, therapeutic treatments adopted, illness duration (days), and health recovery. Moreover, all given medications including vaccines, de-wormers and

metaphylactic treatments, specifying administered dosages, were recorded. Mortality rate was also recorded by day and cause.

Calculations and statistics

Gross feed efficiency (GFE) was calculated as individual energy-corrected milk divided by individual DMI (milk to feed ratio). Energy-corrected milk (**ECM**) was calculated according to the NRC (2001), following the formula:

$$ECM_{(kg)} = 0.327 \times MY_{(kg)} + 12.95 \times FC_{(kg)} + 7.2 \times PC_{(kg)},$$

where MY was the daily milk yield, FC was the milk fat content, PC was the milk protein content, LC was the milk lactose content.

Fat corrected milk (FCM) was expressed as 3.5% fat-corrected, according to the equation reported by Parekh et al. (1986):

$$FCM_{(3.5\%;\ kg)} = 0.35 \times MY_{(kg)} + 18.57 \times FC_{(kg)}.$$

Residual feed intake (**RFI**) per cow was estimated applying a model of regression of DMI on the main energy sinks of lactating cows. The effect of diet was also included in the equation as follow:

$$DMI_i = \beta_0 + \beta_1(MilkE_i) + \beta_2(MBW_i) + \beta_3(\Delta BodyE_i) + Diet + \varepsilon_i,$$

where DMI_i was the observed individual DMI, $MilkE_i$ was the individual milk energy output, MBW_i was the mid-test individual metabolic body weight, ΔbodyE was the estimated change in body energy. RFI was defined as the error term of the model (ε_i).

Milk energy output (**MilkE**) was calculated by following the equation reported by the NRC (2001):

$$MilkE(Mcal/d) = 9.29 \times FC_{(kg)} + 5.63 \times PC_{(kg)} + 3.95 \times LC_{(kg)},$$

where FC, PC and LC were expressed as the daily mean value for the whole trial period.

Estimated change in body energy (**ΔbodyE**) was obtained by modifying the equation of the NRC (2001):

$$\Delta bodyE = (2.88 + 1.036 \times BCS) \times ADG,$$

where BCS is the average individual BCS during the whole trial period, and ADG is the individual average daily gain.

Statistical analysis of calculated parameters and sample data was performed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC v.9.4 2015). Cow was the experimental unit for all the accounted parameters. The model included diet, time and diet nested with time as the main effects. Partially missing covariate data were corrected with the Kenward-Roger approximation method. Significance level was considered for $p < 0.05$ and $p < 0.01$.

RESULTS

No statistically significant difference between the groups was observed for the main productive parameters investigated. Supplementation with *S. cerevisiae* did not affect DMI, that recorded similar values for YEAST and CTR groups across the trial period (Figure 1). In the same way, no significant difference was detected for milk

production and composition, BCS and FE, expressed as both GFE (milk to feed ratio) and RFI (Table 3).

Calculation for RFI resulted in the following equation

$$DMI = -0.486 + 2.088ADG + 0.098MBW - 0.173Diet + RFI$$

Fresh whole blood samples subjected to hemogasanalysis did not evidence difference between groups for pH, pCO_2 and bicarbonate concentrations. A significant time per diet effect was observed for body temperature measured prior to sampling. Compared to CTR group, YEAST group displayed a relevant increase in temperature from d 0 to d 30 (37.95 to 38.24 °C) compared to CTR group that showed more stable temperature value (from 38.16 to 38.13 °C) ($p = 0.04$) (Table 4).

Biochemical profile of plasma and sera showed moderate differences between groups. A significant effect of the dietary treatment was observed on serum total proteins ($p = 0.035$) and globulins ($p = 0.009$), with lower levels in the YEAST group (72.43 and 35.56 g/L respectively) compared to CTR (75.45 and 39.13 g/L respectively). The effect of diet on total proteins was particularly evident on d 56 ($p = 0.017$), when the difference between YEAST and CTR values was of 7.94 (66.28 vs 74.22 g/L; $p = 0.017$) for total proteins and of 6.55 (30.82 vs 37.37 g/L; $p = 0.005$) for globulins. Results illustrated in figures 2a and 2b.

Measurement of pH values of the biological matrices investigated (feces, urine, saliva) revealed an absence of effect of dietary treatment at all timepoints (Table 5). In continuum indwelling measurement of ruminal pH revealed similar pH trend between the two groups. A significant but isolated diet per time effect was observed at d 109, when YEAST had a significant increase at average values above 6.8 (Figure 3). When the effect of the change in the silage used in the diet (April 2019) was

included in the statistical model, a significant effect on ruminal pH value was observed, with the ryegrass-based diet significantly associated to lower pH ($p < 0.05$) (Figure 4). When the effect of the change of silage was nested with the effect of dietary treatment, YEAST group fed the ryegrass silage-based diet displayed overall higher pH values ($p < 0.05$) compared to CTR fed ryegrass silage (Figure 5). Such a significance was not observed when cows were fed the sorghum-based diet.

Dietary treatment did not influence the ruminal mucosa thickness on a significant level. A parallel pattern through time was observed between values of the two groups, with YEAST group displaying constant higher (but not significantly) values for all the experimental period (Figure 6).

DISCUSSION

In our study, the supplementation of *Saccharomyces cerevisiae* in the diet did not affect productive parameters, nor rumen environment in a measurable way. The absence of significant effects on productions was expected, as in line with the results obtained from other authors. Reported effects of S. cerevisiae on milk production and quality are quite inconsistent and often conflicting.

The analysis of blood gas conducted on fresh blood did not result in any significant data. Blood pH values were overlapping in all cows. It must be addressed that circulating blood pH is an extremely strict and finely regulated value, considering the severity of consequences deriving from its modification. Systemic involvement during acute metabolic acidosis – and in particular lactic acidosis – is reported in cattle, though it represents a separate aetiological entity from SARA. Acute ruminal acidosis is more frequently reported in feedlot cattle, as a consequence of severe grain overload causing a production of lactic acid exceeding the ruminal and hepatic capacity of metabolizing it (Hernández et al., 2014). During acute ruminal

acidosis, the reduction in ruminal pH below 4.5-5 can affect blood pH levels (systemic lactic/metabolic acidosis), in turn reducing circulating sodium bicarbonate levels during the buffering of circulating H^+ ions. Simultaneously, a short-term mechanism enacted by the organism to contrast the reduction in blood pH is the increase of the respiratory rate, so as to eliminate more carbon dioxide (CO_2), representing the other component of the sodium bicarbonate-carbon dioxide buffer system. Therefore, an alteration in blood CO_2 gas pressure (lower) and circulating bicarbonate (lower or higher when the organism is able to compensate) are expected only in systemic, severe state of metabolic acidosis. In animals affected by SARA, on the contrary, the moderate, fluctuant reduction in rumen pH is not capable of affecting blood pH, and the buffering activity is mainly deputed to salivary bicarbonates. Sodium bicarbonate concentration in ruminant saliva is significantly higher than that found in monogastric species, from which the markedly alkaline pH of ruminant salivary secretions. The similar values of salivary pH detected in the two groups of our study is likely due to the absence of a real SARA condition, that would cause a reduction in circulating free bicarbonate. Since our study design did not include a challenge of the ruminal environment by feeding a potentially acidogenic diet (Tun et al., 2020), the lack of effect of *S. cerevisiae* on investigated blood analytes is easily explainable. This is furtherly confirmed by the results of the indwelling pH measurement. In continuum recording of rumen content pH and temperature (advanced as an indirect indicator of SARA (Humer et al., 2015) showed similar pH values and daily trends, independently of dietary treatment. Moreover, the changes in rumen mucosa thickness observed for both groups in our study were moderate and not compatible with the parakeratotic lesions associated with SARA (Mirmazhari-Anwar et al., 2013). Therefore, it can be concluded that no episodes of SARA were recorded during the trial period, from which the lack of manifest effect of *S. cerevisiae* in YEAST group.

On the other hand the forced change in the diet formulation occurred in April 2019 could be seen as an unvoluntary challenge for the ruminal environment. The higher pH of YEAST animals compared to CTR when fed low-quality forage is in line with reports found in literature, that seems to underline how *S. cerevisiae* displays a more evident effect in a ruminal environment characterized by challenging conditions. In a study conducted on sheep, Jurkovich et al. (2014) observed a more pronounced ability of reducing ruminal pH when *S. cerevisiae* manifested more pronounced ability of reducing ruminal pH when *S. cerevisiae* was supplemented in animals fed a high roughage-to-concentrate ratio (40:60) compared to a more balanced ratio (49:51). Zhu et al. (2017) evaluated the effect of *S. cerevisiae* fermentation products (SCFP) on ruminal parameters of dairy cows fed low-quality forages. Authors evidenced a significant effect of SCFP on the ruminal microbial composition (increase of the cellulosolytic bacteria and fungine populations) in cows fed low-quality forages but not in those fed a diet of greater quality. Moreover, the shift in microbial composition – translated in better ability to digest the organic matter – seemed to be more marked under environmental conditions favouring heat stress (high temperature-humidity index).

