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Influence of cow diet on nutritional profile of milk and dairy products and effects on alterations of human gut microbiota by an in vitro digestion model

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ABSTRACT

Health-conscious consumers are demanding milk with higher proportions of healthy fatty acids as polyunsatured fatty acids (PUFA), and lower proportion of saturated fatty acids (SFA).

Milk and dairy products contribute significantly to the consumption of essential nutrients in human populations. Despite its important roles in human nutrition, consumption of milk has declined, because nutritional guidelines have limited capita consumption of SFA, which to a significant proportion originate from milk and dairy products (USDA and HHS, 2010). A strategy to improve the FA profile of milk and dairy products is the supplementation of cow's diet with oilseeds, which decrease the proportion of SFA, by decreasing de novo FA synthesis in the mammary gland.

Feeding flaxseed to dairy cows decreases the concentrations of short-chain fatty acids and medium chain fatty acids and increases the long-chain fatty acid content in milk fat. However, oilseeds, and in particular flaxseed, have a very high costs that discourage farmers in their utilization. It's necessary, therefore to find a compromise between costs and the right amount to be administered in the diet to the animals to ameliorate milk yield and composition.

In Italy, about 80% of dairy farms produce milk of Friesian cows both for direct consumption and for cheese production. Jersey breed and it has been used to improve the efficiency of the cheesemaking sector in different part of the world, and is characterized by improved longevity, superior udder health, higher cheese yield, reduced feed and water requirement.

The gastrointestinal tract constitutes the body's largest interface with the external environment and is exposed to a vast amount of foreign material, including pathogenic and commensal bacteria, as well as food antigens. Oral tolerance is an important property of the gut immune system; intestinal homeostasis requires balanced interactions between the gut microbiota, dietary antigens.

At birth, we are colonized with a complex community of microbes that reaches up to a density of 1×10^{12} bacterial cells per grams of content in the adult colon. These microbes live in a symbiotic relationship with the host and they are determinants in health and disease influencing nutrient absorption, barrier function and immune development.

On the basis of the previous considerations and considering that oil seeds are expensive and many farmers are reluctant to use them the aims of this PhD thesis are:

1. trying to reduce the daily amount of flaxseed administered to animals in order to increase the content of polyunsaturated fatty acids in milk at the expense of saturated fatty acids, and to encourage its utilization by farmers as supplements to dairy cows with a reduction of management costs;
2. testing the effects of flaxseed administration on two different dairy cows breeds: Friesian and Jersey;
3. evaluating the transferring of polyunsaturated fatty acids in two different dairy products (Caciotta vs Caciocavallo) at different ripening time;
4. evaluating the effects of dairy products naturally enriched in polyunsaturated fatty acids on human health by an in vitro digestion model with the evaluation of changes in:
    a) fatty acid profile of dairy products after in vitro digestion;
    b) short chain fatty acids (SCFA) produced by gut microbiota;
    c) changes in gut microbiota populations by fecal fermentation followed by pyrosequencing.

The higher milk content of C18:3n3 in milk suggests that the reduction in the amount of flaxseed supplementation can also improve milk fatty acid profile with a consistent reduction of production costs; however, Friesian and Jersey cows replied differently to the same flaxseed supplementation;

Polyunsatured fatty acids are transferred into dairy products, especially in Caciotta cheese, suggesting that probably the different cheese making influenced the transferring. After in vitro digestion, fatty acids remain in the digest; their presence can have beneficial effects on the gastrointestinal tract and consequently on human health. Moreover the presence and the amount of short chain fatty acids (SCFA) could suggest some changes of microbiological populations that could have beneficial effects on human health.

INTRODUCTION

Nutrition plays an important role in the maintenance of human health and prevention of the development of chronic diseases. Developing foods enhancing human health is the topic to dietary approaches for preventing and reducing the economic and social impacts of chronic disease. Numerous studies attested that an high consumption of saturated fatty acids (SFA) and *trans fats* is implicated as a risk factor for different diseases, as cardiovascular diseases (Shingfield et al., 2008).

Milk, milk products, cheese, butter, meat and meat products were found to give a significant but variable contribution to total trans fatty acids (TFA) in the human diet. However, over the years it has shown a decrease in the consumption of ruminant meat and milk to lower SFA and TFA intakes ignoring the value of these foods as a versatile source of high-quality protein, vitamins, minerals and bioactive lipids (Shingfield et al., 2012). Milk and dairy products contribute significantly to the consumption of essential nutrients in human diet. Despite its important role in human nutrition, consumption of milk has declined (Haughes et al., 2007), because nutritional guidelines have limited capita consumption of SFA, which in a significant proportion originate from milk and dairy products (USDA and HHS, 2010).

Altering the fatty acid composition of ruminant meat and milk represents one strategy to lower SFA intakes and increase cis monounsaturated (MUFA) and polyunsatured fatty acids (PUFA) in the human diet, without changes in consumer eating habits, and maintaining the potential benefits associated with the macro- and micronutrients in these foods. Ruminant dairy products are the major dietary sources of Conjugated linoleic acid (CLA) and the isomer cis-9, trans-11 is approximately 80–90% of the CLA in milk fat (Parodi, 1999).

In addition, it has been shown that certain bacteria cultures used in food fermentation possess the ability to generate cis-9,trans-11 CLA (Coakley et al., 2003). Most of the published studies have used a mixture of CLA isomers that contained the two major forms, cis-9, trans-11 CLA and trans-10, cis-12 isomers as in Fig.1.

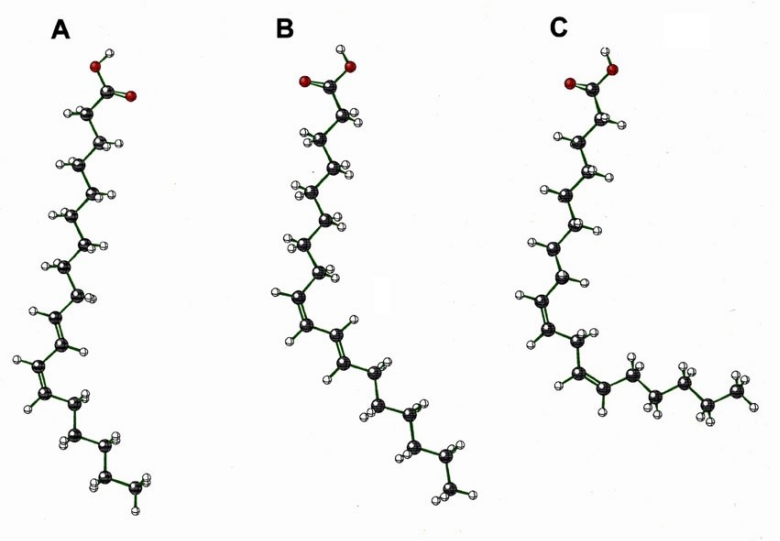


Figure 1. Chemical structure of conjugated linoleic acid isomers and linoleic acid. (adapted from Bauman et al., 1999). Fatty acids are *trans*-10, *cis*-12 octadecadienoicacid (A), *cis*-9, *trans*-11 octadecadienoic acid (B) and *cis*-9, *cis*-12 octadecadienoic acid (linoleic acid) (C).

Health-conscious consumers are demanding milk with higher proportions of healthy fatty acids as PUFA, and lower proportion of SFA. Rumenic acid (RA) and its precursor vaccenic acid (VA) has been shown to have potential health benefits, including anticarcinogenic properties in experimental animals (Hughes and Dhiman 2002).

Digestion of lipids in ruminants

Ruminal lipolysis and biohydrogenation

Tissue lipids and milk fat in ruminants contain much higher proportions of SFA compared with dietary intake, which is partly due to a first lipolysis and subsequent biohydrogenation of unsaturated fatty acids in the rumen. The primarily responsible are different bacteria, which use these processes to reduce the toxic effects of dietary unsaturated fatty acids on bacterial growth (Lourenço et al., 2010). Lipolysis represents the first step in the complete metabolism of dietary lipids, and under normal conditions more than 85% of esterified dietary lipids are hydrolysed (Buccioni et al., 2012). Recent studies in vitro have demonstrated that a diverse range of intermediates are formed during incubations of pure fatty acid substrates with rumen fluid or pure strains of ruminal bacteria (Lourenço et al., 2010; Buccioni et al., 2012). Fatty acids available for absorption are also derived from rumen bacteria and protozoa. Microbial fatty acids appear initially in the form of structural membrane lipids, originate from biohydrogenation. *De novo* fatty acid synthesis is also responsible for the occurrence of odd- and branched chain fatty acids in membrane lipids of rumen bacteria (Vlaeminck et al., 2006).

Lipolysis consists in a release of free fatty acids (FFA) from esters to allow biohydrogenation, which is reduction of the number of double bonds on the carbon chain of the FA. The subsequent hydrogenation can only happens if the carboxyl moiety is free, so lipolysis is a necessary step in biohydrogenation. If only a small quantity of dietary PUFA reach the duodenum, this may be due to a missing lipolysis, and it may be determines the rate of hydrogenation in the rumen. These

processes are utilised by microorganisms to protect themselves from toxic effects of unsaturated fatty acids (UFA) (Dehority, 2003). After ingestion, dietary esterified lipids are hydrolysed. Various bacterial strains of *Butyrivibrio fibrisolvens* and *Anaerovibrio lipolytica* are capable of hydrolysing the ester bond, but *B. fibrisolvens* lipase hydrolyses phospholipids, *A. lipolytica* hydrolyses only tri- and di-glycerides, with rates of hydrolysis.

Mammary lipogenesis

Ruminant milk fat contains more than 400 different fatty acids, but saturated fatty acids with chains with 4 to 18 carbon atoms, cis-9 16:1, cis-9 18:1, isomers of trans 18:1 and 18:2n-6 are the most abundant. Fatty acids incorporated into milk fat tryacilglicerol (TAG) are derived from the uptake of fatty acids from non esterificated fatty acids (NEFA) and TAG in arterial blood and synthesis *de novo* in the mammary gland (Bernard et al., 2008).

Mammary epithelial cells synthesize short- and medium-chain fatty acids using acetate and 3-hydroxy-butyrate in the presence of acetyl-CoA carboxylase (ACC) and fatty acid synthetase (FAS; Figure 2). Fatty acid synthesis de novo accounts for all 4:0 to 12:0, most of the 14:0, and about 50% of 16:0 secreted in milk, whereas all 18-carbon and longer-chain fatty acids originate from the absorption of fatty acids in the small intestine. The activity of stearoyl-CoA desaturase (SCD) in mammary epithelial cells that catalyses the oxidation of fatty acyl CoA esters resulting in the introduction of a cis double bond between carbon atoms 9 and 10 is responsible for secretion in milk of ca. 90%, 55%, 60% and

70% to 95% of cis-9 14:1, cis-9 16:1, cis-9 18:1 and cis-9, trans-11 conjugated linoleic acid (CLA), respectively. (Shingfield et al., 2010).

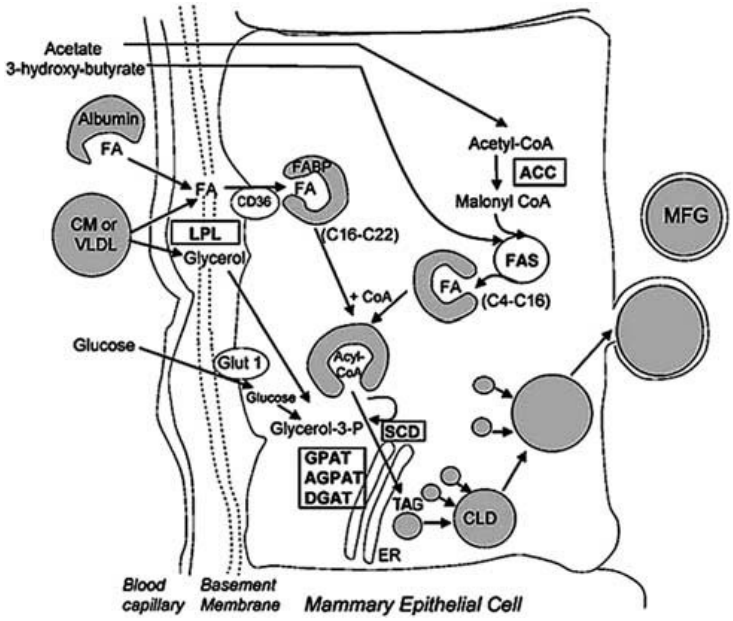


Figure2. Synthesis of milk fat in the bovine mammary epithelial cell (adapted from Bernard et al., 2008). ACC5acetyl-CoA carboxylase; AGPAT5 1-acylglycerol 3-phosphate acyltransferase; CD365cluster of differentiation 36; CLD5cytoplasmic lipid droplet; CoA5coenzyme A; CM5chylomicron; DGAT5diacylglycerol acyltransferase 1; ER5endoplasmic reticulum; FA5 fatty acid; FABP5fatty acid-binding protein; FAS5fatty acid synthase; Glut 15glucose transporter 1; GPAT5glycerol-3 phosphate acyltransferase; LPL5lipoprotein lipase; MFG5milk fat globule; SCD5stearoyl-CoA desaturase; TAG5triacylglycerol; VLDL5very-low-density lipoprotein.

Improving the fat content of food

A strategy to improve the FA profile of milk and dairy products is the supplementation of cow's diet with oilseeds, which decrease the

proportion of SFA, by decreasing de novo FA synthesis in the mammary gland (Glasser et al., 2008a).

Oilseeds are rich of polyunsaturated fatty acids (PUFA), which can be fed to dairy animals to modify the milk fatty acid profile and produce nutritionally beneficial milk for human consumption (Kennelly, 1996). Flaxseed is a rich source of both protein and fat. It contains on average 40% oil, 20% crude protein (CP) and 30% neutro detergent fiber (NDF) (Petit, 2010).

Changes in milk fatty acid composition to plant lipid supplements are dependent on: 1) the amount of oil included in the diet, 2) the fatty acid profile of the lipid supplement, 3) the form of lipid supplement, and 4) the composition of the basal diet. Attempts to improve the concentration of specific fatty acids in milk can change results in other fatty acids (Shingfield et al., 2008).

Linoleic (LA, C8:2ω6) and α-linolenic (ALA, C18:3ω3) acids are the precursors for the ω6 and ω3 FA amilies respectively, and are considered essential fatty acids as mammals do not contain the desaturase enzymes (Δ112 and Δ15 desaturases respectively) to insert double bonds beyond the C9 position. Therefore, a dietary supply is necessary (Minihane A. M. et al., 2006). It is estimated that human requirements for these fatty acids are 1% and 0.2% of daily energy intake (Department of Health, 1994).

Feeding flaxseed to dairy cows decreases the concentrations of short-chain fatty acids and medium chain fatty acids and increases the long-chain fatty acid content in milk fat (Mustafa et al., 2003; Petit, 2003; Caroprese et al., 2010; Neveu et al., 2013). These effects are due to the ruminal biohydrogenation of flaxseed PUFA. It is possible to protect

dietary oilseed fatty acids from ruminal biohydrogenation altering its physical structure; heat treatment for example, to oilseed can denature the protein matrix surrounding the fat drops protecting fatty acids from ruminal biohydrogenation (Kennelly, 1996; Gonthier et al., 2005). Feeding oilseeds to dairy cows can, however, produce a reduction in milk fat concentration and yield due to the formation of several trans and conjugated fatty acids isomers during ruminal biohydrogenation which influenced negatively de novo milk fatty acids synthesis (Glasser et al., 2008; Caroprese et al., 2014). Ruminal pH and the microbial population are influenced by several dietary factors like the type and concentration of supplementary fat: concentrate ratio, type of forage, and composition of basal diet. Modifying the ruminal fatty acid biohydrogenation is possible to alter the duodenal flow of fatty acids, and consequently milk fatty acids composition.

Griinari and Shingfield (2002) suggest that ruminal VA formation is dependent on three interdependent processes: 1) substrate supply, 2) inhibition of trans-18:1 reductase, and 3) prevention of a shift in ruminal biohydrogenation toward trans-10 18:1 to the expense of trans-11 18:11. In the Fig. 3 is represented the "biohydrogenation balance model" that characterizes the impact of diet on PUFA content of ruminant foods through the effects of all three processes on ruminal biohydrogenation simultaneously (Palmquist et al., 2005).

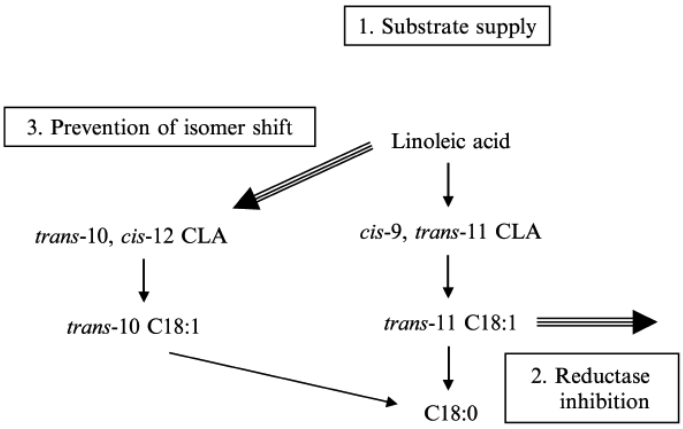


Fig. 3. **Schematic of the ''biohydrogenation balance model"** Palmquist et al., 2005. (Used, with permission, from Griinari and Shingfield, 2002.)

In cheese, CLA content ranges widely and mainly depends on CLA content of raw milk itself Werner et al. (1992). The transfer of fatty acids from milk to dairy products is influenced by their content in the milk. During cheese ripening CLA concentration is subjected to decreases. This bioactive compounds are adsorbed from gastrointestinal tract and they could get beneficial effects on human health.

However, oilseeds, and in particular flaxseed, have very high costs that discourage farmers in their utilization. It is necessary, therefore to find a compromise between costs and the right amount to be administered in the diet to the animals to ameliorate milk yield and composition.

Technological properties of milk: influence of natural variation in milk fat globule size and casein micelles size

The physical functionality of milk can be influenced by composition and structural organization of proteins and fat milk (Logan et al., 2014). The polar lipids in milk are becoming of increasing interest due to their

nutritional and technological properties. These compounds are secondary messengers involved in transmembrane signal transduction and regulation, growth, proliferation, differentiation and apoptosis of cells (Korhonen 2006). Flaxseed administration to lactating ewes during heat stress beside improving milk fatty acid composition, succeed in enhancing milk coagulating properties probably as a result of increasing fat and casein content (Caroprese et al., 2011). The bovine milk proteins are constituted in large part by 4 caseins (CN): α_{s1}-, α_{s2}-, β- and κ- and then by 2 whey proteins: β-Land α-LA. The network of CN micelles is stabilized by a combination of hydrophobic interactions and repulsive electrostatic forces between CN molecules; Ca^{++} mediate these interactions (Jensen et al., 2012). The CN micelles are the base to build the blocks of gel formed during the rennet coagulation during the cheesemaking (Lucey 2009). Milk fat globule (MFG) size is correlated with fat content and could be connected to the quantity of milk fat secreted and the concentration of long chain fatty acids in milk (Wiking et al., 2004). The coagulation process can be divided in two phases; in the first one, the enzymatic rennet-induced cleavage of κ-CN into an insoluble para-κ-CN and a soluble hydrophilic caseinomacropeptide (CMP); in the second one, in which rennet-induced milk coagulation is nonenzymatic. When a major part of κ-CN is hydrolyzed, the destabilized CN micelles aggragate spontaneously in a gel like a network, that is, the curd or coagulum (Lomholt and Qvist, 1997). Timmen and Patton (2008) affirm that MFG size could be influenced by the fatty acids composition's differences. Other variables influence the MFG size like variations among cows, and genetics variations (Logan et al., 2014), and feed (Couvrer et al., 2007). In the same way, casein

micelle (CM) size may be influenced by cow genetics, like casein protein variants (Bijl et al., 2014), and by feed (Devold et al., 2000).

Technological properties of bovine milk measured by oscillatory rheology include curd firmness, Curd firmness is defined by coagulum strength (a30) and curd firming time (k20) that is related to CN concentration and composition (Hallén 2007); whereas rennet clotting time (r) is not completely related (Frederiksen et al., 2011b).

To date the study of the effects of fat supplementation on milk coagulating properties has been neglected. However, it could be hypothesized that fat supplementation, by altering the milk fatty acid composition, could have an effect on milk coagulation properties, thus influencing cheese-making ability.

Friesian and Jersey breed: effects on quality of milk and cheese

In Italy, about 80% of dairy farms produce milk of Friesian cows both for direct consumption and for cheese production. Most of the milk produced in Italy comes from the Italian Friesian breed; milk processing lead to a wide range of products among which some products with the designation of Protected Designation of Origin (PDO). The Holstein-Friesian may be the single most important breed of cattle, not just dairy cattle, in the world (Fig. 4). They have the well-known black-and-white colour patter. The spots should well defined. The popularity of Friesian is due to the extremely high average milk production (25-35 kg day^{-1}). Although other breeds have a higher percentage of fat, protein and solids, the very high milk production of Friesian means that the total quantity of milk components is also superior. The second most important dairy breed in the world is Jersey breed (Fig. 5) and it has

been used to improve the efficiency of the cheesemaking sector in different part of the world, and is characterized by improved longevity, superior udder health, higher cheese yield, reduced feed and water requirement (Bland et al., 2015). Jersey cattle may vary in colour from light grey to a dark fawn. In Southern Italy, beside the use and commercialization of Friesian cow milk, Jersey milk is used in combination to Friesian milk to increase cheese yield production and meet market demand. Jersey cattle yield milk higher in fat and other solids than milk from Friesian cattle (4.1-4.9 vs 3.3-4.1%, respectively), even if the average milk production is lower than Friesian milk production (19-25 vs 25-35 kg day^{-1}) (Buckanan, 2002). White et al. (2001) found that the higher the milk fat the lower is the activity of Δ^9-desaturase. Besides, when comparing digestibility and rate of digest passage of Jersey and Friesian cows, Aikman et al. (2008) found that both digestibility of neutral detergent fiber and rate of digesta passage are higher in Jerseys, probably as a consequence of their increased mastication per unit of feed consumed. The authors also observed differences in N utilization between the Friesian and Jersey breeds during lactation. Beaulieu and Palmquist (1995) tested increasing dietary intake of calcium salts of palm fatty acid distillate in the diet of Friesian and Jersey cows, and found differences among breeds in the composition of milk fat, suggesting that feeding could induce even more effects on milk fat quality than the only differences in milk fatty acid composition.

Fig. 4. Friesian cow (Buchanan 2002)

Fig. 5. Jersey cow (Buchanan 2002)

Role of the gut microbiota

The gastrointestinal tract constitutes the body's largest inter-face with the external environment and is exposed to a vast amount of foreign material, including pathogenic and commensal bacteria, as well as food antigens. Oral tolerance is an important property of the gut immune system; intestinal homeostasis requires balanced interactions between the gut microbiota, dietary antigens. Intestinal homeostasis requires balanced interactions between the gut microbiota, dietary antigens and the host. At birth, we are colonized with a complex community of microbes that reaches up to a density of 1×10^{12} bacterial cells per grams of content in the adult colon (Fig. 6). The composition of gut microbiota changes at three stages: from birth to weaning; from weaning to reach a "normal diet", and during old age. These microbes live in a symbiotic relationship with the host and they are determinants in health and disease influencing nutrient absorption, barrier function and immune development (Verdu et al., 2015). Intestinal health depends on positive or negative modifications of the faecal and colonic microbiota. The gut microbiota is considered to have an important role in the prevention of different disease (Gill et al., 2002).

Several beneficial effects of the microbiota flora within the gut should be deduced from comparisons of germ-free and re-colonised animals. Principally, defence against infections depends by competition for nutrients and epithelial binding sites and production of antimicrobial factors. Defence is also get stronger by the firs effect of the flora on the mucosal immune response which is maintained in state of 2controlled physiological inflammation" on ready alert. In addition, the flora produce short chain fatty acids, a major energy source for colonic epithelia (Shanahan 2003). In certain individuals, under some circumstances, components of the flora may become a risk facor for development of disease (Dugas et al., 1999). Another risk is bacterial translocation into the systemic circulation, which may occur in immunodeficiency, or disruption of mucoasal barrier function (Berg 1999).

Dietary intake determines the metabolic products of the microbial community that are able to modify the species composition. Diet offers a potential way to keep health benefits through manipulation of microbial community (Flint et al., 2012). Modification of the gastrointestinal bacterial flora has become an attractive therapeutic strategy for several infiammatory, infectious and neoplastic intestinal disorders (Shanahan 2003).

Intestinal location	Stomach	Duodenum	Jejunum	Ileum	Colon
Microbes/gram	1×10^{1}	1×10^{3}	1×10^{4}	1×10^{7}	1×10^{12}
Composition	*Lactobacillus* *Helicobacter* *Veillonella*	*Streptococcus* *Lactococcus* *Staphylococcus*	*Lactobacillus* *Streptococcus* *Enterococcus*	SFB Enterobacteriaceae *Bacteroides* *Clostridium*	*Bacteroides* *Clostridium* Lachnospiraceae Proteobacteria Actinobacteria Prevotellaceae

Fig. 6. **Development of the gut microbiota**. The composition and density of the microbiota varies along the length of the intestine as well as with age. Differences in microbial composition and density are observed along the length of the gastrointestinal tract, with much lower densities and greater variability in the proximal intestine (Verdu et al., 2015).

Some members of gut microbiota could differentially modulate host response as explained in Fig. 7 in which is described a gut microbiota shapes and the different host responses to different molecules.

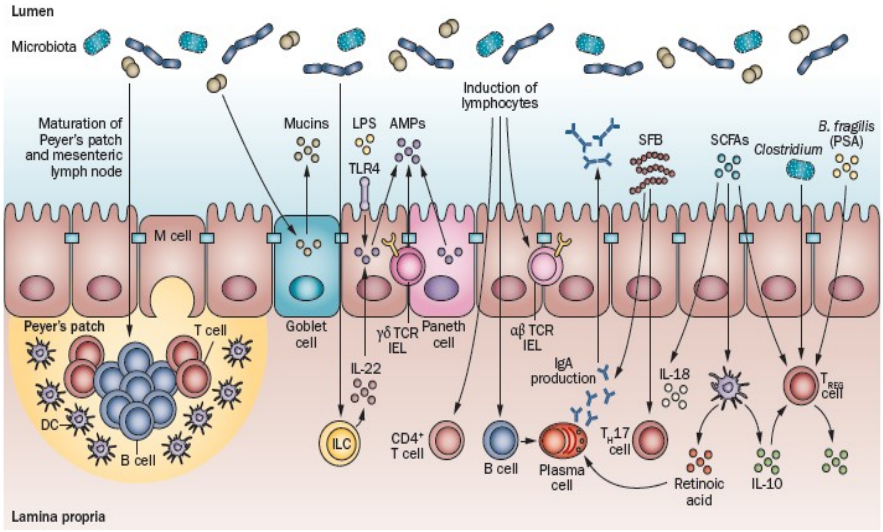


Fig. 7. **Gut microbiota shapes host immunity** (Verdu et al., 2015).

Bacterial products like short-chain fatty acids (SCFAs: acetic acid, propionic acid and butyric acid) have also been shown to induce T_{REG}

cells (Hrneir et al., 2008; Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). Tregs play an essential role in immune tolerance and in their absence both humans and mice spontaneously develop autoimmune disorders at a young age (Rudensky 2011). Natural Tregs develop in the thymus and induced Tregs develop at sites of inflammation in the presence of IL-2 and TGF-b (Davidson 2007). SCFAs is showed that induce IL-18 production from epithelial cells and promote tolerogenic dendritic cells, which produce IL-10 and retinoic acid (Singh et al., 2014). On the basis of these studies, the gut microbiota's response is influenced not only by the presence or absence of live bacteria, but also by the relative abundance of particulars members of gut microbiota and their by products (Verdu et al., 2015).

At the same time the gut community microorganism are considered beneficial for health and can harbor organisms that have the capacity for adverse effects, or potencial for pathogenicity (Blaser et al., 2007). The balance between benefits and damages depends on the state of the microbial community in terms of its distribution, diversity, species composition, and metabolic products (Fig. 8).

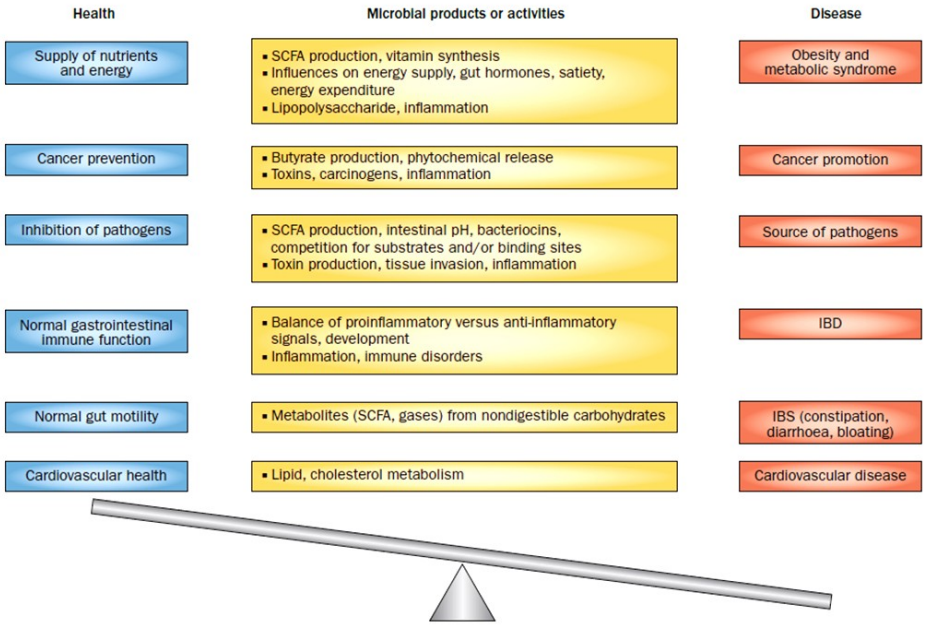


Fig. 8. **Influence of gut microbial communities on health** (Flint et al., 2012).

The major microbially produced short chain fatty acids (SCFAs) are acetate, propionate and butyrate in the molar proportions of 20:1:4 (Zoetendal et al., 2012), compared with approximately 3:1:1 in typical faecal sample. SCFAs have a lot of effects on the host, and as source of energy for the host; in fact the butyrate is the mainly product consumed by the colonic epithelium and acetate becoming available (Pomare et al., 1985). Some studies reported anticancer effects, especially for butyrate, and anti-infiamatory properties (Hamer et al., 2008; Gassull et al., 2006). Therefore the changes of products of SCFAs by the colonic microbiota have important physiological consequences. Moreover other acids like lactate, succinate and formate, normally transform themselves in

intermediates in microbial metabolism in the gut due the conversion like Fig 9 explains.

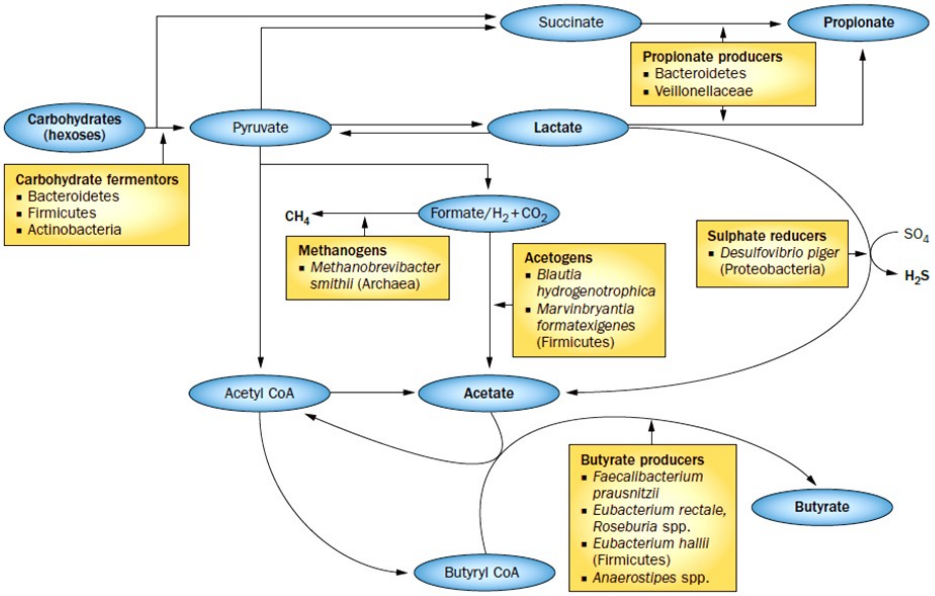


Fig. 9. **Functional and phylogenetic groups of gut bacteria involved in the metabolism of short-chain fatty acids** (Flint et al., 2012).

Among the faecal SCFAs acetate is the mainly recognized having an highest concentration, but is also an intermediate that is consumed by the major butyrate-producing bacteria (Louis et al., 2009). The absorption of microbially produced SCFAs provides energy to the host from dietary components remaining undigested in the small intestine. Therefore, gut microbiote, contribute to the energy from the diet (Turmbaugh et al., 2006).

The informations about the composition of gut microbiota are available from faecal samples amplifying small subunit (16S) ribosomal (rRNA)

sequences (Walker et al., 2011). Faecal microbiota profiles in healthy adults seems to have stability during time (Costello E. K. et al., 2009) and the dominant groups of bacteria can have or not an effect by dietary changes. Dietary substrates have a great influence on the species composition of the microbiota, but their structures determine which metabolic pathways are used for fermentation by individual bacterial species (Flint et al., 2012).

Gastrointestinal lipid digestion is a very complex, but also efficient process. In healthy persons, approximately 95% of dietary lipids are digested and absorbed (Fatouros & Mullertz, 2008). Intestinal lipi digestion needs as prerequisite of precence of pancreatic lipase, co-lipase and bile salts (Bauer, Jakob, & Mosenthin, 2005). Pancreatic lipases hydrolyse ester bonds in triglycerides at positions 1 and 3, releasing a 2- monoacylglycerol and two free fatty acids (Armand, 2007). The knowledge about the digestibility of individual components is very important to predict the nutritional quality of food products (Devle et al., 2012).

AIMS

Feeding flaxseed to dairy cows decreases the concentrations of short-chain fatty acids and medium chain fatty acids and increases the long-chain fatty acid content in milk fat and subsequently in dairy products. These effects are due to the ruminal biohydrogenation of flaxseed PUFA. The gastrointestinal tract is involved in interactions with the external environment exposing itself to a great amount of foreign cells including commensal bacteria as well as food antigens. Food intake is able to modify the microbial community composition.

On the basis of the previous considerations and considering that oil seeds are expensive and many farmers are reluctant to use them the aims of this PhD thesis are:

- trying to reduce the daily amount of flaxseed administered to animals in order to increase the content of polyunsaturated fatty acids in milk at the expense of saturated fatty acids, and to encourage its utilization by farmers as supplements to dairy cows with a reduction of management costs;
- testing the effects of flaxseed administration on two different dairy cows breeds: Friesian and Jersey;
- evaluating the transferring of polyunsaturated fatty acids in two different dairy products (Caciotta vs Caciocavallo) at different ripening time;
- evaluating the effects of dairy products naturally enriched in polyunsaturated fatty acids on human health by an in vitro digestion model with the evaluation of changes in:
    - fatty acid profile of dairy products after in vitro digestion;
    - short chain fatty acids (SCFA) produced by gut microbiota;

- changes in gut microbiota populations by fecal fermentation followed by pyrosequencing.

MATERIALS & METHODS

Experimental design

The experiment was conducted in a farm located in Gravina in Puglia (BA), Apulia, Southern Italy (latitude: 40°49'14"52 N and longitude 16°25'24"96 E). A 30 days trial was performed from May to June of 2014 with 20 Italian Friesian cows and 20 Jersey late lactation cows widely distributed divided into 2 groups of 10 animals each. Treatments involved feeding diets with different types of fat supplementation. Experimental diets were 1) a traditional diet (CON) administrated as unifeed and no supplemental fat 2) a diet containing 0.5 kg/days of whole flaxseed (FS) in substitution of an equal amount of cotton seed administrated in the unifeed. Formulation of experimental diets and chemical composition of the diets are shown in Table1. Cows were housed in tie stalls and individually fed; water was available ad libitum. The diets were fed twice daily and each group of cows was fed separately.

Feed Sampling and Analysis

The chemical composition of diets was determined by standard procedures (AOAC, 1990). The crude fiber and fiber fractions were determined by Fiber Cap, FC 221, FOSS. A representative sample of feed supplementation was collected for fatty acid (**FA**) analysis of feed according to O′ Fallon (2007). The fatty acid methyl esters (FAME) were analyzed on a Agilent 6890N gas chromatograph. Separation of the FAME was performed using a DB 23 fused-silica capillary column [60 m × 0.25 mm (i.d.) with 0.25μm film thickness**.** Operating conditions were: helium flow rate of 1.2 mL/min; FID detector at 250°C; a split-splitless injector at 240 °C and an injection volume of 1μL with a split ratio 1:50.

The initial column temperature was set at 60°C, increased to 180°C at 25°C/min and finally increase to 230°C at 6°C/min and held for 15 min. Individual FAMEs peaks were identified using standards from Matreya (Matreya, Inc., PA). Each fatty acid was reported as a percentage of FAME

Milk Sampling and Analysis

At t0, t15 and t30 of the experiment milk samples from each cow were collected at morning and afternoon milking during 30 days treatment period; a total of three sampling during month. One aliquot was stored at -20°C for fatty acid analysis. Fresh samples were used for chemical analysis consisting in the following measurements: pH (GLP 21 Crison, Spain), total protein, casein, fat and lactose content using an infra red spectrophotometer (Milko Scan 133B; Foss Electric, Hillerød, Denmark) according to the International Dairy Federation standard (IDF, 1990), and somatic cell count (**SCC**) using a Foss Electric Fossomatic 90 cell counter (IDF, 1995); evaluation of the renneting characteristics (clotting time, rate of clot formation, and clot firmness after 30 min) measured by a Foss Electric formagraph (Foss Electric, Hillerød, Denmark). The milk coagulating index (CoI) was calculated as the clot firmness to clotting time + rate of clot formation ratio. Milk energy output was calculated according to Crovetto and Honing's formula (1984).

Milk fat was extracted according to the procedure of Luna et al. (2005) and transesterification of fatty acids according to ISO-IDF (2002) procedures, as reported in Caroprese et al. (2010). Briefly, fatty acid methyl esters were separated and measured using a gas chromatograph (Agilent 6890N) equipped with CP-Sil 88 fused-silica capillary column

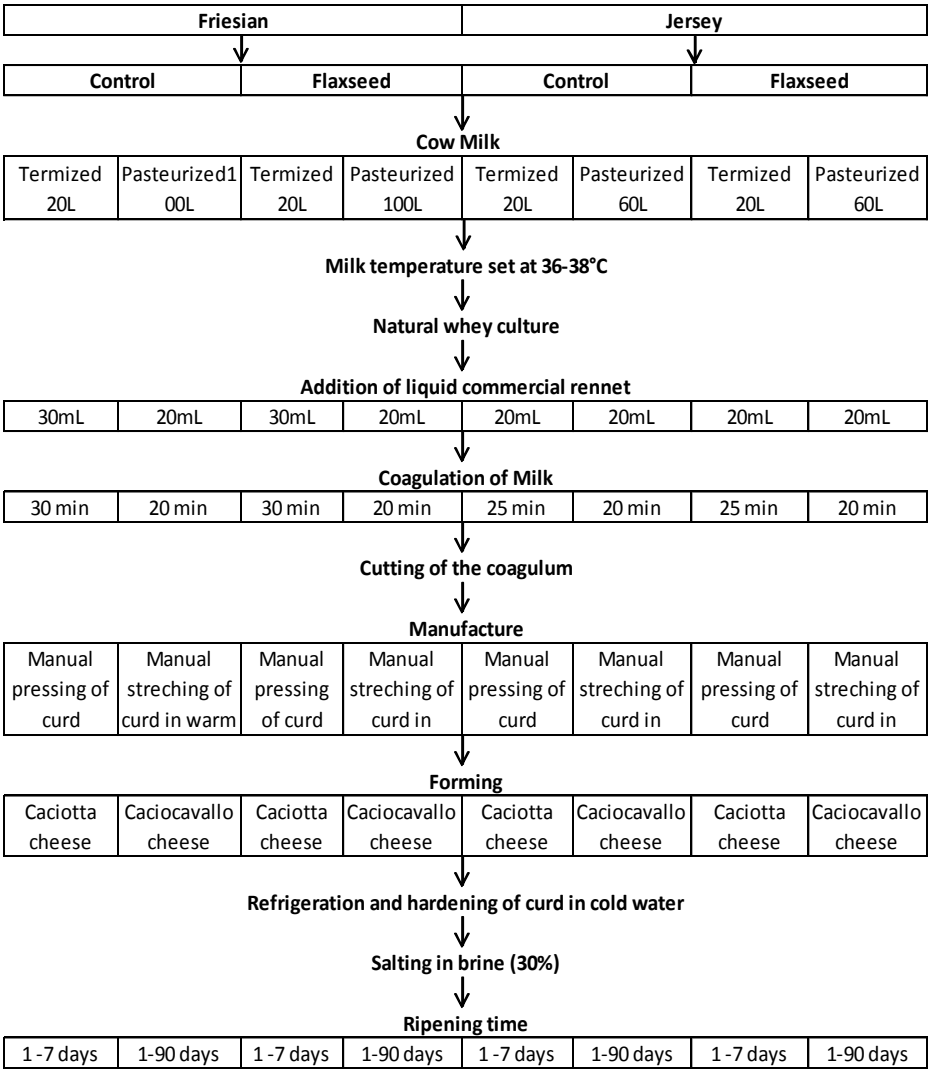
(100 m × 0.25 mm i.d. with 0.25-µm film thickness). Operating conditions were a helium flow rate of 1 mL/min, a flame-ionization detector at 260°C, a split-splitless injector at 260°C, and an injection volume of 1 µL with a split ratio 1:50. The temperature program of the column was set at 100°C with a subsequent increase to 240°C at 3.5°C/ min and held for 15 min. Fatty acid were reported as grams per 100 grams of FA. The content of short-chain fatty acids (C4:0 to C12:1, SCFA), medium-chain fatty acids (C14:0 to C16:1, MCFA) and long-chain fatty acids (>C18:0, LCFA) were calculated. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and PUFA were calculated. The desaturase indexes were calculated as described by Kelsey et al. (2003). Atherogenic (AI) and thrombogenic (TI) indices were calculated according to Ulbricht and Southgate (1991) formula:

AI = (C12:0 + 4 × C14:0 + C16:0)/[Σ MUFA + Σ PUFA(n-6) and (n-3)];

TI = (C14:0 + C16:0 + C18:0)/[0.5 × Σ MUFA + 0.5 × Σ PUFA(n-6) + 3 × Σ PUFA(n-3) + (n-3)/(n-6)].