Another challenge for ruminal environment – and by consequence a potential application of *S. cerevisiae* supplementation – is the weaning period, during which the development of the forestomach and the microbial colonization take place. Supplementing *S. cerevisiae* to neonatal calves has been reported to determine a significant increase in DMI and ADG, together with a slight improvement in rumen mucosa development (Lesmeister et al., 2004). In line with these findings are Terré et al. (2015), that reported greater DMI and ADG, higher rumen pH and greater *Ruminococcus albus* abundance in freshly weaned calves. Chaucheyras-Durand et al. (2020) observed greater proliferation of *Fibrobacter succinogenes* (a cellulosolytic

bacteria) and greater gene expression for hemicellulase in lambs fed *S. cerevisiae* during the weaning period.

The indwelling pH measurement allowed to detect a significant, self-limiting increase of the ruminal pH of the YEAST group around d 109. At present, such a result is difficult to read. Further analysis of correlation with the data at our disposal (environmental temperature) or currently under analysis (ruminal FA profile and microbiota) may help to clear this finding.

The biochemical profiling of YEAST and CTR cows resulted in no differences other than serum concentration of total protein and globulin. Reference intervals for total protein (67.54 ± 11.53 g/l), albumin (31.86 ± 4.60 g/l), α(1)-globulin (5.77 ± 2.20 g/l), α(2)-globulin (5.84 ± 1.90 g/l), β-globulin (7.46 ± 1.94 g/l), and γ-globulin (16.73 ± 4.54 g/l) concentrations as well as for albumin/globulin (A/G) ratio (0.88 ± 0.43) in healthy dairy cows were reported by an Italian research group (Alberghina et al., 2011). Values obtained by liquid chromatography for total protein and globulin of both YEAST and CTR fall within the physiological reference intervals for dairy cows. Variations in serum total proteins and globulins have been found to be positively correlated with the age of cows (Bobbo et al., 2017). Total proteins tend to increase through lactation, reaching a peak value at the fourth month in milk (Bobbo et al., 2017). Interestingly, the same authors found a linear relationship between total proteins and somatic cell count. Such a correlation has not been observed in the present study, though sample size may have affected the accuracy of the correlation analysis. Recent findings suggest that total proteins, globulins and albumin to globulin ratio are traits moderately heritable (≥ 0.20), and evidenced a strong negative genetic correlation between blood β-hydroxybutyrate (BHB) and globulins (-0.70) (Cecchinato et al., 2018). Despite in our study CAME group had lower serum globulins, no difference in BHB, nor in other indicators of energy balance was

observed. A personal interpretation of the results is that lower levels of serum globulins may be put in relation with the alleged immunomodulatory effect of *S. cerevisiae*. Bach et al. (2018) analyzed the gene expression of selected genes involved in the immune response after the bioptical collection of ruminal and colon epithelium samples of dairy cows supplemented or not supplemented *S. cerevisiae*. Identifying treatment-related differences in the expression – among others – of TLR4 (PRR receptors involved in innate immunity), IL1B (pyrogenic, pro-inflammatory cytokine), IL10 (anti-inflammatory cytokine), DEFB1 (secretion of antimicrobial peptides). Knoblock et al. (2019) recorded lower levels of haptoglobin (a positive acute phase protein of the α-globulin fraction) in cows supplemented SCFP. *Saccharomyces cerevisiae* fermentation products were able to contain the inflammatory response of cows challenged with acidogenic diets, as testified by their lower serum TNF-α levels (Li et al., 2016).

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TABLES AND FIGURES

Table 1. Ingredients and nutrient composition of the 1st diet (sorghum; Jan-Apr 2019)

(% as fed)	
Corn silage	25.00
Sorghum silage	9.00
Corn meal	5.00
Soybean meal	4.00
Sodium bicarbonate	0.20
Calcium carbonate	0.15
Salt	0.07
Mineral-vitamin premix	0.05
Calculated composition (% DM)	
Moisture	54.44
Crude protein	15.17
Ether Extract	2.81
NDF	33.80
peNDF	24.37
NFC	41.20
Starch	28.65
$NEL_{(mCal/kg)}$	
Calcium	0.58
Phosphorus	0.34

Table 2. Ingredients and nutrient composition of the 2nd diet (ryegrass; Apr.-Jun 2019)

(% as fed)	
Corn silage	27.00
Corn meal	5.20
Ryegrass silage	5.00
Soybean meal	4.50
Hydrogenated fat	0.35
Calcium carbonate	0.20
Sodium bicarbonate	0.15
Salt	0.10
Mineral-vitamin premix	0.05
Calculated composition (% DM)	
Moisture	48.94
Crude protein	17.20
Ether Extract	4.90
NDF	31.60
peNDF	25.10
NFC	41.40
Starch	30.80
$NEL_{(mCal/kg)}$	
Calcium	0.62
Phosphorus	0.36

Figure 1. Daily DMI for YEAST (T) and CTR (C) groups

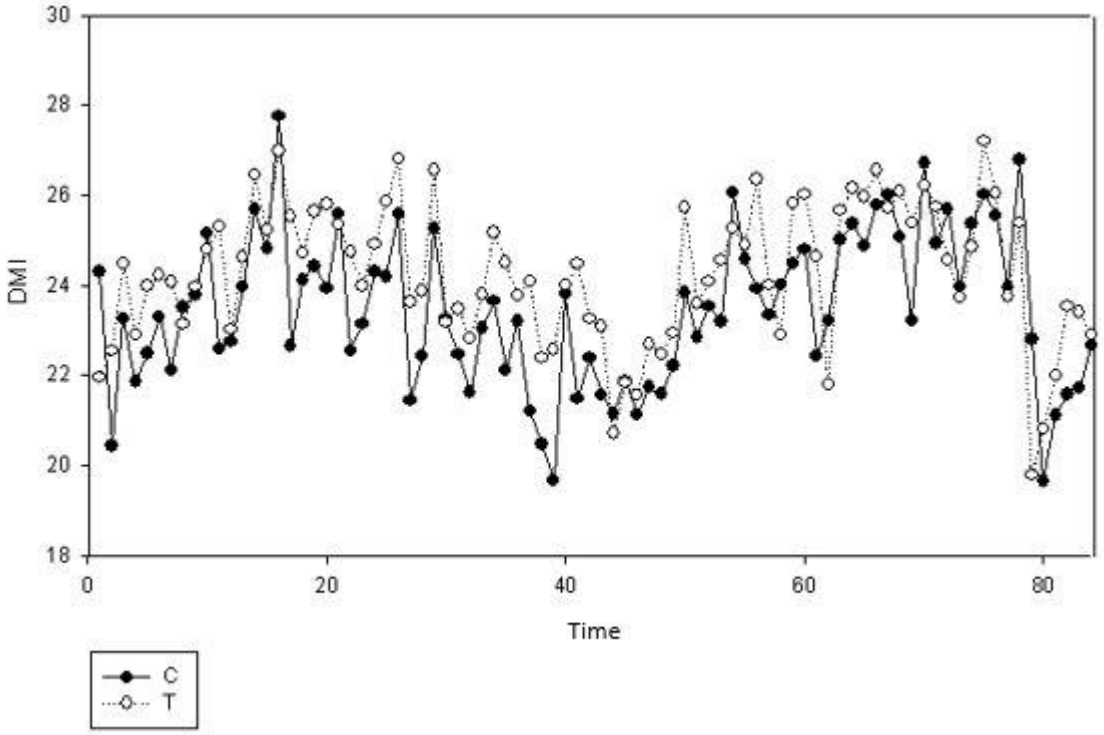


Table 3. Average values of production parameters

	CTR	CAME	SEM	*p-value*
DMI (kg)	27	29.38	1.31	0.21
BCS	3.06	3.07	0.05	0.85
LBW (kg)	577.2	602.11	16.65	0.3
ECM (kg)	30.15	32.85	1.41	0.18
FCM (kg)	28.03	30.81	1.4	0.17
GFE	24.98	25.13	1.23	0.69
RFI(kg)	0.14	0.11	0.32	0.21
Milk yield (kg)	27.00	29.30	1.31	0.20
Milk fat (%)	4.3	4.38	0.12	0.65
Milk fat (kg)	1.15	1.27	0.06	0.17
Milk protein (%)	3.49	3.37	0.06	0.2
Milk protein (kg)	0.93	0.98	0.04	0.38
Milk lactose (%)	5.05	5.1	0.03	0.29
Milk casein (%)	2.73	2.64	0.05	0.23
MUN (mmol/L)	23.82	24.18	0.66	0.7
SCC (x 1000)	82.34	34.26	39.85	0.4
SFA (g/100g)	2.95	2.98	0.09	0.83
UFA	0.98	1	0.03	0.39
MUFA	0.9	0.9	0.02	0.87
PUFA	0.11	0.17	0.08	0.49