Cheese production and cheese composition

The protocol for production of Caciotta and Caciocavallo cheese is reported in the scheme below.



Four cheesemaking were performed in two days, two per each day. Caciotta cheese samples were collected immediately and at 7 days of

ripening, while Caciocavallo cheese samples were collected immediately and at 90 days of ripening and analysed in duplicate. Moisture and ashes were determined according to the International Dairy Federation standards (1986). Total nitrogen (TN), water soluble nitrogen (WSN), non casein nitrogen (NCN), and pH 4.6 soluble-N were determined as described in Albenzio et al. (2004); fat content was measured using a Soxhlet method by using petroleum-ether. Fatty acid profile was performed extracting methyl ester by O'Fallon 2007 method. From all samples were extracted methyl-ester as the method described. The fatty acid methyl ester (FAME) was separated with 1 mL of hexane solvent by mixing for 5 min and centrifuging at 500 rpm for 10 min. For the quantitative determination of the fatty acids has been constructed a calibration curve with a standard mixture of 50 fatty acids (GLC Reference standard 674, Nu-Check Prep, Inc. Elysian MN 56028, USA GLC standard Reference 674, Nu-Check Prep, Inc., Elysian MN 560 , USA) with the addition of standard CLA: C18:2-8t, 10c; C18:2-9c, 11t; C18:2-11c, 13t; C18:2-9t, 11c; C18:2-8c, 10c; C18:2-10c, 12c; C18:2 9c, 11c; C18:2-10t, 12c; C18:2- 8t, 10t; C18:2-9t, 11t; C18:2- 10t, 12t; C18:2-11t, 13t (GLC Reference standard UC-59M, Nu-Check Prep, Inc. Elysian MN 56028, USA). The fatty acids were separated using a capillary column (HP88 ; 100m x 0.25mm id, 0,20 mm film thickness, Agilent Technologies Santa Clara, USA), using a gas chromatograph mod. 6890N (Agilent Technologies Santa Clara, USA). The temperature of the injector and FID detector was 250°C, while the temperature ramp of the column was the following (Eulitz et al., 999): 70°C for 4 minutes, from 70°C to 175 ° C (13°C / min.), maintained at 175°C for 27 minutes, from 175 ° C to 215°C (4 °C / min), maintained at 215°C for 45 minutes. The

injection was performed in split mode 1:20. The pressure of the carrier gas (helium) was maintained constant at 175 kPa. Results were expressed as percentage of the total analysed fatty acids and saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids were determinated. Atherogenic and thrombogenic indices were calculated according to Ulbricht and Southgate (1991) as follows:

Atherogenic Index (AI) = (C12:0 + 4 x C14:0 + C16:0) / [(ΣMUFA + ΣPUFA (ω-6) and (ω-3)];

Thrombogenic Index (TI)= (C14:0 + C16:0 + C18:0) / [(0.5 x ΣMUFA + 0.5 x ΣPUFA (ω-6) + 3 x ΣPUFA (ω-3) + (ω-3)/ (ω-6)]; where MUFA is monounsaturated fatty acid and PUFA is polyunsaturated fatty acid.

Color

Color was measured by Konica Minolta CR400 (Conica Minolta Osaka, Giappone) using illuminant C. Results are expressed as lightness (L*), redness (a*), and yellowness (b*), according to the International Commission on Illumination (CIE) L*a*b* system.

Tbars analysis

MDA quantification was performed by OxiSelect™ TBARS Assay Kit. Before starting the analysis were prepared the following reagents: 1X TBA Acid Diluent, SDS Lysis Solution, TBA Reagent and 1X BHT Solution.

All samples were resuspended at 1-2 x 10^7 cells/ml in PBS containing 1X BHT, than the resuspended cells were sonicate on ice.

Initially was prepared a standard curve preparing a dilution series of MDA standards in the concentration range of 125 µM – 0 µM by diluting the MDA Standard in distilled or deionized water.

After analysis the quantification of MDA was performed by Spectrophotometric Measurement transferring 200 µL of the MDA standards and samples to a 96 well microplate compatible with a spectrophotometric plate reader and reading the absorbance at 532nm.

In vitro digestion Protocol

The simulated in vitro digestion model on cheese samples was performed according to Minekus et al. (2014) with some modification (Fig. 10)

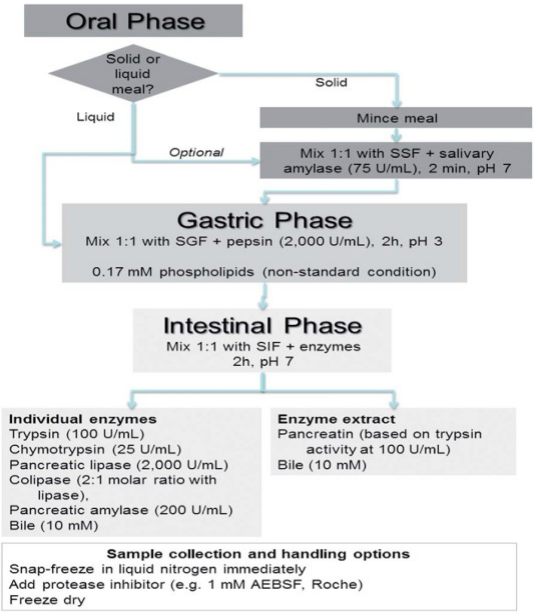


Fig. 10

The sample was minced to simulate the chewing mouth with a mincer. During the simulation of oral phase 5 grams of cheese sample, oral phase stock solution, $CaCl_2$ and 975 µl of water were mixed. During

gastric phase simulation, 10mL of liquid cheese samples after oral phase (oral bolus) were mixed with 7.5 mL of electrolyte stock gastric fluid (SGF), 1 mL porcine pepsine stock solution of 25000U/ml in Simulated Gastric Fluid (SGF) electrolyte stock solution (pepsin from porcine gastric mucosa 3200–4500 U/mg, Sigma), 5µL of 0.3 M $CaCl_2$ and with 1 M HCl and water to reach 20 ml of a pH 3 solution. Time of gastric digestion was 2 hours at 37°C. During the last phase, the intestinal phase, 20ml of samples after gastric phase (gastric chime) was mixed with of Simulated Intestinal Fluid (SIF) electrolyte solution, 4.0 ml of a pancreatin solution 800U/mL made up in SIF electrolyte stock solution based on trypsin activity, 2.5 mL of 160mM fresh bile, 40µL of 0.3 M CaCl2, mL of 1M NaOH to reach pH 7.0 and water up to 40ml. Time of intestinal digestion was 2 hours at 37°C. After the final step the sample was snap freezed immediately and stored at -80 °C until performing the subsequent analysis.

Faecal fermentation

Faecal fermentation were carried out following the method by Fooks and Gibson 2003 with modification. Before to performing analysis were prepared a media consisting of Tryptone water, Yeast Extract, Cysteine HCl, Bile salts, Glucose, Tween80, Hemin, Vitamin K1, Antifoam, NaCl, KH_2PO_4, K_2HPO_4, $CaCl_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, $NaHCO_3$ and 50mM phosphate buffer with KH_2PO_4 and K_2HPO_4.

To perform Faecal fermentation is necessary to produce a pool of faecal slurry in anaerobic hood; the faecal slurry was aliquotate and stored at -80°C.

To perform the analysis was used the fermentators Multifors1

Analysis of faecal fermented cheese samples

Faecal fermented samples were analyzed by a direct injection GC method for a rapid quantification of SCFA's by Zhao et al. (2005). Previously to the analysis is necessary an acidification of the samples as described in the method. Chromatographic analysis was carried out using an Agilent 6890N GC system equipped with a flame ionization detector (FID). A fused-silica capillary column with a free fatty acid phase (DB-FFAP 125-3237, J&W Scientific, Agilent Technologies Inc., USA) of 30 m × 0.25 mm i.d. coated with 0.25 µm film thickness was used. Helium was supplied as the carrier gas at a flow rate of 1.4 mL/min. The initial oven temperature was 100°C, maintained for 0.5 min, raised to 180°C at 8°C/min and held for 1.0 min, then increased to 200°C at 20°C/min, and finally held at 200°C for 5 min. The temperature of the FID and the injection port was 240 and 200°C, respectively. The flow rates of hydrogen, air and helium as makeup gas were 45, 450 and 30 mL/min, respectively. The injected sample volume for GC analysis was 1 µL, with a split 1:50 and the run time for each analysis was 17.5 min.

Pyrosequensing

Dna extraction

Total bacterial metagenomic DNA was extracted using a modified protocol which combined the repeat bead beating method with the QIAmp Fast DNA Stool Mini kit (Qiagen, UK). Briefly, 1ml of lysis buffer (500mM NaCl, 50mM Tris-HCl pH8.0, 50mM EDTA and 4% sodium dodecyl sulphate) was added to the bead beating tubes

containing the faecal sample. Samples were homogenised for 3 mins at max speed using the Mini Beadbeater (BioSpec). Samples were incubated at 70°C for 15mins to lyse the cells. Following centrifugation the supernatant was removed and the bead beating steps repeated. Following pooling of the supernatant, samples were treated with 10M ammonium acetate (Sigma Aldrich, Ireland) and then the DNA was pelleted and washed with 70% ethanol. The DNA was then RNAse and proteinase K treated. Finally the DNA was washed using buffers AW1 and AW2 (QIAmp Fast DNA Stool Mini kit; Qiagen, UK) and eluted in 200 µl of ATE buffer. DNA were determined spec- trophotometrically using the NanoDrop® ND-1000 (Thermo Scientific, Ireland). DNA yield was also determined fluorometrically using the Broad Range (BR) kit on the Qubit® Fluorometer 1.0 (InvitrogenCo.,Carlsbad,USA). Integrity of genomic DNA was determined by visualizing 1µL of extracted DNA on a 1% agarose gel (w/v) containing

SYBR Safe DNA Gel Stain (Invitrogen Co.,Carlsbad,USA) run in TBE bufferat 100V for 30 min. All values were normalized to allow for accurate comparisons between methods.

After DNA extraction of all faecal fermentated samples by QIamp Fast DNA stool Mini Kit (QIAGEN) was performed a pyrosequensing of all DNA extract samples. The following procedure is described by *Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System:*

The V3-V4 variable region of the 16S rRNA gene was amplified from 21 faecal DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). Two PCR reactions were completed on the template DNA. Initially the DNA was amplified with primers specific to the V3-

V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGT GTATAAGAGACAGCCTACGGGN GGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVG GGTATCTAATCC). Each PCR reaction contained DNA template (~10–12ng), 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM), 12.5 µl 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), PCR grade water to a final volume of 25µl. PCR amplification was carried out as follows: heated lid 110°, 95°C x 3mins, 25 cycles of 95°C x 30s, 55°C x 30s, 72°C x 30s, then 72°C x 5mins and held at 4°C. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose, 100V). Successful PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland). A second PCR reaction was completed on the purified DNA (5µl) to index each of the samples, allowing samples to be pooled for sequencing on the one flow cell and subsequently demultiplexed for analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5µl index 1 primer (N7xx), 5µl index 2 primer (S5xx), 25µl 2x Kapa HiFi Hot Start Ready mix, 10µl PCR grade water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 25. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit (Bio-Sciences, Dublin, Ireland), along with the broad range DNA quantification assay kit (BioSciences) and samples were then pooled in an equimolar fashion. The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. The sample pool

(4nM) was denatured with 0.2N NaOH, then diluted to 4pM and combined with 10% (v/v) denatured 4pM PhiX, prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform in the Teagasc sequencing facility, using a 2 x 300 cycle V3 kit, following standard Illumina sequencing protocols.

Statistical and Bioinformatics analysis

Data were processed using ANOVA with the REPEATED statement in PROC MIXED with CV (variance components) as covariance structure of SAS (SAS, 2013). Diet, time of sampling, breed, and their interactions were Fixed factors. When significant differences between means were found ($P < 0.05$), a Tukey post-hoc test was performed to adjust tests in multiple comparison.

200bp paired-end reads were joined using FLASH (fast length adjustment of short reads to improve genome assemblies) (Magoc et al., 2011)). Reads were further processed with the inclusion of quality filtering based on a quality score of >20 followed by subsequent removal of sequences below length threshold using QIIME (Caporaso t al., 2010). USEARCH v7 (64-bit) was then used for denoising and chimera detection ad well as clustering into operational taxonomic units (OTUs) at 97% identity. PyNAST (Caporaso et al., 2010) was used to align OTUs and taxonomy was assigned by using BLAST (Altschul et al., 1990) against the SILVA SSURef database release 111 (Quast et al., 2013). QIIME was used to generate alpha (Shannon, Simpson, Chao1, observed species and phylogenetic diversity) and beta diversities, (Bray Curtis, Weighted and UnWeighted Unifrac) distance matrices. Principal coordinate analysis (PCoA) plots were generated based on the beta diversity distance matrices and were visualised using EMPeror v0.9.3-dev (Vazquez-Baeza et al., 2013).

Phylum, Family and Genus level and the relative abundances of each taxonomic group were compared using the statistical test Mann-Whitney U (also called Wilcoxon or Wilcoxon rank sum test), with

continuity correction and any p-values lower than 0.08 are recorded along with the apparent trend.

The principal coordinate analysis plots (PCoA) generated using a weighted Unifrac distance matrix and the 3D graph is generated using EMPEROR, an open-source softwarepackage with an interactive and hardware-accelerated graphics, implemented with HTML, WebGL, Javascript and Pyton and tightly integrated with QIIME (Caporaso et al., 2010) and Pycogent (Knight et al., 2007). The number of metagenomic reads have been converted into Operational Taxonomic Units, or OTUs, which can then be considered. Mean relative abundance was calculated for each OTU, and the Wilcoxon.Mann-Whitney U test was performed on means.

RESULTS

& DISCUSSIONS

Milk Yield and Milk Composition

Table 2 shows the Least Square Means ± SEM of milk yield and composition of cows fed control diet (CON) and flaxseed (FS). Milk production was influenced only by breed. Fresian cows showed a higher milk production than Jersey cows ($P<0.001$).

The fat content was influenced by breed and, as expected, Jersey cows showed higher fat content than Friesian cows. Protein, casein and lactose contents were affected only by breed with values higher in Jersey cows than in Friesian. The clotting parameters were significantly influenced by diet ($P<0.01$ for clotting time; $P<0.001$ for rate of clot formation and curd firmness). FS diet reduced rate of clot formation and increased curd firmness in Friesian milk; it resulted in increased curd firmness in Jersey milk. As represented in the graph 1, Jersey cows showed lower values of r (rennet clotting time), and k20 (curd firming time) and higher values of a30 (curd firmness) than Friesian cows. Breed comparison revealed that milk from Jersey cows generally exhibited superior coagulation properties when compared with milk from Holstein-Friesian cows, as reflected in higher curd firmness in Jersey milk samples like in our study (Jensen et al., 2012).

Milk Fatty Acid Composition

Fatty acid profile is showed in Table 3; the content of C18:0 was significance influenced by breed and by the interaction breed x diet ($P<0.001$ and $P<0.01$, respectively). C18:0 showed higher values in milk from Jersey cows fed flaxseed than in milk from Friesian fed flaxseed. The increase in the content of C18:0 in the milk fat of cows fed the flaxseed diet has been reported by several authors (Mustafa et al., 2003;

Loor et al., 2005, Caroprese et al., 2010). In the present experiment the supplementation of the diet with flaxseed did not increase its content, even flaxseed administration resulted in higher C18:0 content in Jersey than in Friesian milk. However, the higher C18:0 content in Jersey milk could be attributed to the higher C18:0 content in Jersey milk apart from flaxseed supplementation (13.74 vs 12.19±0.21). C18:1trans11 showed higher values in Friesian milk than in Jersey milk (3.23 vs 1.28 ± 0.11, $P<0.001$) and in milk from flaxseed supplemented cows than in milk from CON cows (1.96 vs 2.55 ± 0.11, $P<00.01$). In addition, an effect of the interaction of breed x diet was found with higher contents measured in milk from Friesian than from Jersey flaxseed fed cows ($P<0.01$). As reported by Bell et al. (2006) the formation of C18:1trans11 in the rumen is a function of the overall increase in trans C18:1 accumulation in the rumen. Whole flaxseed administration succeeded in enhancing milk C18:1trans11 content, which is considered the main health promoting fatty acid in milk together with CLA. The diet affected also milk C18:1cis9 and C18:1cis11 content ($P<0.001$) with increased levels measured in milk from FS diet; in addition, higher values for both for C18:1cis9 and C18:1cis11 content were measured in Friesian milk than in Jersey milk (21.14 vs 19.92 ± 0.25 for C18:1cis9, $P<0.001$). Also Drackley et al. (2001) found higher contents of C18:1cis9 in milk from Holstein than from jersey cows. The increase of C18:1 isomers can be the result of a partial biohydrogenation in the rumen of the increase in linolenic acid by flaxseed diet and of the desaturation of C18:0 in the mammary gland (Kennelly, 1996). C18:2cis9cis12 content was influenced only by breed with higher percentages in Friesian group than in Jersey ($P<0.05$). Interestingly significant differences were found

in C18:3n-3, which was influenced by breed ($P<0.01$) and diet ($P<0.001$). The C18:3n-3 trend is showed in Figure 2. At the end of the experiment both milk from Friesian and from Jersey fed flaxseed displayed significantly higher contents than milk from both Friesian and Jersey CON cows. Differences between Friesian and Jersey milk in C18:3 content emerged also in Drackley et al. (2001) with higher C18:3 contents found in Friesian cows; however, in that study fat supplementation resulted in a reduction in milk C18:3. In the present experiment flaxseed supplementation resulted in an enhancement of C18:3 to such an extent that no differences between supplemented Friesian and Jersey cows were found at the end of the experiment according to Palladino et al. (2010). Among the CLA isomers in milk CLA c911t was significantly influenced by breed with higher contents measured in Friesian than in Jersey milk. In a previous experiment the supplementation of flaxseed in the diet resulted in significantly increase in CLA content of milk in Frisian cows; however, in that experiment the flaxseed supplemented was more than two-fold compared with the present experiment (Caroprese et al., 2010). On the contrary, in the present experiment though measuring a reduced CLA content in milk when compararing with the previous experiment an increase in both C18:3 and in C18:1trans 11 was found, suggesting that the reduction in the amount of flaxseed supplementation can also improve milk fatty acid profile with a consistent reduction of production costs. SFA and MUFA were influenced by breed ($P<0.001$) and diet ($P<0.001$); higher values in milk from Jersey cows then in milk from Friesian cows were found (70.95 vs 64.03 ± 0.32 for SFA and 23.74 vs 29.79 ± 0.28 for MUFA, respectively). Flaxseed supplementation resulted in a reduction of SFA

content (66.28 vs 68.65 ± 0.32) and an increase in MUFA (27.77 vs 25.76 ± 0.28). In addition, an effect of the interaction breed x diet was found for both SFA and MUFA ($P<0.05$); milk from FS group displayed lower SFA and higher MUFA than CON milk both in Friesian and in Jersey milk; in addition milk from Friesian cows supplemented with FS had lower SFA and higher MUFA than milk from Jersey cows supplemented with FS. PUFA content was influenced by breed ($P<0.001$). However, the diet resulted in increased content of n3 FA with increased content in milk from cows supplemented with flaxseed ($P<0.001$). Jersey cows showed lowest values n3/n6; on the contrary Palladino et al. (2010) showed highest values of the same ratio in Friesian cows. The Atherogenic and Trombogenic indexes were influenced by breed ($P<0.001$) and diet ($P<0.001$) with lower values in flaxseed group especially in Friesian cattle. Milk from FS cows was characterized by low Atherogenic and Trombogenic indexes, suggesting that, even at low level of supplementation, cows fed flaxseed yield milk with healthier features from a human perspective.

Cheese Composition

Chemical composition of Caciotta and Caciocavallo cheese produced with milk of Friesian and jersey cows fed with different diet (CON and FS) at two time of ripening (1 and 7) is showed in Table 4 and 5. The percentage of fat was higher in Caciotta from CON milk than from FS milk in Friesian at time 1 of ripening ($P<0.05$). A tentative explanation was that during the cheese-making there was a loss of fat in serum as observed by the higher percentage of fat in the whey from FS caciotta cheese-making in Friesian and Jersey respectively (1.51 ± 0.09 and 1.90 ± 0.09 vs 0.92 ± 0.09 and 1.07 ± 0.09, respectively). The loss of fat in the whey could also be ascribed to the formation of a softer gel after milk coagulation in FS cheese-making, according to Caroprese et al. (2013) leading to FS Caciotta with a lower fat content than CON. Differences in cheese-making between Caciotta and Caciocavallo cheese may account for the absence of differences between the Caciocavallo from the two dietary treatment at 1 day of ripening. In pasta filata cheese, curds were then milled, stretched, and moulded by hands in hot water of about 82°C until they were adequately smooth and elastic.

Protein and casein in Caciotta and Caciocavallo from milk of Jersey FS were higher as the milk from this group presented a slightly higher content of protein than CON. In both cheeses the moisture in Jersey breed was slightly higher due to the lower protein to fat ratio, resulting in lower syneresis according to (Bliss, 1988), related to the higher fat content and larger and more fragile fat globules. However, the cheesemaking process has to adapt to the differences in acidity development and syneresis (Bland et al., 2015)

The level of pH 4.6-soluble nitrogen, expressed as a % of Nsol/Ntot in the cheese, increased as ripening progress, in particular in Caciocavallo cheese because the time of ripening was greater than Caciotta cheese; a similar results was found in Gobetti et al. (2002). In addition, % Nsol/Ntot was higher in Caciotta cheese from Jersey milk at 7 days than in Caciotta cheese from Friesian milk, as a result of higher levels of protein and casein content of jersey than Friesian cheese. Moreover, this event could be also an outcome of the greater content of fat, protein and casein in the processed milk (Albenzio et al., 2010) from Jersey breed. Moreover Water soluble nitrogen contains numerous peptides, free amino acids and their degradation products (McSweeney & Fox, 1997). Aminoacids are important precursor of a range of catabolic reactions which produce volatile compounds essential for cheese flavour (McSweeney & Sousa, 2000).

The fatty acid profile expressed as (g/100g of total fatty acids) of Caciotta and Caciocavallo cheese produced with milk from Fresian cows fed control diet (CON) or flaxseed (FS) at two time of ripening is showed in Table 6 and 7. The flaxseed administration not influenced short chain fatty acids from C4 to C12 as Neveu et al. (2014) reported, even if at time ripening 1 day there was a difference (5.39 vs 2.46 ± 0.31); in this case diet had a small effect on short chain fatty acids synthesis only. Glasser et al. (2008) showed that some oleaginose seeds riched in C18:3ω3, as flaxseed, could have an inhibitor effect on the novo synthesis short chain fatty acids. In the present study it happened the same situation in Caciocavallo cheese. C16:0 was influenced only by breed, considering that Jersey breed produce a milk with an high fat content compared to Frisian breed, as mentioned above; therefore also

the fatty acids profile of some fatty acids is strongly influenced by breed.

C18:0 was not influenced by diet, but only by breed, with slightly higher values in Jersey cows than in Friesian, reflecting the fatty acids profile results of milk. The content of this fatty acid in raw milk could result significantly higher as others studies affirmed (DePeters et al., 1995; Morales et al., 2000). C18:1cis 9 content showed significante results ($P<0.001$) influenced by diet and breed in Caciocavallo cheese but however flaxseed didn't bring great effects in both cheeses; and this behavior could be attribuited to the low quantity of flaxseed administrated in cow's diet. Da Silva et al. (2007) reported that a marked effect of flaxseed on C18:1 fatty acids series it's apparent when the flaxseed administration in the diet is higher than 12% of dry matter in animal diet. In C18:1trans11 is more relevant the breed effect ($P<0.001$); in jersey cows the content of these fatty acid is lower than Friesian in both treatment and in both time of ripening probably due to an higher fat content in jersey breed that produce an early lipolysis (Cooper et al., 1911), infact was present an higher content of C18:0 in cheeses from Jersey breed, suggesting that the transformation of C18:3ω3 is carring out until C18:0, while in cheeses fron Friesian brees is blocked at C18:1, vaccenic acid.

In both cheeses the C18:3ω3 content shown a two fold increment, at bothh ripening time, but in Friesian breed only, suggesting that in this breed the flaxseed administration is resulted more efficacious and that this integration in cow diet brought to upgrade its content in milk and subsequently in cheeses.

The transfer of this fatty acid from animal diet to the milk or dairy products is well documented (Chilliard et al., 2001; Gonthier et al., 2005; Cortes et al., 2010).

The percentage of saturated fatty acids (SFA) resulted lower in both cheeses produced with milk of cows fed with flaxseed in Friesian breed only and at the same time is manifested an increase in monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). A similar trend is shown in literature in dairy products produced with milk of animal fed with flaxseed (Petit 2010; Oeffner et al. 2013; Cattani et al. 2014).

Color

Table 8 and 9 show the color parameters. The $a*$ values (red component) did not change according to diet in Caciotta cheese and data were in agreement with previous results (Hughes et al., 2012). In Caciocavallo cheese the $a*$ values were not influenced by diet in the inner part of cheese; however, a^* values were significantly influenced by time of ripening ($P<0.001$) but only in the outer side of Caciocavallo cheese. The decrement observed in both CON and FS Caciocavallo cheeses and in both breeds could be correlated to the fat content; Sabbagh et al. (2010) found that decreasing the fat content of cheese samples increases the a^* value. The higher values in fat content at 90 days of ripening in Caciocavallo cheese observed could be claimed to explain the reduction in $a*$ values in both breeds.

The b^* values (yellow component) in FS Caciotta cheese showed a significant reduction in both breeds ($P<0.01$ and $P<0.05$, inner and outer side of cheeses respectively). The same results were found in

Caciocavallo cheeses but only in the outer side of Caciocavallo cheeses. Increasing linseed dose in the ration could led to a slightly linear decrease in butter yellow index (Hurtaud et al., 2010). Moreover, the *b** values in Caciocavallo cheese were higher after ripening at 90 days in the outer side of cheese and inside Caciocavallo cheese from FS Jersey milk. The increase in *b** values has been related to the occurrence of proteolysis and the Maillard reaction, which decrease the luminosity due to the production of browning compounds (Lucas et al., 2008). These results were more evident in Caciocavallo than in Caciotta cheese as a result of greater time of ripening, and for the different manufacturing and different temperature used during cheesemaking (Prudencio et al., 2014).

*L** parameter indicates lightness and the capacity of an object to reflect or transmit light based on a scale ranging from 0 to 100. The *L** values in Caciotta cheeses remained constant in Friesian breed in both times of ripening as reported by Verruck et al. (2015) in which this parameter was stable during storage, and diet treatment; on the contrary, in Jersey breed at 7 days there was a reduction of *L** in the inner side and only in CON Caciotta in the outer part of cheese ($P<0.001$, and $P<0.05$, respectively). In Caciocavallo a decrement in both breed during time of ripening was found both inside and outside in the cheese. These results are probably due to both the quantity of moisture that reduced during time of ripening and to time of ripening which in Caciocavallo cheese is longer than Caciotta cheese (90days vs 7 days). As explained by Sabbagh et al., (2010) ripening time affects L values with a decrement of it during the ripening period, but a and b values increased. In our study

the increase in a and b values during ripening time occurred in the outer part of cheeses and in the inner part of Caciotta cheese.

Tbars

Oxidation of cheeses is shown in Table 10 and is expressed as mg MDA (malondialdehyde) per Kg of cheese (TBARS). In Caciotta Cheese no differences during ripening were observed, but only between breeds ($P<0.001$). In Caciocavallo cheese produced from Jersey milk there was a reduction in TBARS number at 90 days of ripening. Although malonaldehyde is a secondary products of lipid oxidation, TBARS could even reduced during ripening or storage time. These lower results found at 90 days in Caciocavallo cheese could be due to a mamonaldehyde reactions with proteins (Zapata et al., 1990) and maybe in Jersey breeds the presence of these interactions was more evident than in Friesian breeds. Fedele et al. (2001) showed that also manufacturing and pasteurization temperature of milk influence the TBARS numbers in cheese products.

Digested Fatty acids composition

Table 11 and Table 12 show the results of digest fatty acids profile of Caciotta and Caciocavallo cheese from Friesian milk. The results, in general, reflected the trend of cheese fatty acids profile suggesting that the digestion did not significantly influence the fatty acids in the digest. Their presence can have beneficial effects on the gastrointestinal tract and consequently on human health.

The C18:1*trans*11 content in digest from Caciotta cheese was slighlty higher in flaxseed group, but only at the beginning of ripening (3.30 vs

3.08 g/100g of FA ± 0.09); a similar trend was observed for C18:3ω3 (0.84 vs 0.31g/100g of FA ± 0.042); as a consequent the total ω3 conent was higher in the same samples. On the contrary, CLA9cis11trans was not significantly affected by flaxseed supplementation.

The increase of two-fold in C18:3ω3 found in Caciocavallo cheese from flaxseed group was found also in Caciocavallo cheese digest from flaxseed group (0.70 vs 0.36 g/100g of FA ± 0.10 in Caciocavallo cheese and 0.78 vs 0.31 ± 0.03 g/100g of FA in Caciocavallo digest). There was also an increment of MUFA in FS group (27.46 vs 24.98 g/100g fatty acids ± 0.37 at the beginning of ripening and 29.05 vs 27.05 ± 0.37 at time 90 of ripening). The differences in Caciocavallo digests were even more evident after 90 days of ripening for totalo ω3 content.

SCFA composition

The graph 2 and 3 show the results of least square means ± SEM of short chain fatty acids composition (g/100g of total short chain fatty acids) of faecal fermenta Caciotta and Caciocavallo cheese produced with milk from Fresian cows fed control diet (CON) or flaxseed (FS) at 0, 6, 12 and 24 hours time sampling. As described in a paper of De Fillippis et al. (2016), there was a relationship between food intake and faecal SCFA profile. Usually the faecal levels of acetic, propionic and butyric acid are correlated with the vegetables consumption, whereas valeric and caproic acid are linked to foods rich in protein and fat. In our study diet didn't significantly influenced the SCFA content except in Caciotta cheese, in which, diet influenced the butyric acid content ($P<0.005$) with lower values in FS group except for time sampling 6 at time 1 of ripening and for time sampling 24 at time 7 of ripening. As in

a paper of Patterson et al., (2013) the FS treatment didn't shown an increasing of butyric acid compared with the other dietary groups, in this case with the control diet. Anyway, both in Caciotta and Caciocavallo, as shown in the graphs the trend of SCFA, during time sampling is very similar, with an increase of acetic acid until time sampling 12h, and a decrement at time sampling 24h, maybe because the microorganism relased during this time have their maximum acid production activity until 12h. Only in Caciotta there was an increment of acetic acid at time sampling 24h in FS diet and also an higher value of propionic acid at time sampling 12h.

Pyrosequensing results

The Principal coordinate analysis plots (Fig. 1) generated using an weighted Unifrac distance matrix; Unifrac similarity is based on the presence and relatedness of bacteria in a sample, and includes the phylogenetic distance between taxa. Weighted Unifrac also accounts for the abundance of those bacteria. Fermenta cheeses samples clustered into relatively distinct groups based on weighted unifrac distance. However, the same bacteria are present in both groups, but in different numbers.

Taxonomy-based analysis of the assigned sequences is showed at the phylum, genus and family level of the DNA fermenta cheeses samples. Fig. 2 showed that the abundance in *Tenericutes* phyla is very low (under 0.02%); only fermenta FS Caciotta cheese at 1day of ripening showed a slightly higher percentage of *Tenericutes* with respect to the percentage in fermenta FS Caciotta cheese at 7 days ripening ($P<0.05$). *Tenericutes* is a bacterial phyla important to predict the liver metabolic

profiles and is associated with high hepatic levels of triglycerides and low hepatic levels of glycogen and glucose (Claus et al., 2011). The positive correlation of *Tenericutes* with hepatic triglycerides seems to be supported by previous work which showed that these bacteria are dominant in the gut ecosystem in an obese mouse model fed on a high-fat/high-sugar diet (Turnbaugh et al., 2008). *Tenericutes* in samples from both Caciotta and Caciocavallo cheeses were characterized by a low abundance, even more marked in fermenta FS Caciotta cheese at 7 days ripening.

A lower abundance of *Bacteroidetes* (Fig. 3) in fermenta FS Caciotta cheese at 1 day of ripening with respect to fermenta CON Caciotta cheese at 1 day of ripening ($P<0.05$), and with respect to FS Caciocavallo at 1 day of ripening ($P<0.01$) was found. These differences could be ascribed to differences in *Bacteroides* (Fig. 4) abundance which was lower in fermenta FS Caciotta cheese at 1 day of ripening than in fermenta from CON Caciotta cheee at 1 day of ripening ($P<0.05$), and FS Caciocavallo cheese at 1 day of ripening ($P<0.01$). In addition *Bacteroides* abundance was lower in fermenta FS Caciotta cheese at 7 days of ripening than in fermenta FS Caciocavallo cheese at 90 days of ripening ($P<0.05$). A decrement in the population of *Bacteroidetes*, which include the *Bacteroides* and *Bacteroidaceae* (Fig. 8) at the genus and family levels, respectively, were observed in other studies in which the decrement in *Bacteroidetes* population was positively correlated with the development of obesity (Ley et al., 2006; Turnbaugh et al., 2009). These bacteria are able to mantain a complex and beneficial relationship with the host when remain in the gut and in literature is well known their role as commensal (Xu and Gordon 2003). Specific members of the gut

microbiota may play a role in obesity and metabolic disorders rather than the phyla distribution (Zupancic et al., 2012). In our study the increase in *Bacteroidetes* in fermenta Caciocavallo cheese at 90 days of ripening, in particular in fermenta FS Caciocavallo cheese than in fermenta Caciotta cheese suggested that, comparing the two different cheese-making, Caciocavallo cheese replied better than Caciocavallo cheese in *Bacteroides* increase. Fermenta FS Caciotta cheese at 1 day of ripening showed the lowest values of *Bacteroides* suggesting that the diet of cows might affect the cheese behaviour in reducing in this phyla the microbiota constituents; this effects is reflected for this treatment only at 1 day of ripening, probably because the time of ripening on this kind of cheese increase the numbers of *Bacteroides* during digestion. On the basis of these considerations it could say that a fresh Caciotta cheese can induce a reduction in number, while Caciocavallo cheese with a greater time of ripening could able to increase the number of them and as Turnbaugh et al. (2006) affirmed, a fewer numbers of *Bacteroidetes* is veryfible in microbiota of obese patients and they are inclined to increase in patients that are going to loss weight. In a previous study propionate proportion among the total SCFA showed a significant positive correlation with the *Bacteroidetes* (Salonen et al., 2014). In fermenta FS caciotta cheese at 1 day of ripening an average increment of proprionate production in SCFA across 24h-sampling was found with respect to fermenta from CON caciotta cheese at 1 day of ripening. Based on previous observation the reduction in the abundance of *Bacteroidetes* in fermenta FS Caciotta cheese at 1 day of ripening can be attributed to the increase in propionic acid in the SCFA produced.

An increase in *Bifidobacterium* is showed in fermenta FS Caciotta cheese at 7 days of ripening and FS Caciocavallo cheese at 90 days of ripening with respect to FS Caciotta cheese at 1 day of ripening ($P<0.05$, Fig. 2B); this result suggests that the cheesemaking and the time of ripening could influence the microorganism distribution. *Bifidobacterium adolescentis* abundance showed in Fig. 5 it is one of the most abundant species of bifidobacteria in the human colon (Apajalahti et al., 2003), and thus has the potential to play a significant role in diet utilization and colonic health. An increase in the number and activity of bifidobacteria in the colon is desirable because the exogenous bacterial remain in a viable form during transit through the gastrointestinal tract and become active on reaching the colon, where appropriate physicochemical conditions for their growth occur. They are able to inhibit the growth of potential pathogen producing SCFA, may have immunomodulators functions and mantein lower blood cholesterol levels (Gibsonn and Roberfroid, 1994). Considering our results fermenta FS Caciocavallo at time of ripening of 90 days was characterized by the highest abundance in this genus, suggesting that its consumption could be able to increase its abundance, with respect to the same cheese at time of ripening 1 day and respect to the other kind in cheese manufacturing as FS Caciotta cheese at both time of ripening.

Significance differences in relative abundance in *Enterococcus* genus and *Enterococcaceae* were showed also in the Fig.6 and 9 Fermenta FS Caciotta cheese at time of ripening 1 day had the highest percentage of this kind of microorganism. In this sample the significance differences ($P<0.05$) are with respect to fermenta from FS Caciocavallo cheese at time 1 day of ripening, fermenta CON Caciotta cheese at 1 day if

ripening, and fermenta FS Caciotta chees at 7 days of ripening. Enterococci has a controversial role; in some studies are ascribed as probiotics (Franz et al. 1999, 2003), improving the intestinal microbial balance and, at the same time, enterococci have been associated with a number of human infections. Althought food products may contain Enterococcus bacteria and influence the intestinal microbiota composition, the consumption of some dairy products such as the Camembert cheese, that does not contain either enterococci or enterobacteria, leads to a significant increase (Firmesse et *al., 2007)*. In mouse models, Enterococcus can contribute to gut inflammation by compromising epithelial barrier integrity (Steck et al., 2011) and stimulating TNF production from macrophages (Kim et al., 2006). Fermenta Caciocavallo cheese at both treatment and time and Fermenta CON Caciotta cheese at 1 day of ripening showed the lowest values with respect to fermenta CON and FS Caciotta cheese at 7 days of ripening and overall respect to FS Caciotta cheese at 1 day of ripening that showed the highest percentage of this genus. These results suggest that Caciocavallo cheese was able to result in a lower level of this microorganism in gut.

As reported in Fig. 10 significance differences ($P<0.05$) in *Lachnospiraceae* family are showed in fermenta CON Caciotta cheese at 1 day of ripening with respect to FS Caciotta cheese at 1 day of ripening and between fermenta FS Caciotta cheese at 1 day of ripening and fermenta FS Caciocavallo cheese at 1 day of ripening. Microbiota preponderance about *Lachnospiraceae* following maintenance of a weight-reduced (WR) state and correlated with hormones known to influence energy homeostasis, suggest that the specific composition of the microbiota

may play a role in host energy balance in weight-perturbed individuals (Ravussin et al., 2012). *Lachnospiraceae*, can use lactate and acetate to produce butyrate (Louis & Flint 2009). These organisms have an important role in stabilizing the microbial ecosystem preventing the accumulation of lactate (Belenguer et al., 2007).

Microorganism belonging to *Lactobacillaceae* (Fig. 11) family showed the hightest values in fermenta from CON Caciocavallo cheese at 90days of ripening that is significantly different ($P<0.01$) with respect to fermenta FS Caciocavallo cheese at 90 days of ripening, with respect to fermenta FS Caciocavallo cheese a t 1 day of ripening ($P<0.05$) and with respect to fermenta CON Caciocavallo cheese at 1 day of ripening. Moreover significantly differences are showed between fermenta FS Caciocavallo cheese at 1 day of ripening and fermenta FS Caciotta cheese at 1 day of ripening ($P<0.05$). The *Lactobacillaceae* family is composed of the genera *Lactobacillus* and other bacteria that are not yet classified by microbial taxonomists; most of the known *Lactobacillaceae* species, which is a diverse group of Gram-positive, facultative anaerobic species commonly known as lactic acid bacteria because most convert sugars into lactic acid. Certain *Lactobacillus* species, are involved in in the anti-inflammatory/immune modulatory effects (Walk et al., 2010).