Table 4. Blood gas analysis on fresh blood samples

Parameters	Time	Diet		*SEM*	*p*-value			
		CTR	YEAST	SEM	*Diet*	*Time*	*DxT*	*P Diff*
Temperature					0.82	0.003	0.04	
	0	38.16	37.95	0.09				0.08
	30	38.13	38.24	0.09				0.40
	60	38.39	38.27	0.09				0.33
	90	38.18	38.35	0.09				0.19
pH					0.23	0.016	0.75	
	0	7.48	7.48	0.01				0.95
	30	7.48	7.49	0.01				0.78
	60	7.46	7.47	0.01				0.44
	90	7.44	7.47	0.01				0.15
pCO_2					0.06	0.15	0.92	
	0	42.93	41.6	1.42				0.51
	30	43.88	42.21	1.42				0.41
	60	46.6	43.97	1.42				0.19
	90	45.27	42.38	1.42				0.15
HCO_3^-					0.11	0.008	0.94	
	0	31.37	30.33	0.6				0.26
	30	32.02	30.99	0.6				0.23
	60	32.23	31.26	0.6				0.26
	90	30.24	29.77	0.6				0.59

* DxT = Diet x Time

Figure 2. Mean serum concentrations of total proteins (a) and globulins (b) in YEAST (T) and CTR (C)

(* $p < 0.05$)

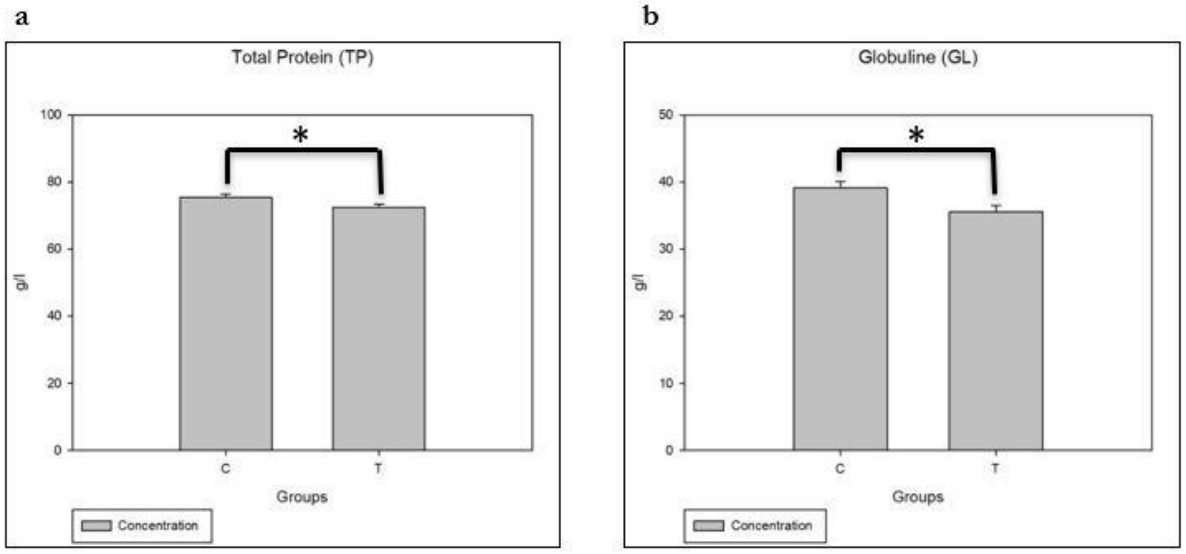


Table 5. pH measured on non-invasive matrices

	Time	Diet			*p*-value			
		CTR	YEAST	SEM	*Diet*	*Time*	*DxT*	*p Diff*
Feces					0.47	<0001	0.7	
	1	6.48	6.42	0.05				0.46
	2	6.41	6.33	0.05				0.29
	3	6.60	6.57	0.05				0.77
	4	6.31	6.34	0.05				0.67
Urine					0.68	<0001	0.38	
	1	8.26	8.32	0.03				0.20
	2	8.17	8.17	0.03				0.98
	3	8.33	8.29	0.03				0.39
	4	8.21	8.25	0.03				0.55
Saliva					0.84	0.94	0.51	
	1	8.38	8.41	0.05				0.64
	2	8.42	8.41	0.05				0.89
	3	8.44	8.35	0.05				0.19
	4	8.40	8.44	0.05				0.54

* DxT = Diet x Time

Figure 3. Fluctuations across the trial of the ruminal pH in YEAST (T) and CTL (C) (* $p < 0.05$)

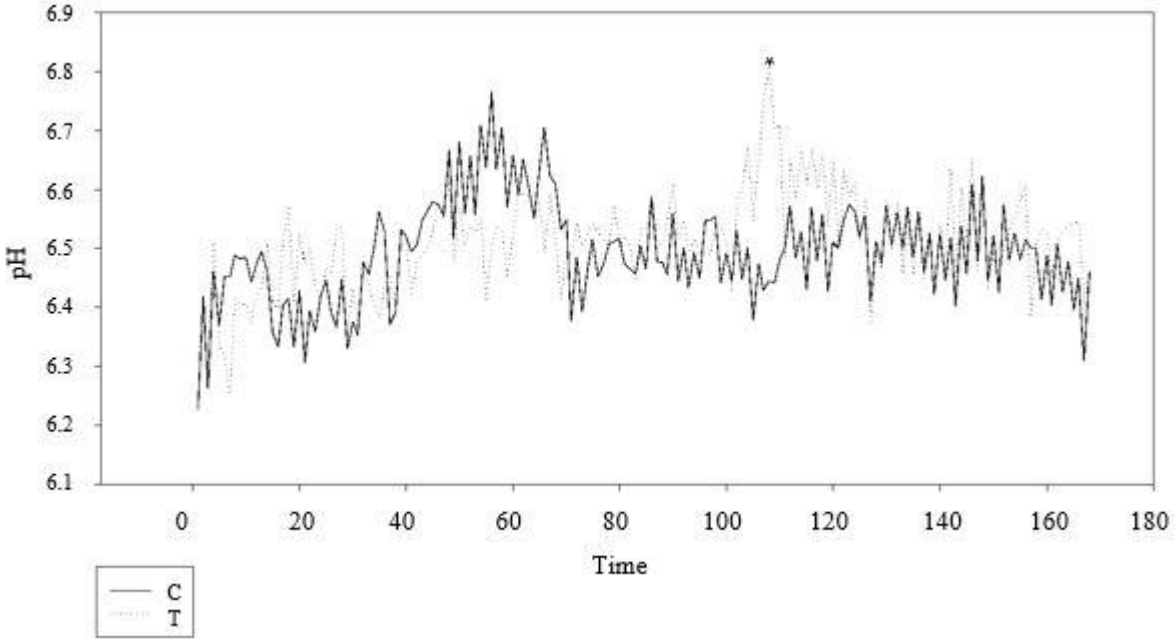


Figure 4. Mean rumen pH values of animals fed ryegrass (in grey) and sorghum (in black) silage (* $p < 0.05$)

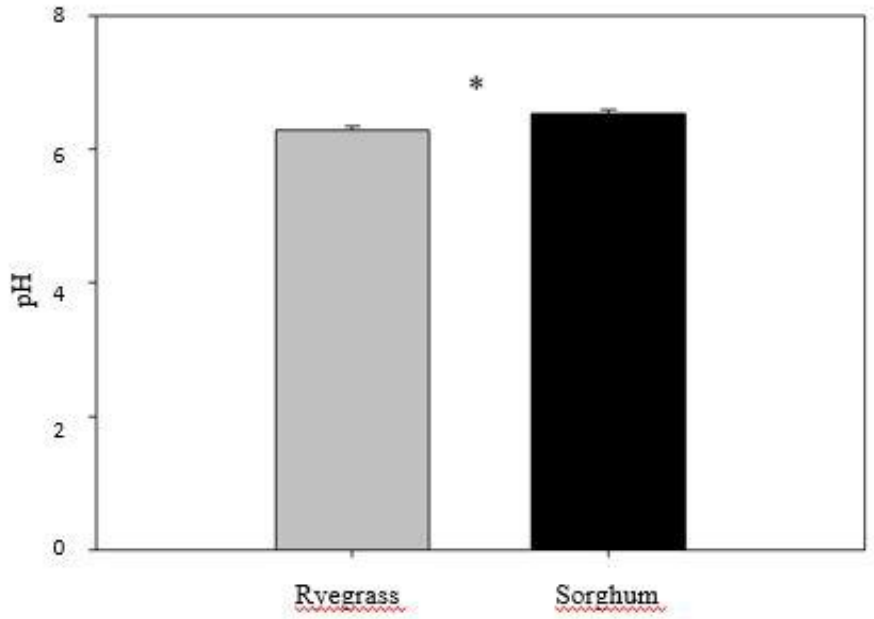


Figure 5. Rumen pH of YEAST (in black) and CTR (in grey) fed ryegrass (left) or sorghum (right) (* $p < 0.05$)