Summarizing the pyrosequensing results, in our study, FS Caciocavallo at 90 days of ripening was the sample that showed better results in microorganisms that may have beneficial effects on gut is, fermenta Caciocavallo cheese at 90 days of ripening presented an increase in *Bacteroidetes*, *Bacteroides* and *Bacteroidaceae*, phyla, genus and family respectively, in *Bifidobacterium, Lachnospiraceae,* whose precence is desiderable. On this sample the diet of dairy cows, had a positive effect

on the development on microorganism that may have a positive effects on gut health. Results suggest that probably the pasta filata cheesemaking associated with a longer time of ripening contributed to develop of above mentionated bacteria during gastrointestinal digestion and consequently during the passage in colonic tract.

The desiderable presence of *Lactobacillaceae* in not available in our samples, apart from fermenta CON Caciocavallo cheese at 90 days of ripening that showed slightly higher values respect to the other samples. Probably to increase the presence of this kind of microorganism in the gut is necessary to include them during the manufacturing cheeses.

CONCLUSIONS

The reduction in the amount of flaxseed supplementation can also improve milk fatty acid profile with a consistent reduction of production costs as suggested by The higher milk content of C18:3n3 in milk

Friesian and Jersey cows replied differently to the same flaxseed supplementation; Polyunsatured fatty acids are transferred in dairy products especially in Caciotta cheese, suggesting that probably the different cheese making influenced the transferring.

After in vitro digestion, fatty acids remain in the digest; their presence can have beneficial effects on the gastrointestinal tract and consequently on human health.

The presence and the amount of short chain fatty acids (SCFA) could suggest some changes of microbiological populations that could have beneficial effects on human health.

Fermenta Caciocavallo cheese at 90 days of ripening presented an increase in *Bacteroidetes*, *Bacteroides* and *Bacteroidaceae*, phyla, genus and family respectively, and in *Bifidobacterium, Lachnospiraceae,* whose precence is desiderable. The diet of dairy cows, with flaxseed administration, had a positive effect on the development on microorganism producing a positive effects on gut health. It could be supposed that pasta filata cheesemaking associated with a longer time of ripening contributed to increase the of above mentioned abundance bacteria during gastrointestinal digestion and consequently during the passage in colonic tract.

The introduction in the human diet of animal based food products obtained from animal fed flaxseed can influence the gut microbiota by

affecting both distribution and abundance of microbiota at family and genus level.

TABLES, GRAPHS & FIGURES

Table 1
Chemical composition, and NE_L of the experimental diets (DM basis).

	Diet	
	CON	**FS**
DM, %	95.14	93.11
Ether extract, %DM	3.39	5.01
CP, %DM	14.87	14.9
Ashes	8.43	7.27
ADF, %DM	24.42	23.27
NDF, %DM	37.85	37.56
ADL, %DM	4.4	2.8
Emicellulose	13.43	14.28
NE_L, Mcal/kg	1.51	1.51
Fatty acids, % total of fatty acids		
C14:0	1.84	1.81
C16:0	32.88	32.39
C16:1	0.93	0.92
C18:0	3.11	3.11
C18:1 *cis*-9	19.23	19.10
C18:2 *cis*-9, *cis*-12	29.99	29.73
C18:3n-3	1.05	2.07
C20:5n-3 (DHA)	0.85	0.83
C22:6n-3 (EPA)	0.32	0.31

[1] Lin Tech (Tecnozoo srl, Torreselle di Piombino Dese, Italy).
[2] CON diet ingredients: Non enzimatically browened soybean meal, sunflower oil seeds, whole, dried golden corn gluten feed, dried sugar beet pulp, yellow corn cobs, oat hay headed, wheat straw and middlings, whole cotton seeds with lint, solvent-extracted sunflower meal, $Ca(HCO_3)_2$, $NaHCO_3$, NaCl.

Table 2. Least Square Means ± SEM of pH, milk yield and composition of Fresian and Jersey cows fed control diet (CON) or flaxseed (FS)

	Friesian		Jersey			Effects,P		
	CON	**FS**	**CON**	**FS**	**SEM**	**Breed**	**Diet**	**Breed x Diet**
Production, Kg/d								
Milk yield	16.87a	16.05a	11.06b	11.51b	0.46	***	NS	NS
Fat yield	0.53	0.52	0.52	0.55	0.02	NS	NS	NS
Protein yield	0.58a	0.56a	0.45b	0.47b	0.02	***	NS	NS
Casein yield	0.45a	0.43a	0.35b	0.38b	0.01	***	NS	NS
Milk composition, %								
Fat	3.20b	3.31b	4.79a	4.81a	0.18	***	NS	NS
Protein	3.42b	3.50b	4.07a	4.12a	0.55	***	NS	NS
Casein	2.65b	2.69b	3.14a	3.28a	0.06	***	NS	NS
Lactose	5.01a	4.96ab	4.88b	4.90ab	0.03	**	NS	NS
pH	6.64	6.64	6.64	6.65	0.01	NS	NS	NS
SCC, cells10^3/ml	86.23	323.87	197.41	383.6	91.71	NS	*	NS
Reological parameters								
r (rennet clotting time)	11.84a	11.11a	8.79b	7.83b	0.41	***	**	NS
k20 (curd firming time)	4.24a	2.69b	2.37bc	1.74c	0.22	***	***	*
a30 (curd firmness)	27.91c	37.74b	38.16b	46.50a	1.55	***	***	NS

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0.001

Table 3. Least Square Means ± SEM of fatty acid composition (g/100g of total fatty acids) of Friesian or Jersey milk from cows fed control diet (CON) or flaxseed (FS).

	Friesian		Jersey			Effects,P		
	CON	**FS**	**CON**	**FS**	**SEM**	**Breed**	**Diet**	**BreedxDiet**
C4:0	6.52	6.35	6.89	6.82	0.20	*	NS	NS
C6:0	2.37b	2.16b	2.97a	3.02a	0.12	***	NS	NS
C8:0	1.45b	1.35b	1.88a	1.85a	0.06	***	NS	NS
C10:0	3.01b	2.62c	3.95a	3.65a	0.09	***	***	NS
C11:0	0.11b	0.12b	0.13a	0.13a	0.01	*	NS	NS
C12:0	3.30b	3.00b	4.29a	4.00a	0.10	***	**	NS
C13:0	0.13	0.15	0.16	0.16	0.01	NS	NS	NS
C14:0	9.99b	9.48b	10.68a	10.28a	0.16	*	**	NS
C16:0	25.48b	24.96b	26.96a	25.48b	0.34	**	**	NS
C17:0	0.16	0.26	0.22	0.21	0.02	NS	NS	*
C18:0	12.64bc	11.74c	13.22ab	14.16a	0.29	***	NS	**
C20:0	0.21a	0.20a	0.18a	0.14b	0.01	***	**	NS
C22:0	0.13a	0.09b	0.08b	0.10b	0.01	NS	NS	***
C14:1t	0.22	0.21	0.20	0.23	0.01	NS	NS	*
C14:1c	1.04b	1.15a	0.93c	0.99bc	0.02	***	***	NS
C15:1t	0.05b	0.04b	0.13a	0.09ab	0.01	***	*	NS
C16:1t	0.68b	0.91a	0.64b	0.61b	0.03	***	**	***
C16:1c	0.04b	0.05a	0.04b	0.04b	0.00	*	**	NS
C17:1t	0.12	0.07	0.06	0.06	0.02	NS	NS	NS
C18:1t6	0.00	0.00	0.08	0.10	0.02	***	NS	NS

C18:1t9	0.39	0.41	0.28	0.37	0.03	**	NS	NS
C18:1t11	2.67b	3.80a	1.24d	1.31c	0.15	***	**	**
C18:1c6	0.13a	0.02b	0.05ab	0.06a	0.02	NS	*	*
C18:1c9	21.55a	22.79a	18.29b	19.49b	0.35	***	***	NS
C18:1c11	0.68b	0.82a	0.45d	0.52c	0.02	***	***	NS
C19:1t7	0.17b	0.26a	0.14b	0.17b	0.01	***	***	***
C19:1t10	0.57	0.24	0.33	0.17	0.12	NS	*	NS
C20:1c11	0.05	0.05	0.07	0.06	0.01	NS	NS	NS
C20:1t11	0.18c	0.27a	0.11d	0.23b	0.01	***	***	NS
C18:2t9t12	0.09	0.08	0.09	0.08	0.01	NS	**	NS
C18:2c9c12	4.08	4.38	3.78	3.92	0.16	*	NS	NS
C20:2	0.03b	0.05a	0.03b	0.03b	0.02	***	NS	NS
C22:2	0.02b	0.03a	0.02c	0.03a	0.0001	***	***	NS
C18:3n3	0.26b	0.31a	0.22c	0.28bc	0.01	**	***	NS
C18:3n6	0.03b	0.02b	0.06b	0.10a	0.01	***	NS	*
C20:3n3	0.29a	0.27a	0.23b	0.23b	0.01	***	NS	NS
C20:3n6	0.12b	0.13b	0.17a	0.12b	0.10	*	NS	**
CLA9c11t	0.70a	0.79a	0.40b	0.40b	0.03	***	NS	NS
CLA10t12c	0.02b	0.04a	0.01c	0.01c	0.001	***	***	***
CLA11c13c	0.04a	0.04a	0.02b	0.03b	0.0001	***	NS	NS
CLA11c13t	0.004a	0.006a	0.001b	0.003ab	0.0001	***	*	NS
CLAt,t	0.01	0.01	0.02	0.02	0.0001	*	NS	NS
C20:4n6	0.01	0.01	0.01	0.01	0.0001	NS	NS	NS
C22:4	0.09a	0.07b	0.06c	0.06c	0.0001	***	***	**

C20:5n3	0.015a	0.015a	0.011b	0.013b	0.0001	***	NS	NS
C22:5n6	0.020a	0.017b	0.015b	0.015b	0.0001	***	NS	NS
C22:5n3	0.05	0.05	0.03	0.04	0.0001	***	**	NS
C22:6n3	0.02	0.02	0.02	0.02	0.0001	*	NS	NS
SFA	65.54c	62.51d	71.75a	70.04b	0.45	***	***	*
MUFA	28.53b	31.05a	23.00d	24.49c	0.40	***	***	*
PUFA	5.93b	6.41a	5.24b	5.46b	0.18	***	NS	NS
n6	5.27ab	5.70a	4.69ab	4.83ab	0.17	***	NS	NS
n3	0.61a	0.65a	0.49b	0.57a	0.01	***	***	NS
A.I.	2.01c	1.80d	2.68a	2.41b	0.07	***	***	NS
T.I.	2.56c	2.29d	3.32a	3.06b	0.04	***	***	NS

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0.001.

Table 4. Least Square Means ± SEM of chemical composition Caciotta cheese produced with milk of Fresian and Jersey cows fed control diet (CON) or flaxseed (FS) at two time of ripening (1-7d).

	time of ripening	Friesian		Jersey			Effects, P						
		CON	FS	CON	FS	SEM	Breed	Diet	time	Breed x Diet	Diet x time	Breed x time	Breed x Diet x time
pH	1	5.38c	6.55a	6.44a	6.50a	0.048	***	***	***	***	NS	*	***
	7	5.33c	5.73b	5.90b	6.40a								
Moisture %	1	52.58a	50.95ab	52.02a	53.14a	0,36	***	*	***	NS	***	NS	**
	7	44.15b	44.05b	52.66a	50.43ab								
Fat%	1	18.36c	16.66d	29.13ab	27.12b	0,56	***	**	*	*	**	NS	*
	7	15.06d	15.54d	30.46a	26.27b								
Ashes %	1	2.43d	2.58cd	2.00e	2.24d	0.04	***	***	***	***	***	NS	***
	7	3.68b	4.44a	2.94c	2.74c								
Protein %	1	20.26c	21.77bc	24.80b	29.75d	0.35	**	***	***	**	***	*	*
	7	22.41b	23.84b	17.86d	19.94c								
Casein %	1	18.52c	20.24bc	23.37b	27.90a	0.35	***	***	***	**	***	**	NS
	7	20.37bc	21.19b	16.33d	18.30c								
Whey Protein %	1	1.10b	0.89b	0.66b	1.07b	0.09	**	**	***	NS	NS	*	***
	7	1.02b	1.89a	1.02b	1.22b								
Nsol/Ntot %	1	3.23c	3c	2.96c	1.50d	0.07	***	***	***	***	***	*	***
	7	3.42c	2.81c	4.91a	4.32b								

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0,001

Table 5. Least Square Means ± SEM of chemical composition Caciocavallo cheese produced with milk of Fresian and Jersey cows fed control diet (CON) or flaxseed (FS) at two time of ripening (1-90d).

	time of ripening	Friesian		Jersey			Effects, P						
		CON	FS	CON	FS	SEM	Breed	Diet	time	Breed x Diet	Diet x time	Breed x time	Breed x Diet x time
pH	1	5.85a	5.74a	5.66a	5.28b	0,31	***	***	***	**	*	NS	NS
	90	5.43b	5.35b	5.30b	5.08c								
Moisture %	1	37.10b	43.06a	41.14a	42.71a	0,4	***	NS	***	***	NS	***	NS
	90	35.86b	35.01b	40.90a	36.11b								
Fat%	1	17.9d	16.32d	24.44bc	23.94c	0,56	***	***	***	***	***	***	***
	90	24.41ab	27.21b	20.33d	30.45a								
Ashes %	1	2.82d	2.46e	2.34e	2.29e	0,03	***	***	***	NS	***	NS	***
	90	3.40ab	3.32b	3.51a	3.01c								
Protein %	1	19.54d	18.58d	23.77c	27.43b	0,27	***	NS	***	***	***	***	***
	90	29.19a	28.39a	29.11a	28.15a								
Casein %	1	18.21c	17.15c	22.37b	26.00a	0,35	***	NS	***	***	***	*	**
	90	23.98ab	23.08ab	24.62a	24.58a								
Whey Protein %	1	0.70cd	0.79cd	0.41de	0.15e	0,09	***	NS	***	**	***	NS	NS
	90	2.78a	3.01a	1.81b	1.28c								
Nsol/Ntot %	1	1.71e	1.80e	2.28e	2.67e	0,09	***	***	***	NS	NS	***	NS
	90	9.89b	11.63b	10.69c	12.18a								

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0,001

Table 6. Least Square Means ± SEM of fatty acid composition (g/100g of total fatty acids) of Caciotta cheese produced with milk from Friesian and Jersey cows fed control diet (CON) or flaxseed (FS) at 1 and 7 days of ripening .

		Friesian		Jersey			Effects,P						
	time of ripening	**CON**	**FS**	**CON**	**FS**	**SEM**	**Breed**	**Diet**	**time**	**Breed x Diet**	**Breed x time**	**Diet x time**	**Breed x Diet x time**
C4	1	5.39a	2.46b	3.09b	1.84b	0.31	*	**	*	NS	**	***	*
	7	2.12b	2.89b	2.72b	2.32b								
C6	1	3.13a	1.9b	2.56a	1.68b	0.19	NS	NS	*	NS	NS	***	NS
	7	1.67b	2.2a	1.77b	2.11a								
C8	1	1.91a	1.21b	1.78ab	1.28b	0.11	NS	NS	*	NS	NS	***	NS
	7	1.13b	1.41ab	1.28b	1.57ab								
C10	1	3.69a	2.76b	4.04a	3.04b	0.17	**	NS	*	NS	NS	***	NS
	7	2.64b	3.11ab	3.02b	3.96a								
C12	1	3.83b	3.17c	4.29ab	4.22ab	0.1	***	NS	NS	**	NS	***	NS
	7	3.05c	3.38bc	3.86b	4.64a								
C14	1	10.73bc	10.59c	10.64bc	11.06b	0.07	***	***	NS	NS	***	***	**
	7	9.68d	10.55c	11b	11.58a								
C14:1	1	0.25d	0.88b	0.68c	0.72c	0.02	**	***	***	***	**	***	**
	7	0.63c	0.9a	1.02a	0.5								
C15	1	0.007f	1.19a	0.89e	1.02d	0.004	***	***	***	***	***	***	***
	7	1.09c	1.15b	0.005f	0.007f								
C16	1	26.8b	27.32b	27.65ab	28.73a	0.33	***	NS	*	NS	NS	**	NS
	7	27.99ab	26.48b	29.29a	28.85a								

C16:1	1	0.22b	1.89a	1.08a	0.95a	0.08	***	**	NS	***	***	**	**
	7	1.19a	0.95a	0.42b	0.2b								
C18	1	11.96b	12.54b	14.06ab	14.65a	0.24	***	NS	**	NS	**	**	NS
	7	13.95ab	13.1b	14.69a	13.97ab								
C18:1trans11	1	2.65ab	3.52a	1.91b	0.99b	0.23	***	NS	**	*	NS	NS	*
	7	3.82a	3.99a	1.92b	1.49b								
C18:1cis9	1	20.16b	23.18a	20.46ab	21.65ab	0.38	*	*	*	NS	NS	**	NS
	7	22.66a	22.38a	21.87ab	21.46ab								
C18:2trans9trans12	1	0.3a	0.06c	0.06c	0.12b	0.008	***	**	***	***	***	***	***
	7	0.09bc	0.07c	0.02d	0.13b								
C18:2cis9cis12	1	4.52c	5.13ab	4.68bc	5.11ab	0.05	*	***	*	***	**	**	***
	7	5.28a	4.92b	4.46c	5.21a								
C20	1	0.06bc	0.1b	0.12ab	0.15a	0.009	*	NS	*	NS	***	***	NS
	7	0.17a	0.11b	0.06bc	0.01c								
C18:ω3	1	0.28c	0.71a	0.4b	0.65a	0.02	**	***	**	***	***	**	NS
	7	0.39b	0.7a	0.34bc	0.43b								
C18:3ω6	1	0.002c	0.06b	0.06b	0.08ab	0.02	*	**	**	**	NS	NS	***
	7	0.12a	0.07ab	0.004bc	0.21a								
CLA9cis11trans	1	0.53a	0.56a	0.27b	0.35b	0.02	***	*	**	*	*	***	**
	7	0.55a	0.52a	0.35b	0.11c								
CLA10trans12cis	1	0.02	0.01	0.003	0.006	0.01	NS	NS	NS	NS	NS	NS	NS
	7	0.03	0.02	0.003	0.01								
CLA9cis11cis	1	0.004	0.006	0.007	0.01	0.01	NS	NS	NS	NS	NS	NS	NS
	7	0.04	0.01	0.008	0.007								
C20:2	1	0.009	0.06	0.01	0.02	0.01	*	NS	NS	NS	NS	*	NS

	7	0.05	0.03	0.01	0.007								
C20:3ω3	1	0.28a	0.01c	0.009c	0.01c	0.002	***	***	***	***	***	***	***
	7	0.01c	0.01c	0.23b	0.007c								
C20:3ω6	1	0.2a	0.01c	0.06c	0.07bc	0.01	NS	***	**	**	**	NS	***
	7	0.12b	0.09bc	0.19a	0.08bc								
C22	1	0.02	0.01	0.02	0.02	0.002	NS	NS	NS	NS	NS	NS	NS
	7	0.02	0.02	0.02	0.01								
C20:5ω3	1	0.02c	0.05a	0.03c	0.05a	0.0009	***	***	***	***	***	***	***
	7	0.03c	0.04b	0.03c	0.009d								
SFA	1	67.69a	63.82b	69.71a	68.83a	0.38	***	NS	*	**	NS	***	NS
	7	64.21b	64.99b	67.85a	69.65a								
MUFA	1	25,47c	29.36a	24.56c	24.73c	0.42	***	NS	*	**	NS	***	NS
	7	28,87ab	28.39b	26.32bc	24.54c								
PUFA	1	6.14bc	6.76a	5.67c	6.52ab	0.12	***	**	NS	*	NS	**	NS
	7	6.82a	6.56ab	5.8c	6.1b								
n3	1	0.64b	0.77a	0.45c	0.71a	0.02	***	***	***	***	*	***	***
	7	0.43c	0.76a	0.65b	0.44c								
n6	1	5.79a	6.04a	5.29b	5.92a	0.11	***	*	NS	***	*	*	*
	7	5.49a	5.86a	5.17b	5.78a								
AI	1	2.31b	2.01c	2.46ab	2.46ab	0.03	***	NS	*	**	**	***	NS
	7	1.95c	2.06c	2.4b	2.59a								
TI	1	2.8c	2.57d	3.2ab	3.09b	0.03	***	**	NS	***	NS	***	NS
	7	2.71c	3.09b	3.08b	3.28a								

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0.001.

Table 7. Least Square Means ± SEM of fatty acid composition (g/100g of total fatty acids) of Caciotta cheese produced with milk from Friesian cows fed control diet (CON) or flaxseed (FS) at 1 and 90 days of ripening .

		Friesian		Jersey			Effects,P						
	time of ripening	CON	FS	CON	FS	SEM	Breed	Diet	time	Breed x Diet	Breed x time	Diet x time	Breed x Diet x time
C4	1	1.68c	1.55c	1.94ab	2.06a	0.04	NS	***	NS	*	**	NS	NS
	90	1.83bc	1.72c	1.98ab	1.9ab								
C6	1	1.28bc	1.16c	1.67a	1.79a	0.04	***	NS	NS	**	**	NS	NS
	90	1.41b	1.31bc	1.65a	1.71a								
C8	1	0.88bc	0.77c	1.24a	1.33a	0.03	***	NS	NS	***	**	NS	NS
	90	0.99b	0.88bc	1.22a	1.31a								
C10	1	2.24bc	1.94c	3.21a	3.89a	0.06	***	NS	**	***	*	NS	NS
	90	2.53b	2.23bc	3.11a	3.55a								
C12	1	2.83d	2.55d	3.95b	4.08b	0.06	***	NS	***	***	*	*	**
	90	3.17c	2.87cd	3.8b	4.47a								
C14	1	9.69c	9.77c	10.99ab	11.15a	0.12	***	**	**	*	**	NS	NS
	90	10.39bc	10.51b	10.69ab	11.59a								
C14:1	1	0.6e	0.75c	0.71cd	0.45f	0.01	***	***	***	***	NS	***	***
	90	0.74c	0.9a	0.67d	0.83b								
C15	1	1.11b	1.15b	0.95d	0.96d	0.008	***	***	***	*	*	**	**
	90	1.18ab	1.21a	0.93d	1.05c								
C16	1	28.97a	28.05b	29.17a	29.64a	0.18	***	**	*	*	NS	NS	*
	90	28.75a	27.93b	29.07a	28.4ab								

C16:1	1	1.18b	1.17b	1.13c	0.21e	0.007	***	***	***	***	***	***	***
	90	1.23a	1.22a	1.1c	0.97d								
C18	1	14.23ab	14.28a	14.45a	14.96a	0.18	***	NS	***	NS	*	**	**
	90	12.86b	13.01b	14.84a	13.48b								
C18:1trans11	1	3.88a	3.83a	1.6ab	1.04b	0.37	***	NS	NS	NS	NS	NS	NS
	90	3.7a	2.94a	1.62ab	1.1b								
C18:1cis9	1	23.27b	24.86a	21.95cd	22.24c	0.11	***	***	**	***	NS	*	*
	90	22.83b	24.41a	22.19c	21.47d								
C18:2trans9trans12	1	0.17b	0.07d	0.07d	0.1c	0.007	***	***	**	*	***	***	***
	90	0.07d	0.28a	0.07d	0.09cd								
C18:2cis9cis12	1	5.37ab	5.11b	4.98c	5.1bc	0.03	***	NS	**	NS	NS	NS	NS
	90	5.47a	5.23b	4.95c	5.17b								
C20	1	0.15a	0.15a	0.13a	0.011b	0.005	***	***	***	***	***	***	***
	90	0.12a	0.13a	0.14a	0.14a								
C18:ω3	1	0.34d	0.7ab	0.43c	0.3e	0.007	***	***	***	***	***	***	***
	90	0.35d	0.72a	0.42c	0.6b								
C18:3ω6	1	0.08b	0.07b	0.07b	0.2a	0.01	**	**	*	**	**	*	**
	90	0.08b	0.09b	0.07b	0.09b								
CLA9cis11trans	1	0.52b	0.55b	0.05e	0.1d	0.06	***	***	***	***	**	***	***
	90	0.65a	0.67a	0.05e	0.44c								
CLA10trans12cis	1	0.01	0.02	0.005	0.01	0.003	**	**	NS	NS	NS	NS	NS
	90	0.01	0.02	0.004	0.008								
CLA9cis11cis	1	0.01	0.03	0.007	0.003	0.004	NS	**	NS	NS	NS	NS	NS
	90	0.008	0.02	0.006	0.01								
C20:2	1	0.004c	0.03bc	0.04b	0.008c	0.005	NS	NS	***	***	**	*	*

	90	0.005c	0.08a	0.04b	0.05b								
C20:3ω3	1	0.01b	0.03a	0.009b	0.002b	0.001	***	***	*	***	***	NS	***
	90	0.008b	0.01b	0.01b	0.01b								
C20:3ω6	1	0.1b	0.12a	0.004d	0.07c	0.003	***	***	NS	*	***	***	***
	90	0.09b	0.1b	0.06c	0.05c								
C22	1	0.02b	0.04a	0.02b	0.006b	0.003	**	NS	NS	**	*	NS	*
	90	0.02b	0.02b	0.02b	0.008b								
C20:5ω3	1	0.03c	0.04b	0.04b	0.007e	0.0004	***	***	***	***	***	***	***
	90	0.04b	0.06a	0.04b	0.06d								
SFA	1	63.72b	61.99b	68.26a	69.81a	0.4	***	NS	NS	**	NS	NS	NS
	90	63.9ab	62.44b	67.99a	68.22a								
MUFA	1	29.45a	31.06a	25.87b	24.55b	0.4	***	NS	NS	**	NS	NS	NS
	90	29.07a	30.1a	26.13b	24.98b								
PUFA	1	6.65c	6.88bc	5.78e	5.83d	0.04	***	***	***	**	*	***	***
	90	6.95b	7.18a	5.8e	6.72c								
n3	1	0.38c	0.79a	0.48b	0.31d	0.008	***	***	***	***	***	***	***
	90	0.41c	0.81a	0.47b	0.75a								
n6	1	6.44a	6.16b	5.37d	5.62c	0.03	***	***	***	***	NS	***	NS
	90	6.61a	6.65a	5.4d	6.06b								
AI	1	1.94b	1.83b	2.43a	2.57a	0.04	***	NS	NS	**	*	NS	NS
	90	2.04b	1.94b	2.36a	2.49a								
TI	1	2.76c	2.47d	3.18b	3.47a	0.04	***	**	**	***	**	**	**
	90	2.72c	2.46d	3.16b	2.99bc								

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0.001.

Table 8. Least Square Means ± SEM of color in Caciotta cheese produced with milk of Fresian and Jersey cows fed control diet (CON) or flaxseed (FS) at two time of ripening (1-7d)

		time of ripening	Friesian		Jersey			Effects, P						
			CON	FS	CON	FS	SEM	Breed	Diet	time	Breed x Diet	Diet x time	Breed x time	Breed x Diet x time
Inner	*a**	1	-2.50a	-2.43a	-2.83b	-3.09b	0.085	***	NS	**	**	***	*	***
		7	-2.17a	-2.56a	-3.94c	-3.08b								
	*b**	1	8.64cd	7.45d	9.27c	9.34c	0,36	***	**	***	NS	***	NS	***
		7	8.73cd	9.09c	16.70a	13.79b								
	*L**	1	94.53a	94.005a	95.28a	94.93a	0,5	***	NS	***	NS	***	NS	NS
		7	94.61a	93.40a	88.28b	90.02b								
outer	*a**	1	-2.40a	-2.23a	-2.71ab	-2.80b	0.12	***	NS	NS	NS	NS	NS	NS
		7	-2.39a	-2.51a	-2.86b	-2.73ab								
	*b**	1	8.60ab	7.49b	9.27a	8.89ab	0,31	***	*	NS	NS	NS	NS	NS
		7	8.16ab	8.19ab	10.19a	9.39a								
	*L**	1	95.05a	94.76a	96.90a	96.58a	0,54	NS	NS	*	NS	*	NS	NS
		7	95.72a	95.14a	94.24b	94.85a								

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***,P<0,001

Table 9. Least Square Means ± SEM of color in Caciocavallo cheese produced with milk of Fresian and Jersey cows fed control diet (CON) or flaxseed (FS) at two time of ripening (1-90d)

		time of ripening	Friesian		Jersey			Effects, P						
			CON	FS	CON	FS	SEM	Breed	Diet	time	Breed x Diet	Diet x time	Breed x time	Breed x Diet x time
Inner	*a**	1	-3.61a	-4.59b	-4.61b	-3.36a	0.18	**	NS	NS	***	***	NS	***
		90	-3.81ab	-3.66a	-4.71b	-4.72b								
	*b**	1	9.50b	9.59b	11.35ab	8.92bc	0,48	***	NS	**	NS	**	**	***
		90	6.89bc	6.16c	7.39bc	13.18a								
	*L**	1	90.42a	82.40b	91.12a	94.20a	1,31	**	NS	***	**	*	*	*
		90	65.22c	66.31c	64.76c	68.92c								
outer	*a**	1	-2.82a	-3.14a	-3.66a	-3.56a	0.21	**	NS	***	NS	NS	*	NS
		90	-4.80b	-4.56b	-5.51b	-4.66b								
	*b**	1	9.63b	7.83c	10.92b	10.82b	0,28	***	***	***	***	***	NS	NS
		90	13.63a	12.51a	14a	13.47a								
	*L**	1	92.27a	89.76a	94.96a	95.4a	1,14	*	NS	***	NS	*	NS	NS
		90	81.85b	82.81b	81.03b	83.04b								

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0,001

Table 10. Least Square Means ± SEM of TBARS Caciotta and Caciocavallo cheese produced with milk of Fresian and Jersey cows fed control diet (CON) or flaxseed (FS) at two time of ripening (1-7) and (1-90 d)

time of ripening	Friesian		Jersey			Effects, P						
	CON	FS	CON	FS	SEM	Breed	Diet	time	Breed x Diet	Diet x time	Breed x time	Breed x Diet x time
1	0.79a	0.78a	0.56b	0.63b	0,23	***	NS	NS	*	NS	*	NS
7	0.75ab	0.72ab	0.60b	0.65b								
1	0.46b	0.51b	2.28a	2.91a	0,78	***	NS	***	NS	NS	***	NS
90	0.66b	0.66b	0.71b	0.56b								

Significance: NS, not significant; *, P <0.05; **, P <0.01; ***, P<0,001

Table 11. Least Square Means ± SEM of fatty acid composition (g/100g of total fatty acids) of digested Caciotta cheese produced with milk from Friesian cows fed control diet (CON) or flaxseed (FS) at 1 and 7 days of ripening

					Effects, P		
	time of ripening	**CON**	**FS**	**SEM**	**Diet**	**time**	**Diet x time**
C4	1	7.54a	8.84a	0.41	NS	*	NS
	7	6.92b	8.04a				
C6	1	2.59	2.63	0.45	NS	NS	NS
	7	3.09	2.20				
C8	1	1.44	1.43	0.18	NS	NS	NS
	7	1.44	1.15				
C10	1	3.44	3.36	0.32	*	NS	NS
	7	2.37	3.17				
C12	1	3.73	3.46	0.42	NS	NS	NS
	7	2.76	3.54				
C14	1	10.63	10.9	0.87	NS	NS	NS
	7	9.73	10.43				
C14:1	1	0.76	0.97	0.10	NS	NS	NS
	7	0.78	0.74				
C16	1	26.48	24.63	0.47	NS	NS	*
	7	26.23	26.59				
C16:1	1	1.14	1.13	0.08	NS	NS	NS
	7	1	1.12				

C18	1	11.61	10.73	0.99	NS	NS	NS
	7	13.58	11.80				
C18:1trans11	1	3.08b	3.30b	0.10	NS	NS	**
	7	3.4a	3.13b				
C18:1trans9	1	0.043b	0.28a	0.02	**	**	**
	7	0.017b	0.022b				
C18:1cis9	1	19.94	20.26	0.72	NS	NS	NS
	7	21.56	20.4				
C18:2trans9trans12	1	0.25b	0.44a	0.03	*	*	**
	7	0.27b	0.23b				
C18:2cis9cis12	1	5.37	5.13	0.19	NS	NS	*
	7	4.79	5.49				
C20	1	0.04	0.03	0.02	NS	NS	NS
	7	0.07	0.04				
C18:3ω3	1	0.31b	0.84a	0.08	NS	NS	**
	7	0.58ab	0.29b				
CLA9cis11trans	1	0.3	0.33	0.01	NS	NS	*
	7	0.31	0.29				
C20:2	1	0.03	0.06	0.02	NS	NS	NS
	7	0.03	0.03				
C20:3ω3	1	0.37a	0.31b	0.01	NS	*	***
	7	0.29b	0.38a				
C22	1	0.15a	0.11b	0.002	*	NS	***
	7	0.11b	0.15a				

SFA	1	68.09	66.52	0.52	NS	NS	*
	7	66.7	67.532				
MUFA	1	25.26	26.25	0.59	NS	NS	NS
	7	26.99	25.75				
PUFA	1	6.4	6.78	0.19	NS	*	NS
	7	6.03	6.49				
n3	1	0.7b	1.2a	0.08	*	NS	**
	7	0.88b	0.68b				
n6	1	5.95	6.01	0.17	NS	*	NS
	7	5.42	6.04				

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0,001

Table 12. Least Square Means ± SEM of fatty acid composition (g/100g of total fatty acids) of digested Caciocavallo cheese produced with milk from Friesian cows fed control diet (CON) or flaxseed (FS) at 1 and 90 days of ripening

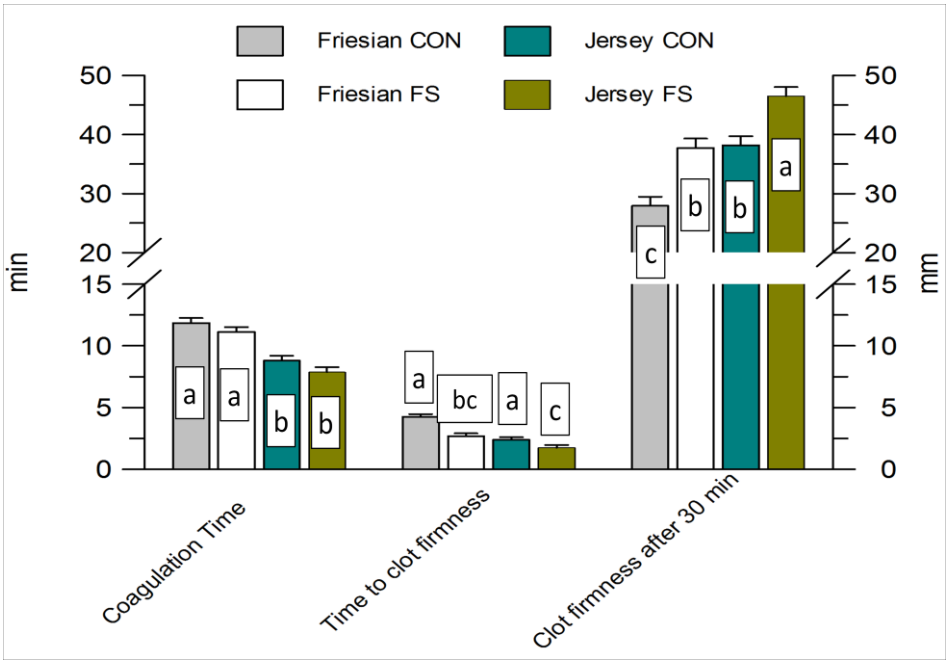
					Effects, P		
	time of ripening	**CON**	**FS**	**SEM**	**Diet**	**time**	**Diet x time**
C4	1	10.32a	4.71b	0.82	*	*	**
	90	4.76b	5.22b				
C6	1	7.00a	1.85b	0.74	**	**	**
	90	1.6b	1.34b				
C8	1	1.56a	1.19a	0.15	NS	*	NS
	90	1.03a	0.95b				
C10	1	2.13ab	3.07a	0.18	*	NS	*
	90	2.55a	2.62a				
C12	1	2.44	3.46	0.18	*	NS	*
	90	2.97	3.10				
C14	1	8.78b	10.25a	0.33	*	NS	NS
	90	9.48ab	10.30a				
C14:1	1	0.72b	0.71b	0.02	***	NS	**
	90	0.64b	0.89a				
C16	1	23.75c	26.98b	0.70	NS	*	**
	90	28.14a	26.17b				
C16:1	1	0.90b	1.09a	0.03	**	*	NS
	90	1.03a	1.10a				

C18	1	12.43b	13.58b	0.52	NS	*	*
	90	15.09a	13.68b				
C18:1trans11	1	3.46	3.68	0.09	NS	NS	NS
	90	3.50	3.60				
C18:1trans9	1	0.16	0.32	0.15	NS	NS	NS
	90	0.10	0.27				
C18:1cis9	1	19.44b	21.02b	0.42	*	**	NS
	90	21.48b	22.65a				
C18:2trans9trans12	1	0.35b	0.36b	0.03	*	NS	*
	90	0.3b	0.46a				
C18:2cis9cis12	1	4.43c	5.29a	0.05	***	**	***
	90	5.02b	5.14a				
C20	1	0.03c	0.04b	0.002	***	***	**
	90	0.04b	0.08a				
C18:ω3	1	0.57b	0.32c	0.03	*	*	***
	90	0.31c	0.78a				
CLA9cis11trans	1	0.27	0.08	0.12	NS	NS	NS
	90	0.45	0.27				
C20:2	1	0.04b	0.04b	***	***	***	
	90	0.04b	0.07a				
C20:3ω3	1	0.25c	0.37a	0.008	**	*	***
	90	0.34a	0.32b				
C22	1	0.11c	0.15b	0.01	NS	*	**
	90	0.17a	0.13b				

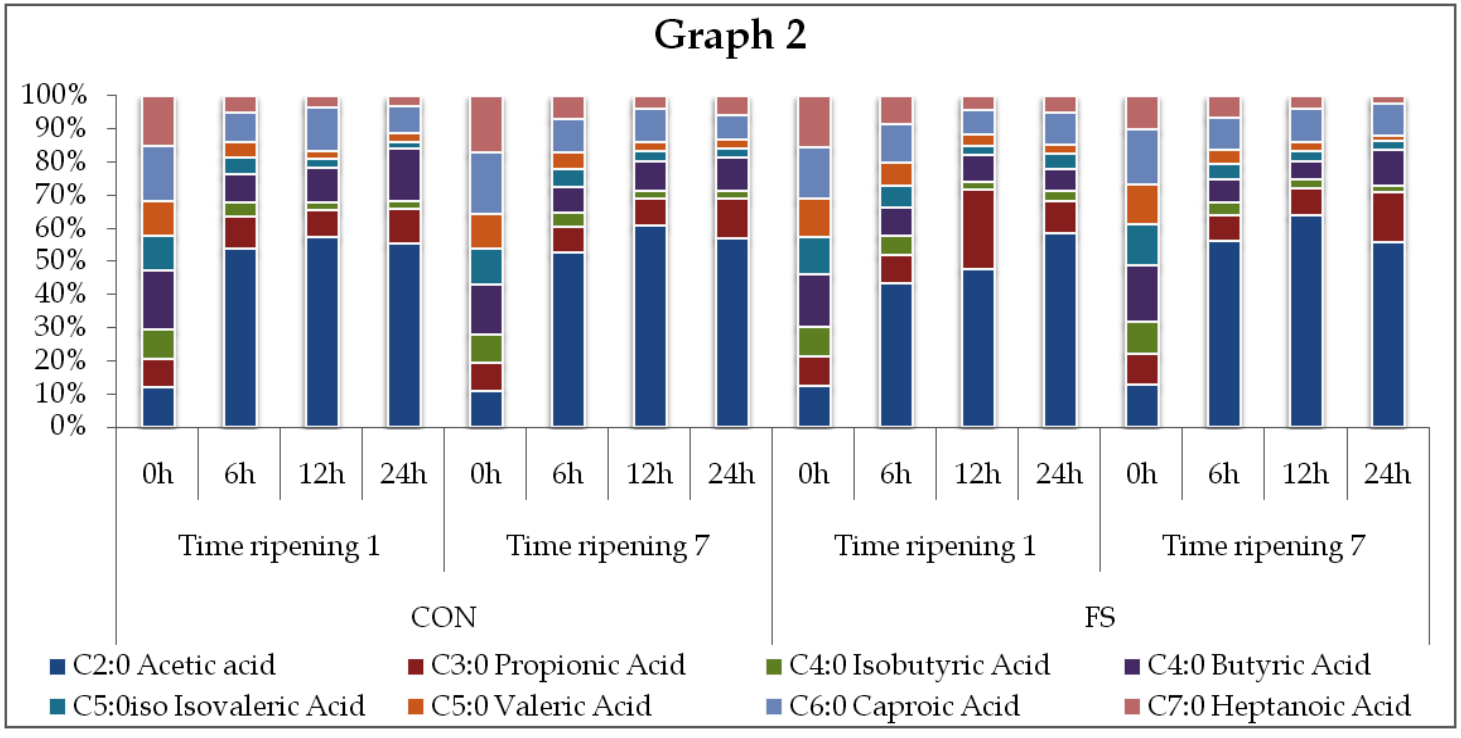
C20:5ω3	1	0.04	0.04	0.01	NS	NS	NS
	90	0.02	0.04				
SFA	1	68.95a	65.96b	0.52	**	**	NS
	90	66.42b	63.74c				
MUFA	1	24.98c	27.46b	0.37	**	**	NS
	90	27.05b	29.05a				
PUFA	1	5.67b	6.29a	0.20	*	*	NS
	90	6.23a	6.74a				
n3	1	0.87b	0.68c	0.04	**	**	***
	90	0.68c	1.13a				
n6	1	5.15b	5.89a	0.21	*	*	NS
	90	5.85a	6.07a				