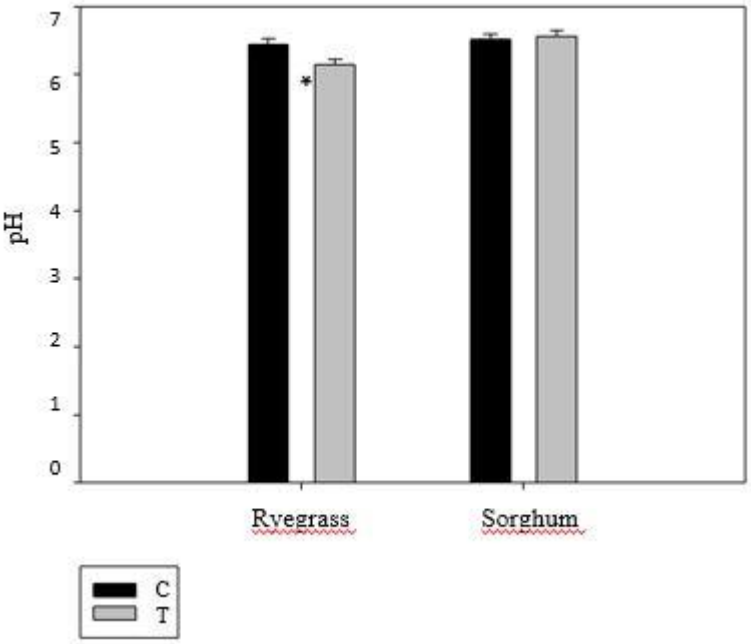
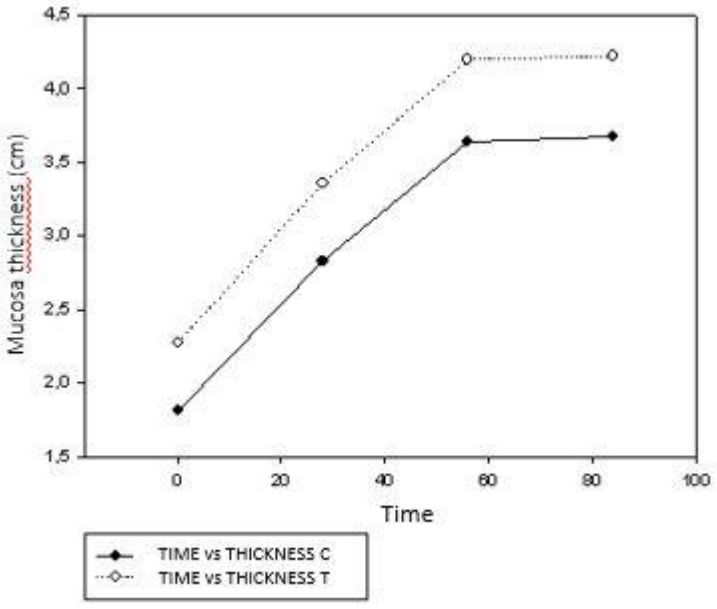


Figure 6. Trend of ruminal mucosa thickness in YEAST (T) and CTL (C)



Chapter 4 |

Impact of *Camelina sativa* on rumen microbiota and gene expression of follicular cells in Italian Holstein-Friesian heifers

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ABSTRACT

Omega-3 PUFAs have unique role in several biological systems in mammals, notably reproductive activity and inflammatory status. Moreover, their impact on rumen microbial environment could represent a critical point for both rumen welfare and their bioavailability. The aim of the study was to evaluate the effects of the inclusion in the diet of a terrestrial vegetable and sustainable source of omega-3 PUFAs (*Camelina sativa* cake) on rumen microbiota and gene expression of fertility markers in follicular cells of dairy heifers. The trial was conducted at the tie-stall dairy barn of the Experimental Farm of Animal Production Research and Teaching Centre of Lodi (CZDS), University of Milan, Lodi, Italy, and lasted 56 days. Sixteen Italian Holstein-Friesian dairy heifers 17 months old were divided in two homogenous groups randomly allocated to 2 treatments: CAME (n=8) receiving the basal diet supplemented with 800 g/head/day of camelina cake and CTR (n=8) receiving the basal diet supplemented with an isonitrogenous and isoenergetic soybean-based premix. Basal diet consisted of a dry TMR composed by alfalfa hay, durum wheat middlings, sugar cane molasses, corn meal, soybean hulls, rice bran, sunflower meal and minerals (15.15 CP, 42.63 NDF, 19.43 starch on DM basis). Performances were recorded weekly, feed intake daily. Rumen and follicular content samples were collected at day 0, 28 and 56 of the trial. Performance data were analyzed by PROC MIXED of SAS for repeated measures. No differences were detected for live body weight, RFI, FCR and BCS, but there was a significant diet x time interaction effect ($p < 0.05$) on DMI with higher values for CAME at day 34 and 55 compared with CTR (14.44 kg vs. 10.47 kg; 13.99 kg vs. 11.15 kg). Preliminary data on the mRNA expression of genes related to follicular development and maturation seem to support a positive effect of CAME compared to CTR, with higher expression levels of selected molecular markers. Rumen microbiota was influenced by dietary treatment both at 28 and 56 days, showing significant alpha diversity values.

INTRODUCTION

While the success of modern breeding programs in increasing milk production is undeniable (VandeHaar & St.Pierre, 2006; Borawski et al., 2020), a worrisome decrease in the reproductive performance of dairy cows have been reported through the last few decades. This trend – transversally affecting all countries benefiting from high yielding dairy herds – is to be imputed to the widely accepted unfavorable correlated response of reproductive performance traits to selection for milk production (Walsh et al., 2011; Dobson et al., 2007). As a successful reproductive capacity is paramount for dairy industry profitability, the improvement of fertility plays a critical role in defining economic feed efficiency on a herd level (Miglior et al., 2017)

Negative energy balance (NEB) is classically considered one of the main factors affecting the reproductive performance of dairy cattle (Leroy et al., 2008). Cows facing NEB enact a complex homeorhetic response in order to better sustain the energy requirements of milk production, among which is the mobilization of the adipose tissue (Martens et al., 2020). As highly producing dairy cows are at higher risk of experimenting NEB in early lactation (de Vries & Veerkamp, 2000), the role of energy partitioning and efficiency in ensuring reproductive success seems to be endorsed.

Incorporation of lipids is generally added to dairy cattle diets and fed during the transition period to increase energy intake and contain lipid mobilization, while improving production and reproductive performance (Useni et al., 2018; Funston, 2004). Energy supplied through the inclusion of lipid in the diet of gestating cows have been proposed as a key source for oocyte maturation and embryo development (Ferguson et al 2006). However, supplementation of fat as an energy source during the transition period is a questionable practice, as it is frequently associated to

reduced DMI and DM digestibility, leading little to no improvement in the energy balance of animals (Santos et al., 2008). Nonetheless, the modulation of dietary FA has been reported to exert beneficial effects on animal reproductive capacity other than that offered by an increased energy intake (Staples et al., 1998, Williams & Amstaldem, 2010). The effects of FA supplementation (and in particular the modulation of the dietary n-3 to n-6 ratio) on reproductive performance of dairy cows – included the effect on oocyte gene expression and quality – have been largely investigated, though with discording results (Freret et al., 2019; Amini et al. 2016; Sharma et al. 2020; Petit et al 2008). However, little data are available on the effect of camelina cake as a source of PUFA on the cumulus-oocyte complexes (COCs) quality of growing dairy heifers.

Camelina sativa is an oilseed crop that is recently generating interest for the low economic and environmental impact of its cultivation and for its potential application in the field of industrial biofuel production. The nutritional profile of camelina seeds, characterized by high levels of protein and α-linolenic acid (an n-3 PUFA) make it particularly suitable for farm animal feeding. Camelina cake, a by-product of camelina oil, may represent a good candidate as a PUFAs source usable in animal nutrition for improving both the reproductive performance of cattle and the nutritional value of dairy products.

MATERIALS AND METHODS

The trial was performed at the Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy). The trial lasted for a period of 56 days (beginning of May 2019-beginning of July 2019). Animals used in this study were treated in accordance with the European Commission recommendation 2007/526/EC and 2010/63/UE on revised guidelines for the accommodation and care of animals used for experimentation and other scientific

purposes. All the experimental procedures included in the trial were reviewed and approved by the Animal Care and Use Committee of the University of Milan.

A total of 16 healthy Holstein-Friesian dairy heifers of the same age (17 ± 2.5 months) were selected from a commercial herd and moved to a tie-stall facility of the CZDS. All animals enrolled have previously had been genotyped by the Italian Holstein-Friesian and Jersey Breeder Association (ANAFIJ). Heifers were allocated at random into two experimental groups (n = 8) stratified for age, live body weight (LBW) and wither height. Groups were fed a basal dry total mixed ration with the addition of a soybean-based premix (CTR) o camelina cake, included at 3% as fed (CAME) (Table 1). Diets were formulated to be isoenergetic and isonitrogenous, and to meet the nutritional requirements for maintenance and growth of dairy heifers, as reported by the NRC (2001) (Table 2). All animals had ad libitum access to the diet, delivered once per day into individual feed bunks equipped with electronic scales. Heifers had also continuous access to water.

Diet samples

On d 0, 28, 56 representative samples of the rations were collected to determine the nutrient profile of the diets. Multiple grabs of the TMR were collected from each feed bunk and composited in a final sample (500 g), vacuum-packed and stored at -20 °C before analysis.

Feed samples were dried in a 60 °C forced air oven for at least 48 hours and analyzed for DM (procedure 4.1.06; AOAC, 2000). Samples were ground to 1 mm particle size in a Wiley mill and used for chemical analysis.

Ovum-pickup and COCs quality markers

On d 0, 28, 56 transvaginal ultrasound-guided follicles aspiration was performed for each heifer. In preparation for the collection, heifers received a synthetic

prostaglandin F2 alpha and equine chorionic gonadotropin treatment. Epidural anesthesia with 4 mL 2% lidocaine was administered prior follicle aspiration. A 5 MHz convex array probe attached to a needle guide containing a 60 cm sterile disposable needle was used to identify the ovary and transvaginally guide the needle into the follicle. The needle was connected to a 50 mL sterile tube placed in a 37 °C water warmer. Follicular fluid was aspirated and directly transferred to the sterile tube through an electronic suction system connected to needle line. After every sample collection the disposable needle was changed and the suction line rinsed with D-PBS solution.

RNA was isolated from COCs using the DynabeadsTM mRNA Purification Kit (ThermoFisher Scientific, USA), and subsequently quantified with NanoDrop 300 spectrophotometer (ThermoFisher Scientific, USA) according to the instructions of the manufacturer. First strand cDNA was synthesized using the SuperScriptTM III First-Strand Synthesis System for RT-PCR (ThermoFisher Scientific, USA) in accordance with the protocol of the manufacturer. After DNA binding with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Italy), quantitative real-time PCR (qRT-PCR) for gene expression analysis was carried out using CFX Connect RT-PCR Detection System (Bio-Rad Laboratories, Italy) with gene-specific primers for FSHR, LHCGR, HAS2 and GREM1. Gene expression levels were quantified using the 2ΔΔ-Ct method and normalized to the reference housekeeping genes GAPDH and ACTB.

Rumen fluid sampling and analysis

Rumen content samples were collected on d 0, 30, 60, 90 using an oro-ruminal probe (Ruminator, Profs-Products). The metallic probe was inserted through the oral cavity and moved forward until reaching the caudal ventral blind sac of the rumen. A suction pump was connected and used to collect the fluid into the sterilized glass case

that completed the set. After removing the first 100 mL of fluid in order to avoid saliva contamination, one aliquot was used immediately for pH measurement using a portable digital pH meter with a combination electrode (HI2002-02 edge, Hanna Instruments) while the remaining fluid was transferred into sterile tubes (Falcon™ 50mL Conical Centrifuge Tube) in triplicates and freezed at -80 °C until processing and metagenomic analysis.

DNA extraction was performed from 0.25 g of lyophilized rumen fluid samples, following a protocol previously reported (Yu and Morrison, 2004), and stored at -80 °C until use. DNA concentration and purity were assessed using a NanoDrop ND-1000 spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE, USA). Specific primers targeting the 16S rRNA V3-V4 hypervariable regions were used for bacterial DNA amplification, together with the Thermo Master Mix 2× (Life Technologies, Italia, Monza, MB, Italy). A first amplification step was performed in an Applied Biosystem 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA), as follows: denaturation at 98°C for 30 s; 25 cycles with a denaturing step at 98°C for 30 s, annealing at 56°C for 1 min and an extension at 72°C for 1 minute; final extension at 72°C for 7 minutes. Following Illumina 16S Metagenomic Sequencing Library Preparation protocol (https://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html), amplicons were cleaned up with Agencourt® AMPure® XP (Beckman, Coulter Brea, CA, USA), and checked for size with a 2100 Bioanalyzer Instruments (Agilent Technologies, Santa Clara, CA, USA). Libraries were prepared by a second PCR amplification step using the Nextera XT Index Kit (FC-131-1001 or FC-131-1002), following the previously reported protocol. Libraries were quantified by qRT-PCR with KAPA Library Quantification Kits (KapaBiosystems, Inc., Wilmington MA, USA), pooled in equimolar proportion and sequenced in one Miseq (Illumina) run with 300-base paired-end reads.

Demultiplexed paired-end reads from 16S rRNA gene sequencing were first checked for quality using FastQC for an initial assessment. Forward and reverse paired-end reads were joined into single reads using the C++ program SeqPrep. After joining, reads were filtered for quality based on: i) maximum three consecutive low-quality base calls (Phred < 19) allowed; ii) fraction of consecutive high-quality base calls (Phred > 19) in a read over total read length ≥ 0.75; iii) no "N"-labeled bases (missing/uncalled) allowed. Reads that did not match all the above criteria were filtered out. All remaining reads were combined in a single FASTA file for the identification and quantification of operational taxonomic units (OTUs). Reads were aligned against a reference 16S rRNA gene database (Greengenes, version 13.8), with 97% cluster identity, applying the CD-HIT clustering algorithm. A pre-defined taxonomy map of reference sequences to taxonomies was then used for taxonomic identification along the main taxa ranks down to the genus level (domain, phylum, class, order, family, genus). By counting the abundance of each OTU, the OTU table was created and then grouped at each phylogenetic level. OTUs with total counts lower than 10 in fewer than 2 samples were filtered out. All of the above steps, except the FastQC reads quality check, were performed with the QIIME open-source bioinformatics pipeline for microbiome analysis.

The rumen microbial diversity was assessed for alpha-diversity (variability level within samples) and for beta diversity (variability level across samples). Alpha and beta diversity were estimated from the complete OTU table, filtered for OTUs with more than 10 total counts distributed in at least two samples. Besides the number of observed OTUs directly counted from the OTU table, within-sample microbial richness and diversity were estimated using the following indices: Chao1 and ACE (Abundance-based Coverage Estimator) for richness, Shannon, Simpson and Fisher's alpha for diversity, Simpson E and Pielou's J (Shannon's evenness or equitability) for evenness. Beta diversity of the rumen microbiota was assessed using the non-metric

multidimensional scaling of the Bray-Curtis matrix. Prior to the calculation of Bray-Curtis dissimilarities, OTU counts were normalized for uneven sequencing depth by cumulative sum scaling (CSS).

Non-invasive matrices

On d 0, 28, 56 individual samples were collected from a series of non-invasive biological matrices: urine, feces, saliva and cerumen.

Urine samples were collected by spontaneous micturition and trasnferred into 15 ml sterile collection tubes. Sterile rectal swabs were used and stored in transport medium pending metagenomic analysis, while faeces aseptically collected from the rectal ampulla were stored in sterile tubes (Falcon™ 50mL Conical Centrifuge Tube). All fecal samples were readily freezed at -80 °C. Disposable spatulas were used to collect cerumen samples for lipidomic analysis and stored at -20 °C. Saliva samples for urea dosage were collected by making each heifer chew a saliva sampling sponge. Sponges were centrifuged and harvested saliva was transferred into 1 mL microvials stored at -20 °C.

All samples were collected in duplicates, with one aliquot per sample type used straight for pH measurement, using a digital pH meter with combination electrode (HI2002-02 edge, Hanna Instruments).

Measurements and sample collections

Individual daily FI was measured as the weight difference between the ration offered the day before minus the residual in the feed bunk.

Starting from day 0 and on a weekly basis individual LBW was measured – before the ration was delivered –with an electronic scale located outside the facility.

Simultaneously, individual BCS were determined as the mean of the evaluation sites on a 1 (very thin) to 5 (very fat) point scale with 0.25-point resolution.

Fecal consistency on fresh feces collected directly from the rectal ampulla was evaluated weekly by means of a 1 (liquid) to 5 (dry and segmented) point scale with a 1-point resolution.

Calculations and statistics

Average daily gain (**ADG**) for a heifer – expressed in kg – was calculated as the mean of the weekly ADG values obtained by the difference between two consecutive LBW values divided by seven days. Metabolic body weight (**MBW**) was estimated as mid-test $LBW^{0.75}$, where mid-test LBW was the heifer LBW measured at mid-time of the trial (d 28).

Individual feed conversion ratio (**FCR**) was calculated as mean of the ratio of individual observed DMI divided by individual ADG. Residual feed intake (**RFI**) was modelled as the residuals of DMI regressed on ADG, MBW and the diet effect:

$$DMI_i = \beta 0 + \beta 1(ADG_i) + \beta 2(MBW_i) + diet + \varepsilon_i,$$

where RFI was defined as the error term in the model (ε_i).

RFI results were used for an ex post reassignment of the heifers into two groups composed of 8 heifers with the lowest RFI values (L-RFI) and the highest RFI values (H-RFI).