Significance: NS, not significant; *, P<0.05; **, P<0.01; ***, P<0,001

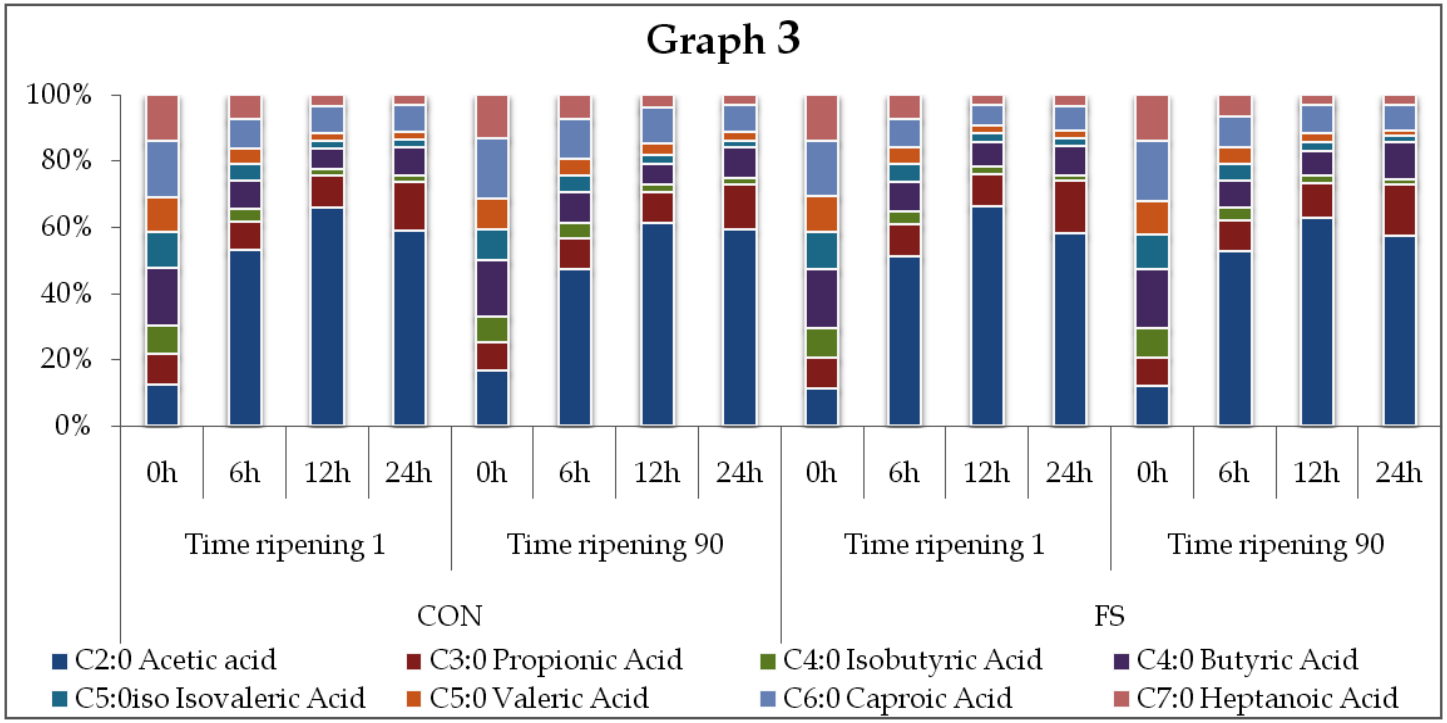
Graph 1



Graph 1. Reological parameters of milk produced from Friesian and Jersey cows fed control diet (CON) or flaxseed (FS)



Graph 2. Percentage of short chain fatty acids (SCFA) present in fermenta from Caciotta cheese produced with milk from Friesian cows fed control diet (CON) or flaxseed (FS) at 1 and 7 days of ripening



Graph 3. Percentage of short chain fatty acids (SCFA) present in fermenta from Caciocavallo cheese produced with milk from Friesian cows fed control diet (CON) or flaxseed (FS) at 1 and 7 days of ripening

Fig. 1

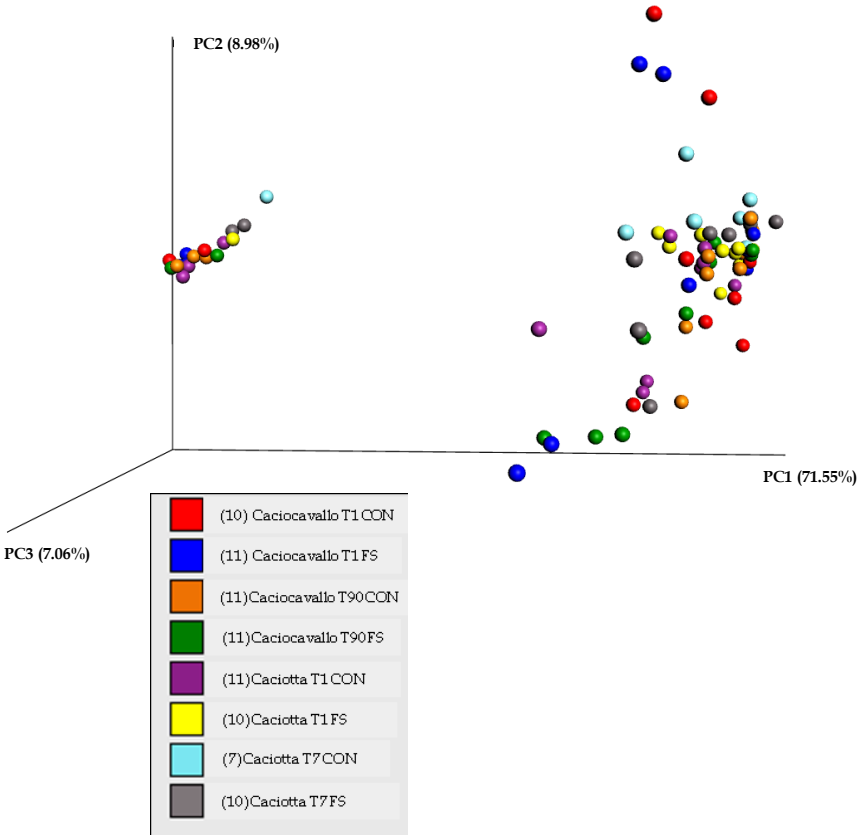


Fig. 1. Principal coordinate analysis **(PCoA)** using weighted UniFrac distances from DNA samples of Fermenta of Caciotta and Caciocavallo cheese (show in the legend)