Statistical analysis of calculated parameters and sample data was performed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC v.9.4 2015). Heifer was the experimental unit for all the accounted parameters. The model included diet,

time and diet nested with time as the main effects. Partially missing covariate data were corrected with the Kenward-Roger approximation method. Significance level was considered for ^{a,b} $p < 0.05$ and ^{A,B} $p < 0.01$.

For *the ex* post analysis, the same statistical model was applied, with RFI, time and RFI nested with time as the main effects.

RESULTS

The inclusion of camelina in the diet did not affect overall DMI, that was not significantly divergent between groups. A significant effect of treatment was found for DMI on d 34 ($p < 0.01$) and d 55 ($p < 0.05$) of the trial, when CAME displayed higher intakes (Figure 1).

Calculation for RFI resulted in the following equation

$$DMI = 3.271 - 0.033MilkE + 0.073MBW + 0.012\Delta BodyE + 0.184Diet + RFI$$

No significant effect of diet or diet per time was observed in the growth performance of animals, as evidenced by the absence of difference for all indicators (final LBW, ADG, MBW, BCS, heart girth and wither height gain). Feed efficiency was not affected by diet, as CAME displayed lower FCR and RFI values, but not in a significant way (Table 3)

The pH values of feces, urine and saliva did not differ between CAME and CTR groups at any timepoint (Table 4).

Results from qRT-PCR on COCs samples evidenced a marked up-regulation in all four molecular markers. Cells from COCs of CAME group displayed higher levels of

HAS2 (p = 0.012), GREM1(p = 0.046), LHCGR (p = 0.042) and FSHR (p = 0.011) (Figure 2).

The most abundant phyla across treatments were the *Bacteroidetes* and *Firmicutes*, accounting for 58 and 25% of the total sequences respectively (Figure 3).

Camelina cake had minimal to nil effect on the bacterial community composition, with a significant effect of time nested with treatment on three isolated bacterial genera (Table 5). No overall changes in the taxonomical distribution of rumen bacteria were found.

No effect of dietary treatment was observed on overall alpha diversity of the ruminal bacterial population. This could suggest a moderate to nil effect of camelina cake on rumen microbiota. However, caution should be paid in reading metagenomic data conducted on small sized statistical samples. For this reason, statistics was run again after correcting results for baseline timepoint, so as to reduce potential confounding factors due to the physiological range of biological variability. Results from the corrected statistical analysis showed a significant difference in alpha-diversity between the groups, with CAME group displaying lower average values. Results were statistically significant at d 0 and 28 for all the investigated alpha-diversity indices of richness (CHAO1, ACE, observed OTUs) ($p < 0.05$) and diversity (Shannon, Simpson, Fisher alpha) ($p < 0.05$), while no significant eveness (measured by Pielou's J and Simpson E indices) was detected (Figure 4). A significant effect of time was seen on alpha diversity of all animals, that followed overlapping trends characterized by a marked reduction at d 28 and return to the detected baseline levels at d 56. Causes behind this trend are currently unknown to the authors, although are likely imputable to factors unrelated to dietary treatment, given its uniform effect across experimental groups.

Beta diversity was found no different between the two experimental groups (Figure 5b). Percentage of variations were not significantly divergent for dietary treatment, whereas a significant time effect was detected for all timepoints (p = 0.01) (Figure 5a).

The *ex post* reassignment of animals to two groups created on the basis of RFI values determined no significant difference between groups for growth parameters. As for CAME vs CTL groups, a significant effect of the interaction of time and treatment was observed for DMI at d 29 (p = 0.015), when L-RFI ate -3.4 kg of DM on average (p = 0.015); and at d 34, when L-RFI ate -2.9 kg of DM (p = 0.034). A significant difference in the pH of ruminal fluid was found in heifers diverging for RFI at d 28 (p = 0.018), with more efficient heifers (L-RFI) having 0.31 point higher average pH values.

DISCUSSION

In our study the inclusion of camelina did not affect overall DMI values. However, DMI of CAME group was found to be higher at d 34 (when also more efficient heifers had higher DMI) and 55. Since palatability is an important factor affecting DMI, it may be inferred that camelina did not affect the palatability of the diet in a negative way. More classical PUFA sources, in particular fish oil, are characterized by low palatability. Lacasse et al. (2002) recorded a reduction of the FI by 25% when cows were fed fish oil at 3.7% DM. This factor, together with the alteration of milk flavor, discourages the use of fish oil in ruminant nutrition. Data on the effect of flaxseed on DMI and palatability are rather inconsistent. Gonthier et al. (2005) found no effect of flaxseed at 12.7% DM on DMI of transition cow. Chillard et al. (2009), on the contrary, recorded a decrease in FI with an inclusion of 21.2% DM. Despite the reduced intake following the administration of previously cited feedstuff might be consequence of their poor palatability, some findings seem to indicate that

factors other than the latter are likely involved in the higher feed refusal rates of high-PUFA fed animals, as abomasal infusion of UFA was similarly able to reduce voluntary intake of cows. Whether saturated or unsaturated FA are more effective in reducing DMI is another debated question, with results propending for the former (Petit et al., 2007) or the latter (Harvatine and Allen, 2006).

As already mentioned, the d 34 of the trial was associated with higher intakes in CAME group compared to CTR. It is interesting to note that the week from d 29 to d 35 was characterized by higher environmental temperature. Though a statistical correlation between DMI and THI values is still to be performed, speculation may be advanced on an alleged beneficial effect of camelina cake on heat stressed animals. Improvements of health status and productions have been reported in ruminants – even under heat stress conditions – fed flaxseed, an oilseed crop sharing several nutritional characteristics with camelina (Caroprese et al., 2012; El-Diahy et al 2016).

The inclusion of camelina cake in the diet did not alter the pH of the ruminal fluid, nor that of feces and saliva. An increase of ruminal pH as a result of unsaturated FA supplementation has been recored previously (Rennó et al., 2014), and according to some authors it could be put in relation with the increased rate of utilization of free H+ during the biohydrogenation process (Jenkins et al., 2008). However, despite the physiological soundness of such a theory, it must be reminded that the percentage of the free ruminal H+ exploited during the biohydrogenation of unsaturated FA has been quantified in 1 to 2% of the total (Czerkawski, 2004). In accordance with our results, other studies did not record significant effect of dietary FA on ruminal pH (Vandoni et al., 2010; Boeckaert et al., 2008), while others detected a reduction in ruminal pH after supplementation of soybean oil as a source of PUFA (Bateman and Jenkins, 1998; Baumann et al., 2016). However, attention must be paid to the physical form of FA when considering their effect on rumen environment.

Different forms of rumen-protected FA (fat prill, calcium soaps) have been found to alter ruminal pH at variable degrees. Since the theoretical poor dissociation influence of these products at physiological values of ruminal pH, the observed effect on rumen pH might be due to an alteration in the feeding behavior of the animals (Ngidi et al., 1990) as consequence of the altered palatability or the increased hepatic oxidative-signaling activity (Allen et al., 2020).

Trying to make a comparison with available data on camelina-derived feedstuff, Hurtaud & Peyraud (2007) detected lower pH in dairy cows fed camelina meal and seeds (9.5 and 2.9% DM, respectively). Feeding camelina also led to changes in VFA concentration, with reduced acetate to propionate ratio. Inclusion percentage of camelina products in the study above are significantly higher than those used in our study, that could explain the absence of effect we recorded. In another study, camelina oil displayed only a tendency to lower rumen pH while not affecting other rumen fermentation parameters (Bayat et al., 2015). Differences between experiments may relate to the form in which camelina lipid is included in the diet and the composition of other feed ingredients. The effect of other feed ingredients seems to be on the basis of the results from a recent study conducted on lactating buffaloes, in which camelina oil caused a reduction in rumen pH only when fed with a low roughage to concentrate ratio (30:70) (Ebeid et al., 2020).

Results from the metagenomic analysis suggests limited effects of camelina cake on the microbial community of the rumen. No significant differences in rumen microbial composition between the groups (beta diversity between groups) was observed. Overall, great changes in bacterial taxonomical composition were not expected, as it is now well established how the rumen 'core microbiome' is a conserved, highly resilient entity characterized by the dominance of phyla *Bacterioidetes* and *Firmicutes* (Weimer, 2015). The ruminal microbiome also displays

signs of elevated redundancy (overlap of functions among multiple species). In this sense, the reduction of the alpha diversity observed in the CAME group may be seen as an adaptative strategy to inhibit the unnecessary microbial activity, directing available substrates and nutrients to specific metabolic pathways. Lower alpha diversity of the ruminal bacterial populations has been reported in highly efficient beef steers (Li & Guan, 2019) and dairy cows (Shabat et al., 2016). The evaluation of the alpha diversity in heifers with low (L-RFI) and high (H-RFI) calculated RFI is currently ongoing.