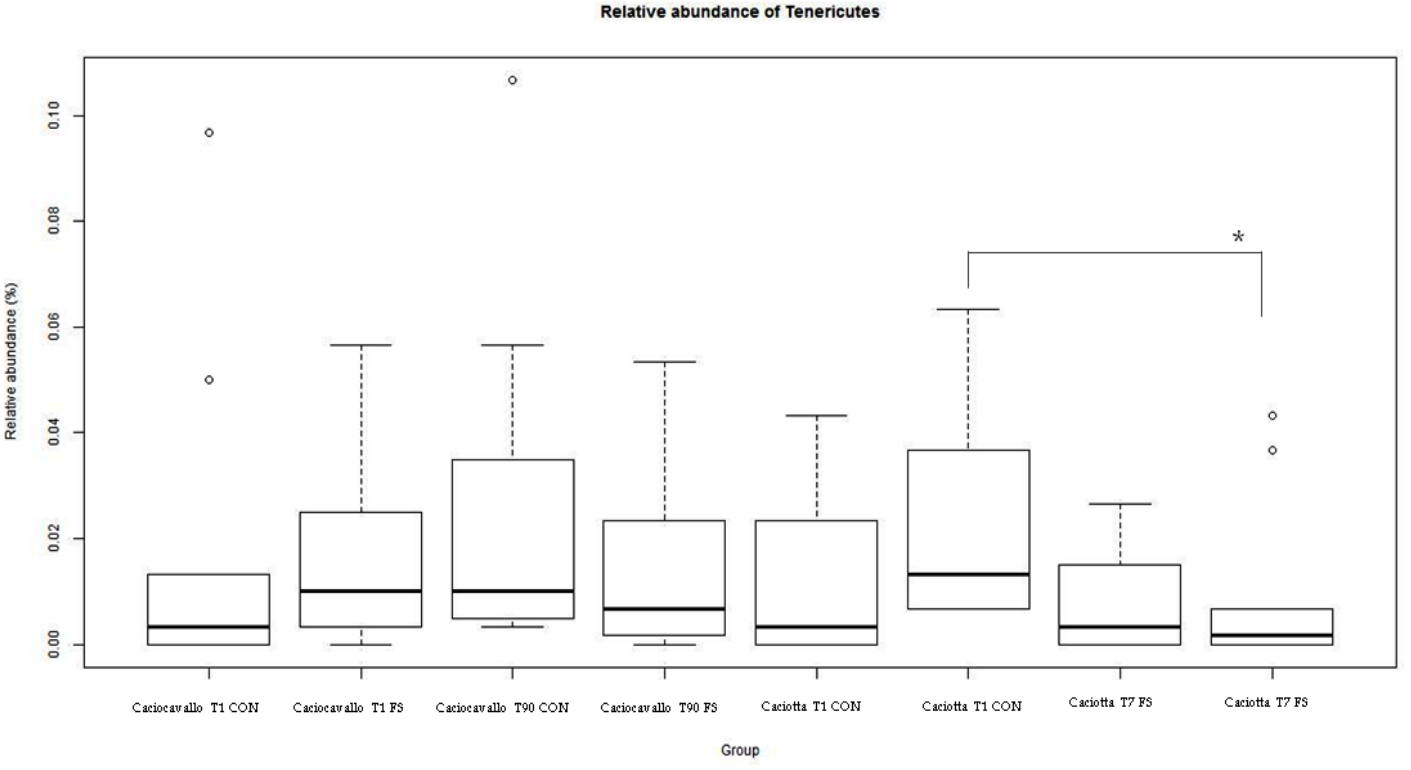


Fig.2 Phyla *Tenericutes* abundance in Fermenta samples

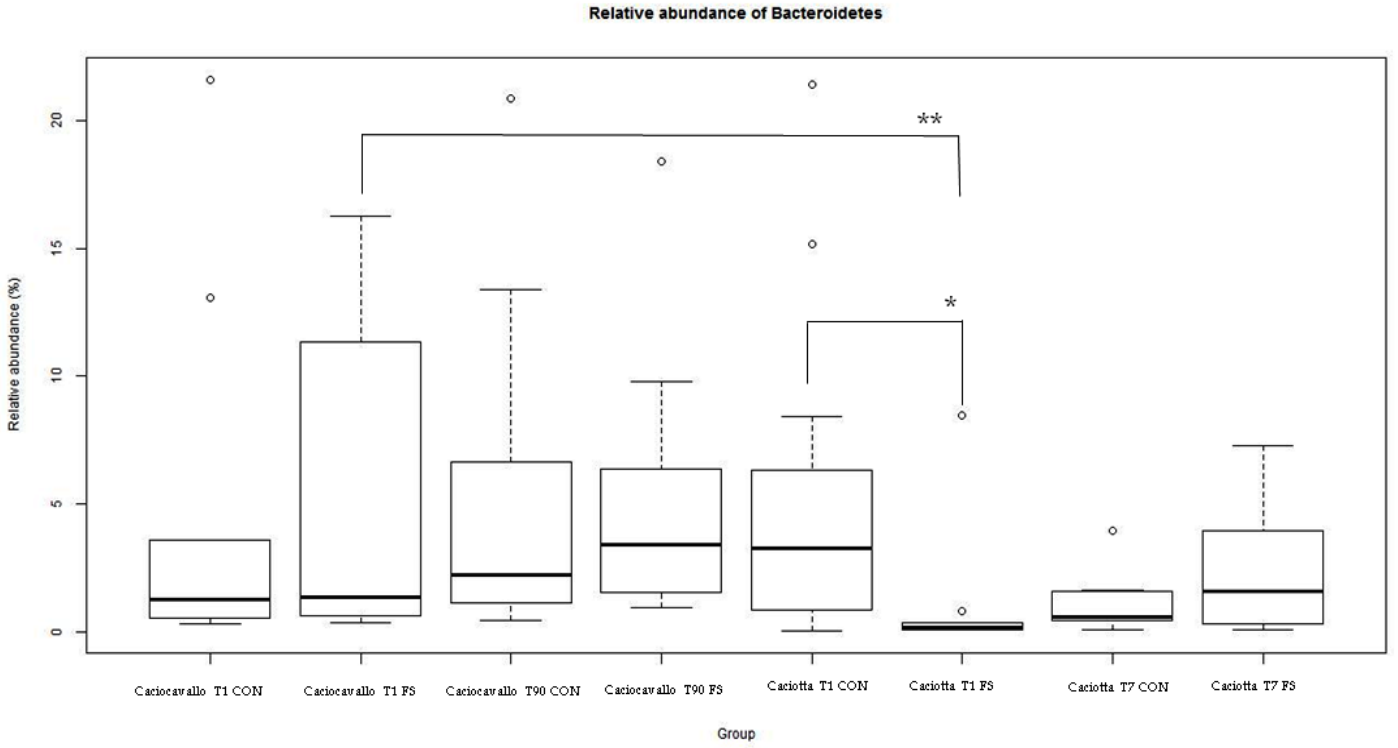


Fig.3 Phyla *Bacteroidetes* abundance in Fermenta samples

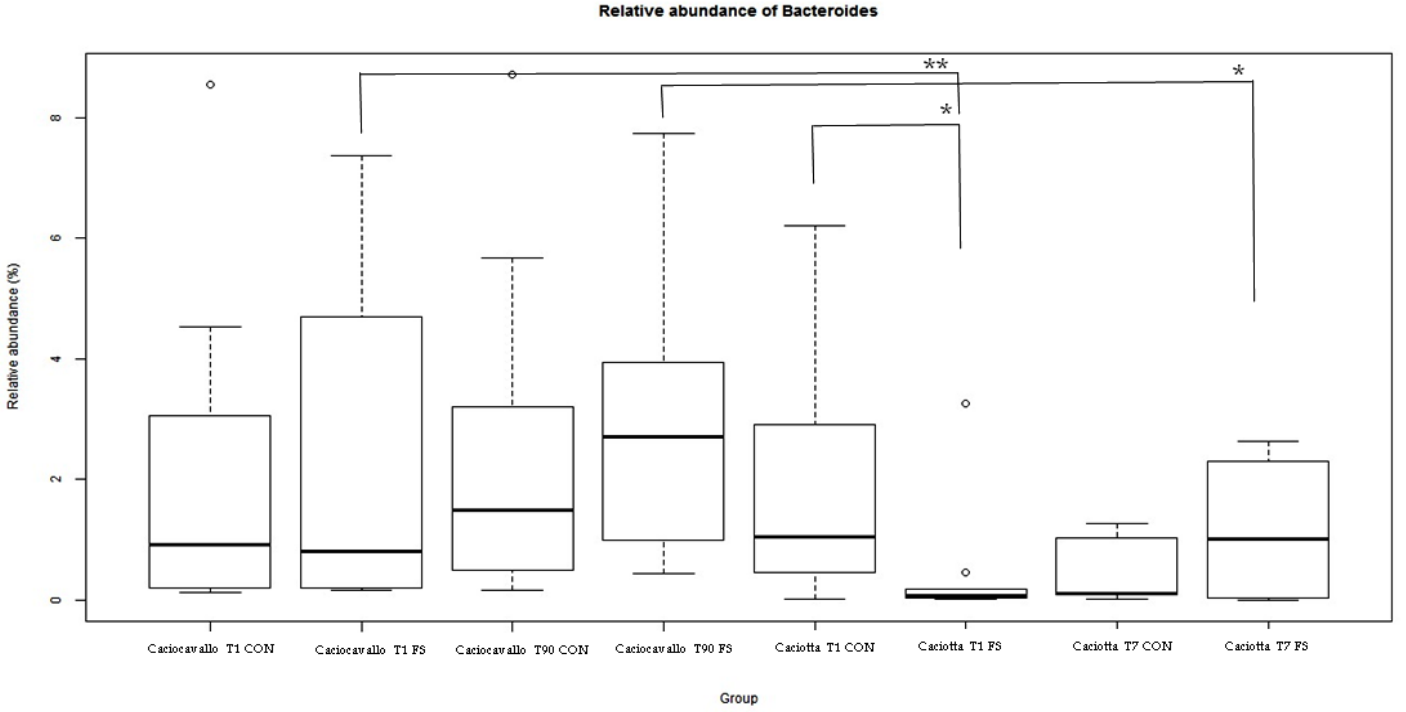


Fig.4 Genus *Bacteroides* abundance in Fermenta samples

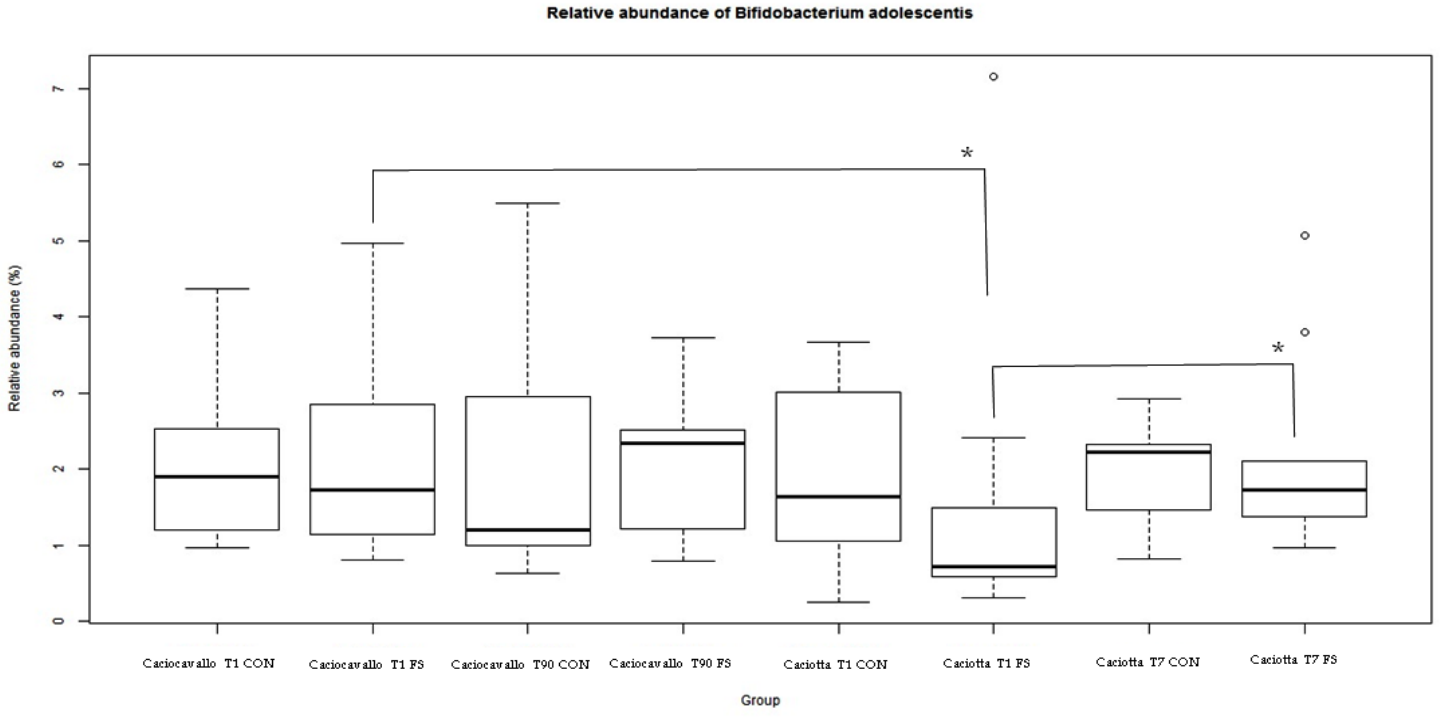


Fig.5 Genus *Bifidobacterium adolecentis* abundance in Fermenta samples

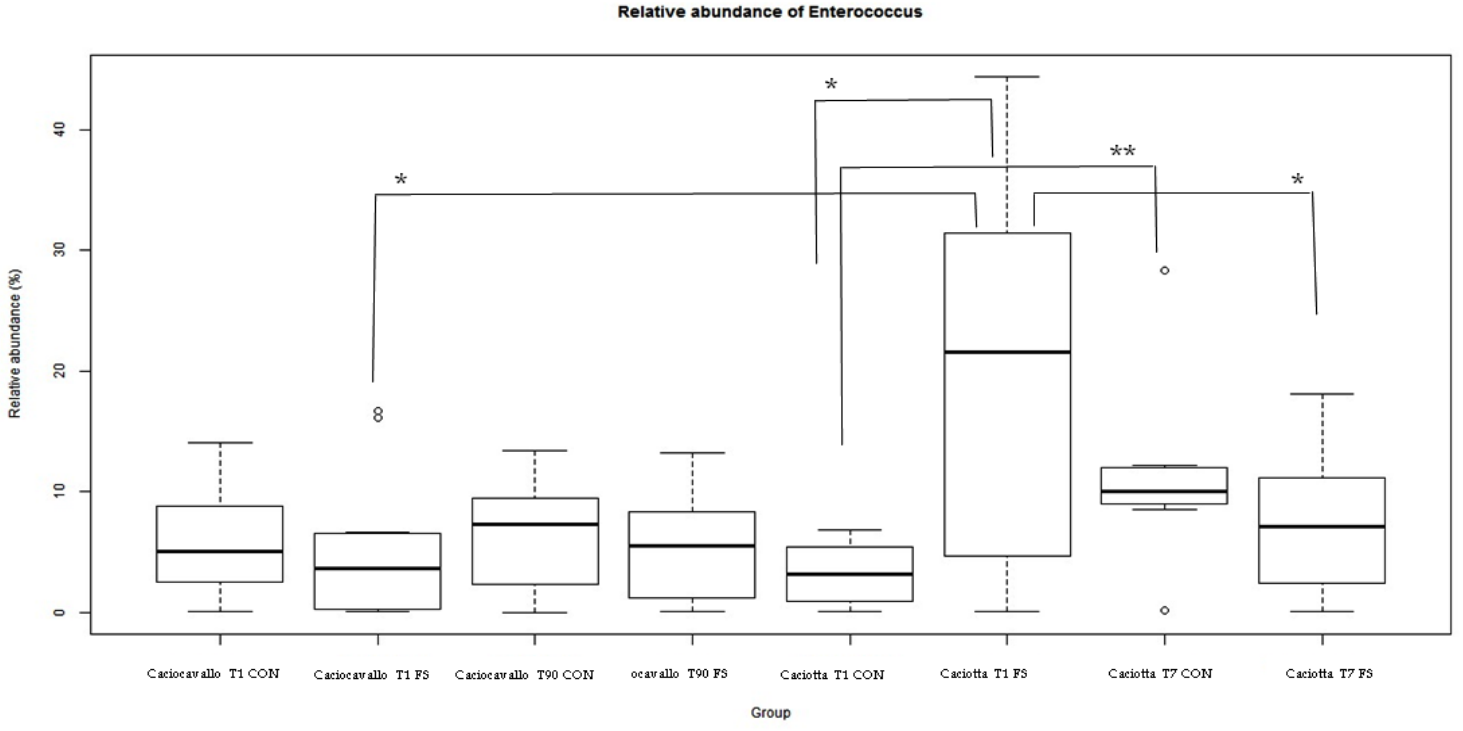


Fig. 6 Genus *Enterococcus* abundance in Fermenta samples

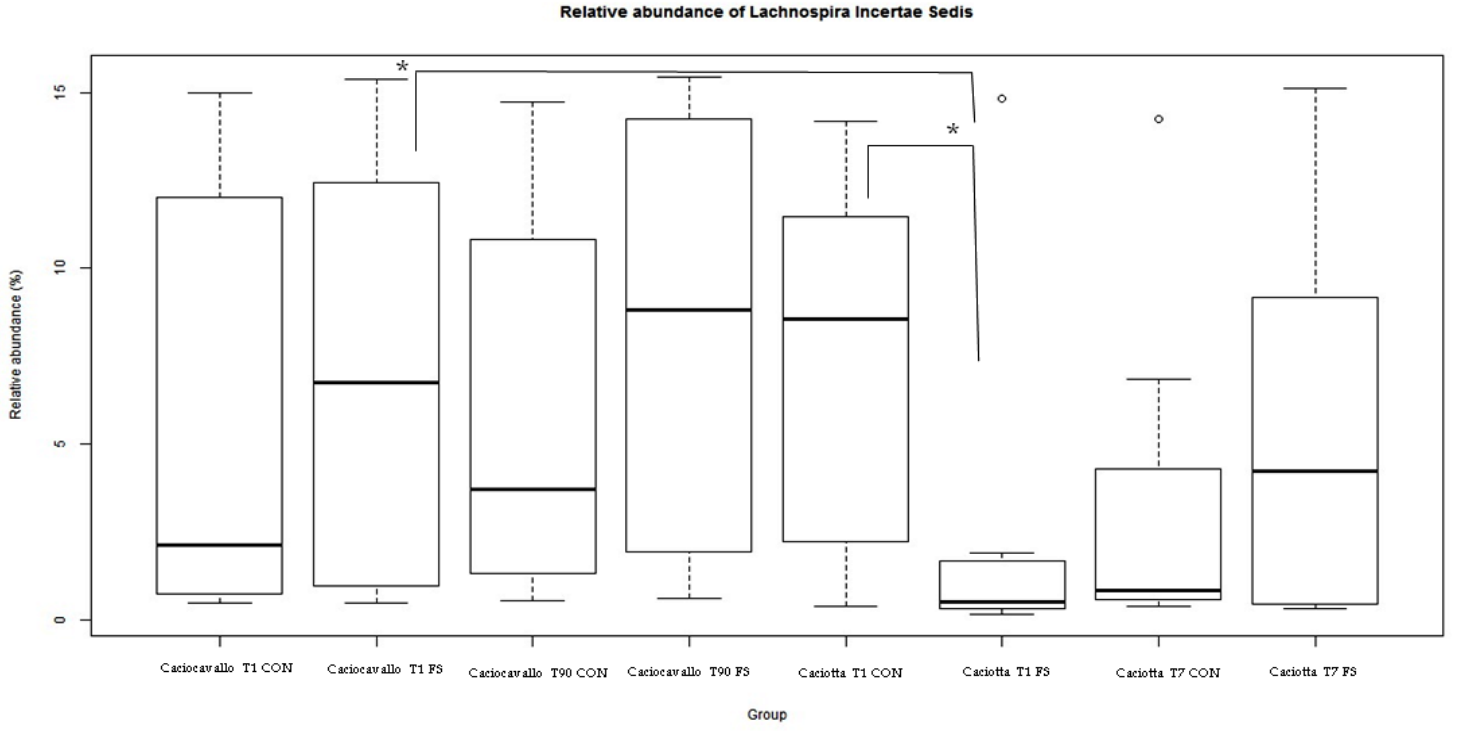


Fig. 7 Genus *Lachnospira incertae sedis* abundance in Fermenta samples

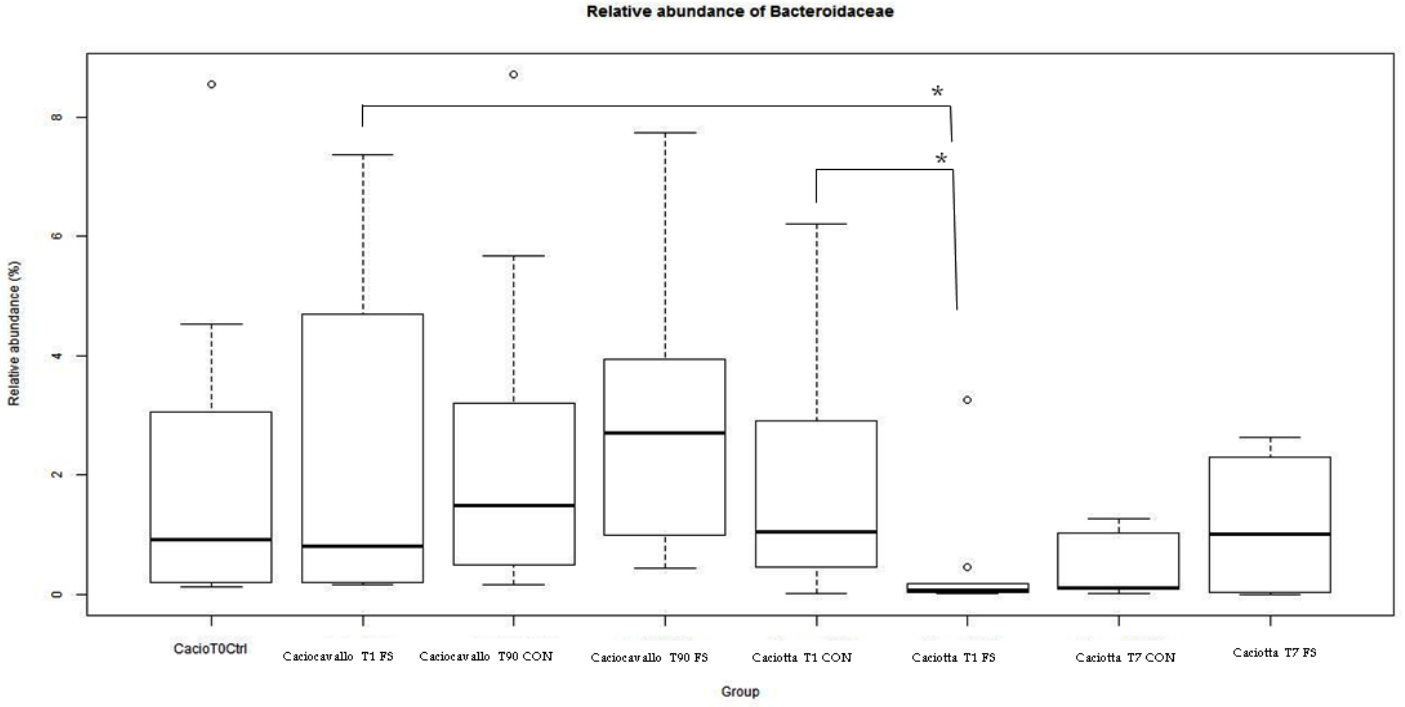


Fig. 8 Family *Bacteroidaceae* abundance in Fermenta samples

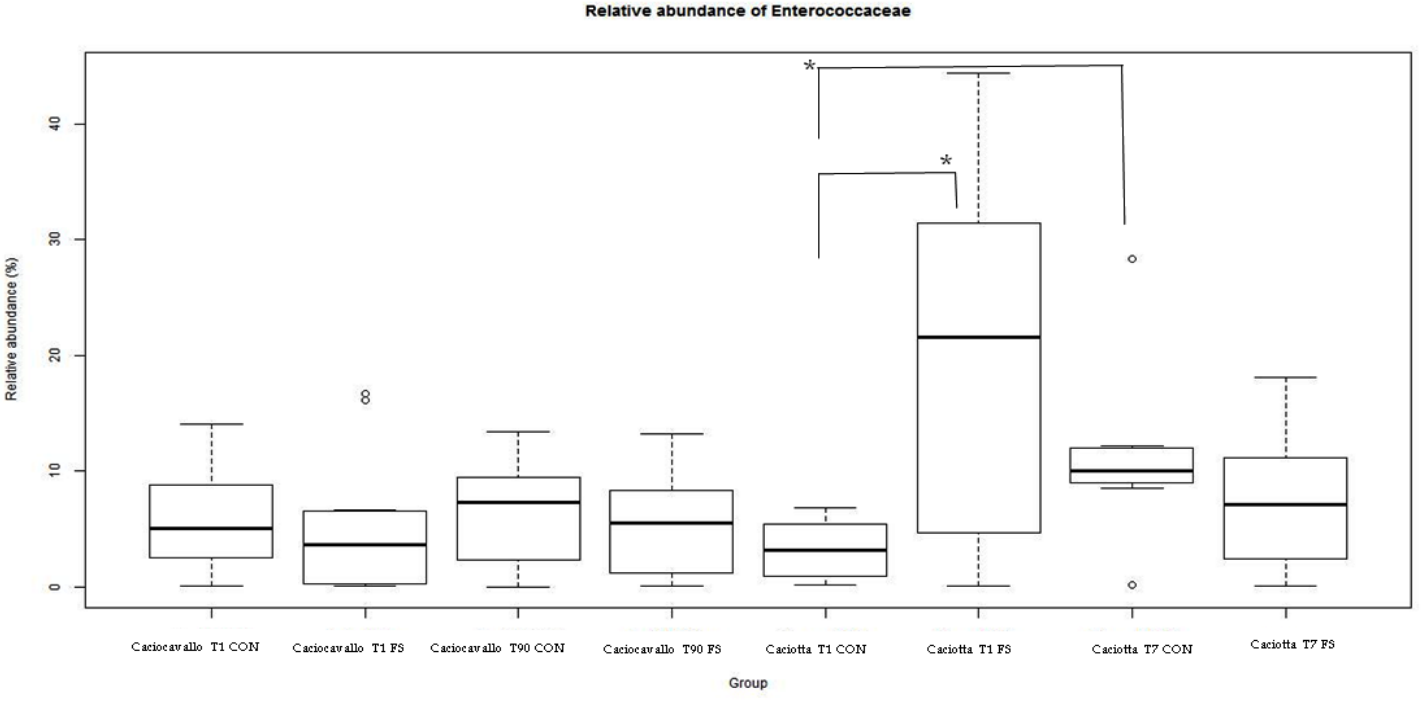


Fig. 9 Family *Enterococcaceae* abundance in Fermenta samples

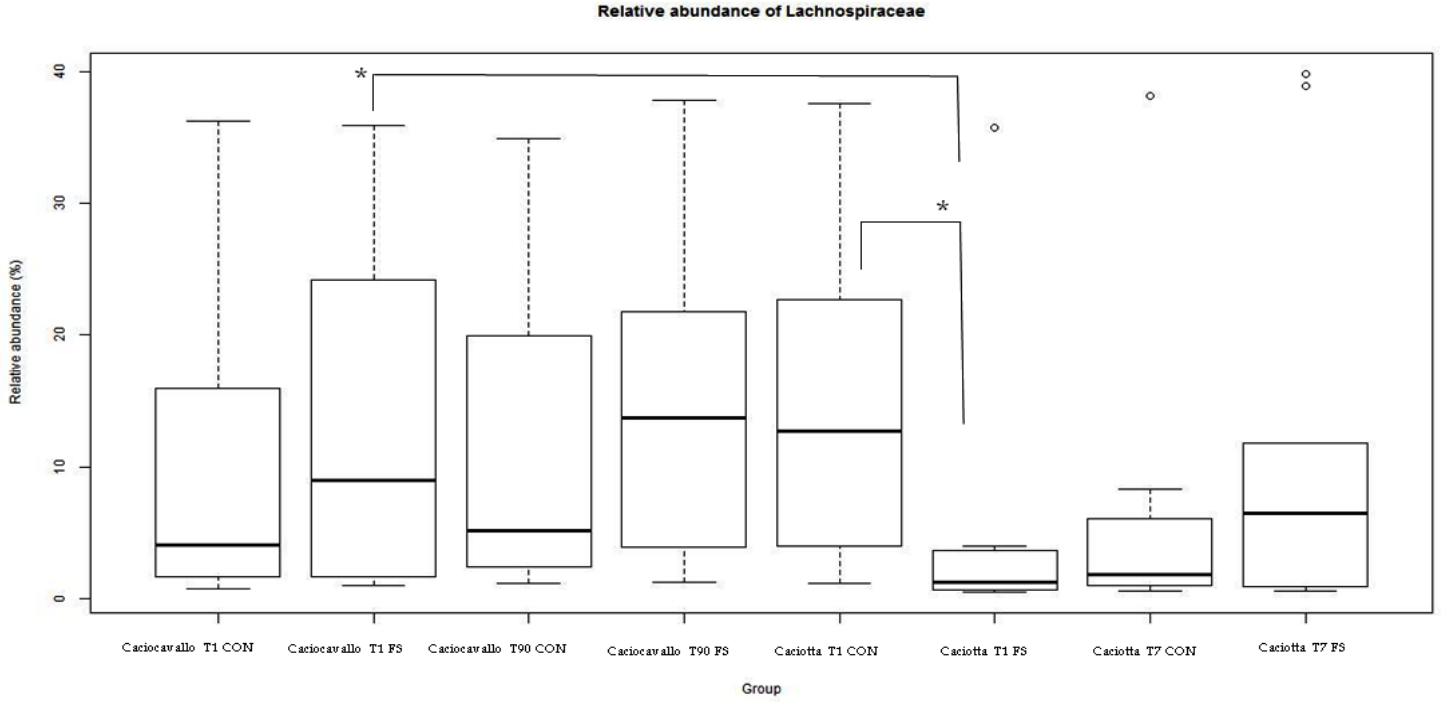


Fig. 10 Family *Lachnospiraceae* abundance in Fermenta samples

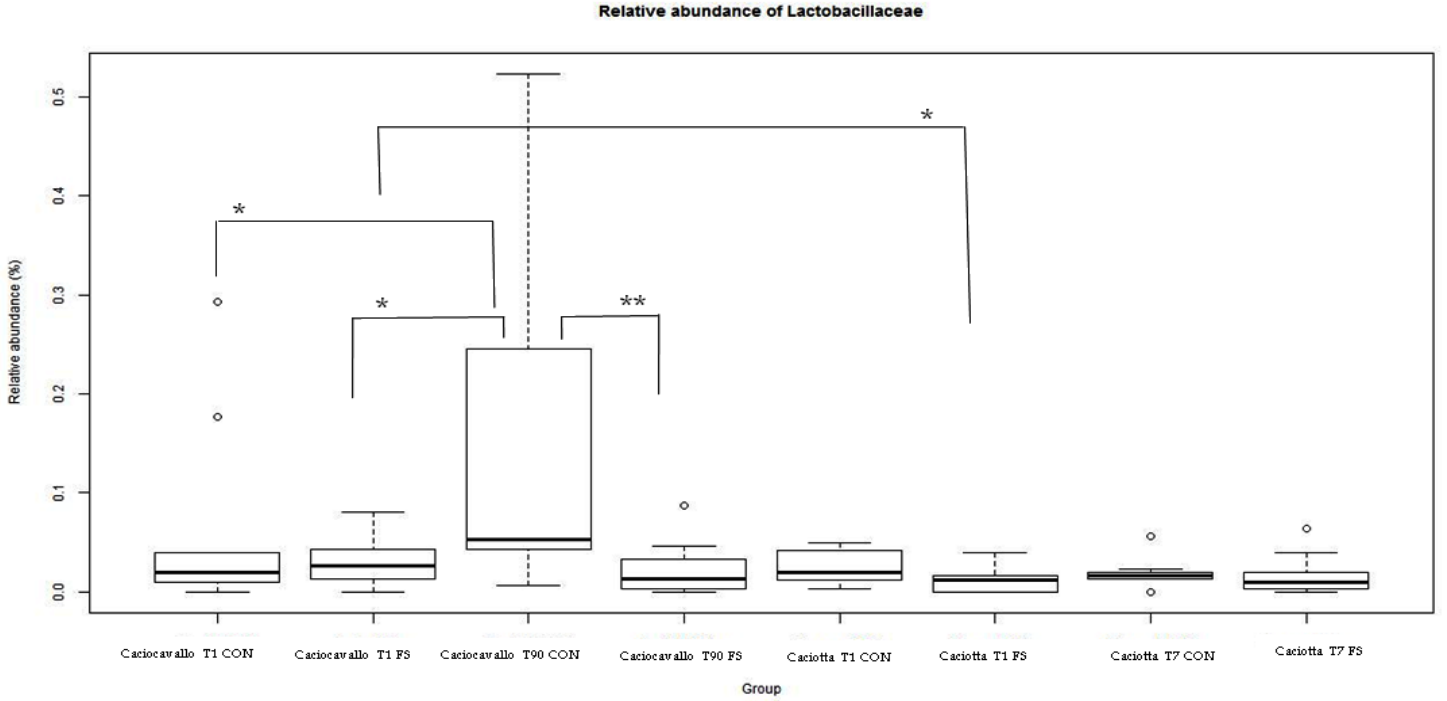


Fig. 11 Family *Lactobacillaceae* abundance in Fermenta samples

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