Animals fed camelina cake displayed significantly higher levels of expression for all four genes selected as markers of COCs quality (HAS2, GREM1, LHCGR, FSHR). Hyaluronan synthase-2 gene (HAS2) is essential for the FSH-induced synthesis of the muchopolysaccharide hyaluronan during the COCs expansion (Schoenfelder & Einspanier (2003), while gremlin-1 gene (GREM1) have been shown to have roles in regulating cumulus cell expansion and granulosa cell luteinization during later stages of follicle development (Pangas et al 2004). Both HAS2 and GREM1 have been identified and used as molecular markers of oocyte competence in bovine species and buffalo (Assidi et al. 2008; Bhardwaj et al 2016). Follicle stimulating hormone receptor gene (FSHR) and luitenizing hormone/corionic gonadotropin receptor gene (LHCGR) are essential for the biological activity of the two main gonadotropins (FSH and LH), enabling the ovulatory process, the selection of dominant follicles and the estrogens synthesis (Luo et al., 2011). Specific FA proved able to modulate the FSH and LHCGR gene expression and activity. The increase in the expression of such genes observed in the present study could be attributable to the α-linoleic acid content of camelina cake. In the bovine species, dietary supplementation with n-3 PUFAs has often been associated with improved embryo survival rate and oocyte quality in both in vitro (Marei et al., 2017) and in vivo (Freret et al., 2019; Zachut 2010;) studies. The n-3 PUFA content of the diet may affect maturation and development of oocytes by

altering the lipidic structure of their cellular membranes (Bender et al., 2010) or by influencing the concentrations of prostaglandins and other lipid-derived mediators detectable in the surrounding follicular fluid (Fouladi-Nashta et al., 2009). Pending investigations on the FA composition of ruminal content and plasma (individually sampled at d 0, 28 and 56) may provide new insights on the alleged effect of n-3 PUFAs on COCs gene expression and the biological mechanisms behind it. In addition, current results must be integrated with those from COCs sampled at d 0 and 26. This would allow to rule out the possibility of an uneven gene expression level between the two groups prior the start of the trial and unrelated to the dietary treatments. qRT-PCR assay for COCs from d 0 and 26 are currently underway.

To conclude, though it is important to remember that gene expression levels do not necessarily imply corresponding protein expression or functionality (Glubb et al 2014), the preliminary results suggest a positive effect of camelina cake on COCs quality, opening for debating on the suitability of Camelina sativa as a functional feed ingrediets capable of improving the reproductive performance of ruminants.

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TABLES AND FIGURES

Table 1. Chemical composition of the camelina cake

(% DM)	
Crude protein	38.41
Ether extract	8.11
NDF	20.24
Ash	6.07
Calcium	0.51
Phosphorus	0.73
ME (kcal/kg)	2230

Table 2. Ingredients and nutrient composition of the basal diet

Ingredients (% as fed)	
Alfalfa hay	35.00
Durum wheat middlings	18.00
Sugarcane molasses	12.00
Ground maize	10.00
Soy hulls	8.00
Rice middlings	7.30
Sunflower meal	7.00
Calcium carbonate	1.40
Calcium phosphate	0.70
Sodium chloride	0.50
Sulfur	0.10
Calculated composition (% DM)	
Moisture	13.17
Metabolizable protein	10.07
Ether extract	4.16
NDF	42.63
NFC	51.04
Starch	19.43
Calcium	1.04
Phosphorus	0.62

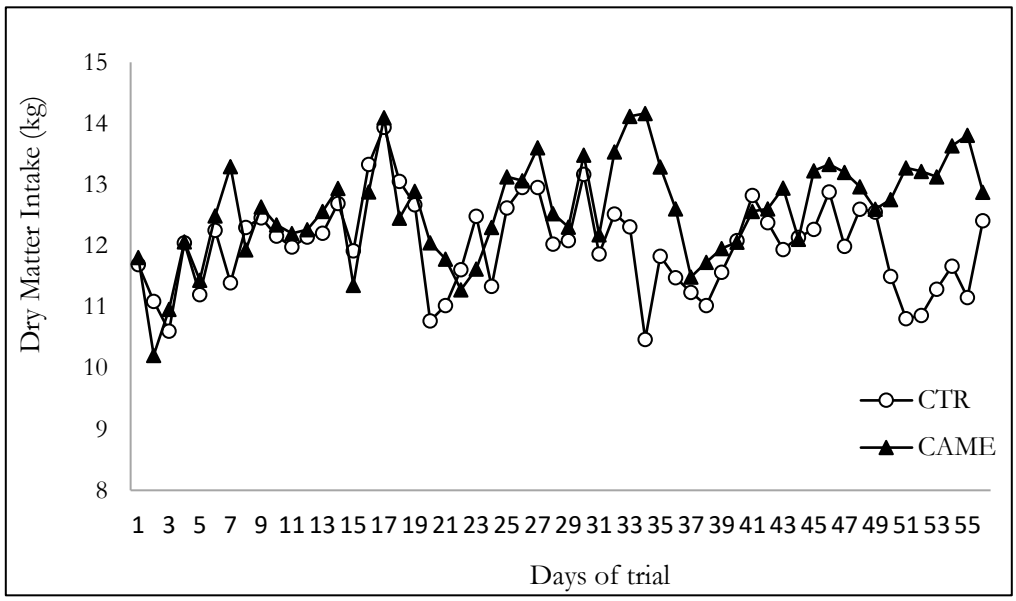


Figure 1. Daily DMI of CTR and CAME groups

Table 3. Mean growth performance of CAME and CTR groups

	Diet		**SEM**	***p-value***
	CTR	CAME		
DMI	12	12.61	0.73	0.56
LBW	494.81	501.47	47.43	0.89
ADG	1.05	1.3	0.18	0.18
BCS	2.99	3.1	0.08	0.55
Heart girth	191.87	193.81	6.43	0.77
Wither height	138	140.25	3.06	0.47
RFI	0.17	-0.17	0.51	0.5
FCR	13.27	9.88	2.24	0.15

Table 4. Values of pH recorded in non-invasive matrices

	Time	Diet		SEM	*p*-value			
		CTR	CAME		Diet	Time	DxT	P Diff
Feces pH					0.89	0.29	0.18	
	0	6.71	6.57	0.01				0.31
	26	6.65	6.55	0.01				0.51
	52	6.39	6.59	0.01				0.16
Urine pH					0.83	0.16	0.93	
	0	7.86	7.87	0.07				0.93
	26	7.73	7.77	0.07				0.67
	52	7.82	7.82	0.07				1.00
Saliva pH					0.65	0.44	0.69	
	0	8.27	8.34	0.08				0.57
	26	8.26	8.33	0.08				0.52
	52	8.41	8.36	0.08				0.67
Rumen fluid pH					0.98	<0.01	0.99	
	0	6.67	6.67	0.09				0.97
	26	6.86	6.87	0.09				0.95
	52	7.10	7.1	0.09				0.98

* DxT = Diet x Time

Figure 2. Gene expression of the four molecular markers (* $p < 0.05$)

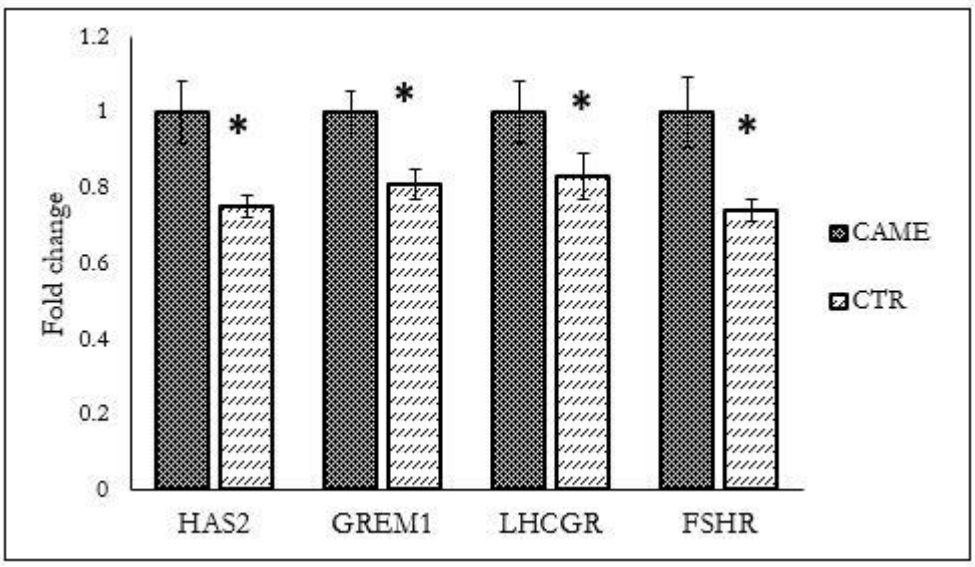


Figure 3. Pie charts of the taxonomic distribution in CTR and CAME

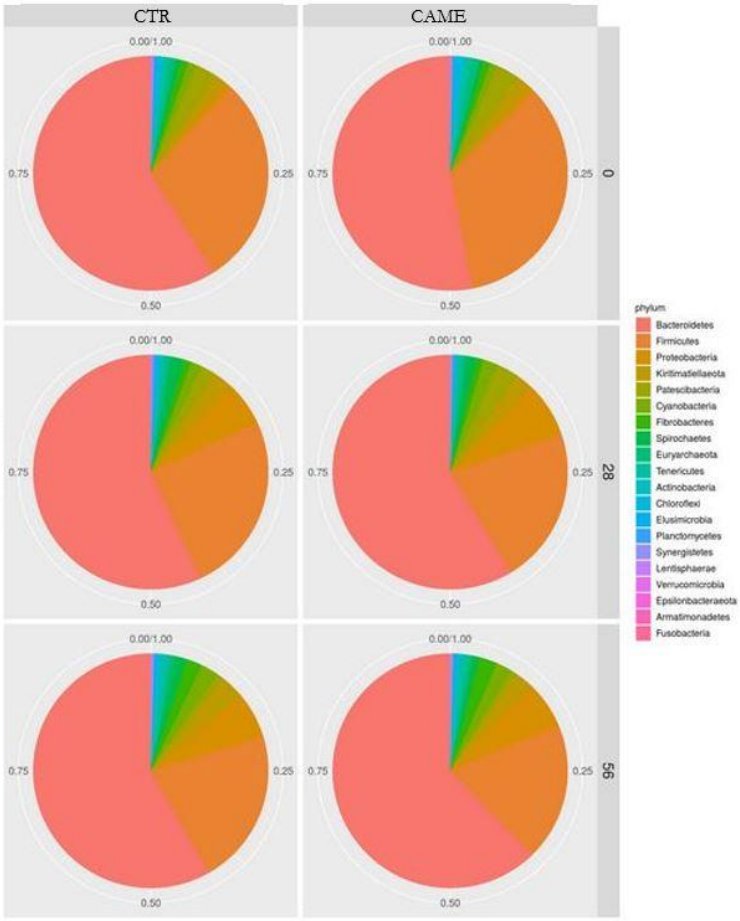


Table 5. Significant differences between CAME and CTR at a taxa level

Taxa	SEM	*p*-value
Tenericutes; *Mycoplasmatales*; *Mycoplasmataceae*[f]; *Mycoplasma*[g]	7.78	0.02
Firmicutes; *Clostridiales*; *Ruminococcaceae*; *Ruminococcaceae* UCG-001[g]	14.71	0.04
Lentisphaerae; Z20[g]	3.57	0.02

Figure 4. Alpha diversity corrected for d 0 (* $p < 0.05$; ** $p < 0.01$)

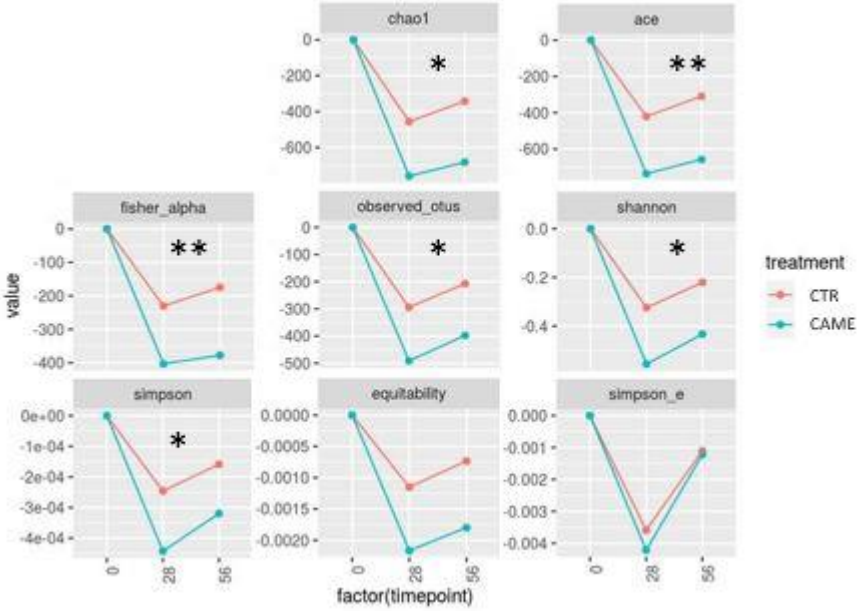
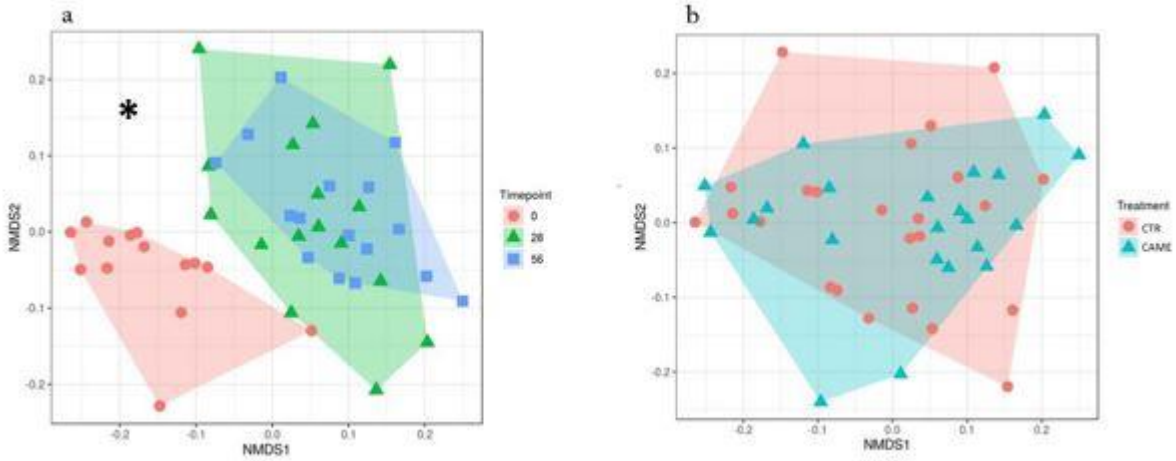


Figure 5. Beta diversity for time (5a) and dietary treatments (5b) (* $p < 0.05$)



Chapter 5 |

CONCLUSIONS

We all are living an era characterized by ever increasing concerns regarding future availability of food resources and the increasingly fragile environment. The quadratic increase of the global population growth curve, and the now evident consequences of the climate change that we are witnessing fuel the primary need to investigate new approaches to animal production. Researchers and scientists involved in the veterinary and animal science field should drive and direct futures developments of dairy industry on the street for a more sustainable and efficient food supply chain.

Apart for the evident economic benefit for entrepreneurs and farm workers, the importance of improving feed efficiency (FE) of cattle for the sustainability of the whole dairy industry cannot be stressed enough. This elaborate had the aim to introduce the extremely broad and complex concept of feed efficiency, from its biological and genetic aspects to the more practical economic impact. Since simple solutions hardly follow complex problems, a multimodal approach comprising international data sharing and a choral spirit of collaboration is required to determine significant improvements in energetic, as well as economic and environmental, efficiency of animals.

As evidenced by a consistent body of literature, the inclusion of FE traits into future breeding plans plays a leading role in the multimodal, multidisciplinary approach to efficiency improvement. Genomic selection proves an invaluable tool for reaching the objective of a low impact, high producing agriculture.

It is important to remind that all animals included in this PhD project were previously genotyped. Individual phenotypic data on dry matter intake (DMI) collected throughout the period covered by the PhD course will be made available to international research groups, contributing to the expansion of the dataset needed to

increase the prediction accuracy of genomic estimated breeding value (gEBV) for DMI and related FE measures.

The trials conducted during the PhD course also investigated some nutritional factors that may contribute to improving FE on a herd level.

Camelina cake, a by-product of the oil extraction from *Camelina sativa*, proved a valid feedstuff characterized by low environmental footprints and a good nutritional value, capable of sustaining milk production while enriching the functional profile of milk for human consumption and improving the oocyte quality of growing heifers. Despite our results proved less evident than those reported in literature for other camelina products, it should be noted that – unlike camelina oil and seeds – camelina cake is a non-human-edible, non-exploitable for energetic purpose industrial by-product, excluding the risk inter-sectorial competition for its use.

In our personal experience, supplementation of lactating cow diet with *Saccharomyces cerevisiae* gave mild results in term of productivity and efficiency. A significant effect on ruminal pH was observed when cows were fed a forage with a lower nutritional value, endorsing the already reported increase in the biological activity of live yeast in challenging environments. However, the change of diet was not an effect planned in the experimental design, therefore caution should be exercised when interpreting the results. Though inconsistent, the reported positive effect of *S. cerevisiae* on dry matter digestibility and rumen environment stability deserves further study, as the association between healthy animals, optimal ruminal functionality and feed efficiency is quite intuitive. Lack of results on our trials could rise from the absence of a factor challenging the rumen, a condition during which *S. cerevisiae* seems to manifest its biological activity at full.

It is in the hopes of the author that results from the present research line may prove a small step towards the objective of a dairy sector that makes of animal health, productivity and environmental sustainability three equals reasons of pride.