Universita' degli Studi di Foggia

DIPARTIMENTO DI SCIENZE AGRARIE DEGLI ALIMENTI E DELL'AMBIENTE

(SAFE)

PhD course on Management of Innovation in the Agricultural and Food Systems of the Mediterranean Region (XXVIII cycle)

Strategies for improving the nutritional quality of milk and dairy products from different species reared in the Mediterranean area.

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ANNO ACCADEMICO 2016 — 2017

FIRST TRIAL

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ABSTRACT

The aim of the first trial was to evaluate the effects of flaxseed supplementation on the yield and quality of buffalo milk. In particular, the fatty acid profile of milk from buffalo cows subjected to different diets has been investigated.

Although buffalo milk is the second most produced milk in the world, and of primary nutritional importance in various parts of the world, few studies have focused on the chemical properties of buffalo milk. The larger size of buffalo fat globules, 5 vs. 3.5 μm, was related to the higher amount of fat in the buffalo milks: 73.4 ± 9.9 vs. 41.3 ± 3.7 g/kg for cow milk. Buffalo milks contained significantly lower amount of polar lipids expressed per gram of lipids (0.26% vs. 0.36%), but significantly higher amount of polar lipids per litre of milk (+26%).

For these reasons, considered the interesting potential of this milk and the growing need for functional products it seemed interesting to evaluate the fatty acid composition of buffalo milk obtained from a supplementation of flaxseed in the diet of animals.

The experimental design provided for flaxseed supplementation to buffalo dairy cows in mid lactation. Treatments were: (1) no flaxseed supplementation (**CO**); (2) low flaxseed supplementation (500 g/d; **FS500**); (3) moderate flaxseed supplementation (1,000g/d; **FS1000**).

Milk protein and casein were affected by flaxseed supplementation resulting lower in flaxseed-supplemented diets. Furthermore, the results revealed that flaxseed supplementation positively affected the milk fatty acids Short-chain fatty acids, in particular C8:0 and C10:0, were the lowest in milk from buffalo cows fed the highest level of flaxseed supplementation. Medium-chain fatty acids were the lowest in FS1000, intermediate in FS500. Long-chain fatty acids were the highest in FS1000, intermediate in FS500 groups, and the lowest in milk from buffalo receiving no flaxseed supplementation. Total conjugated linoleic acid content evidenced the same trend of long-chain fatty acids, with an increase of about 7% in FL500 and of 22% in FL1000 than the control. Atherogenic index, thrombogenic index, and n-6/n-3 were the lowest in FS1000 groups; thrombogenic index and n-6/n-3 were intermediate in milk from animals receiving FS500. Nutritional value of the acidic profile in buffalo milk is influenced by flaxseed supplementation, and its improvement reflects the level of dietary flaxseed supplementation.

Key words: buffalo cow, flaxseed, fatty acids, conjugated linoleic acid

INTRODUCTION

The domestic water buffalo (Bubalus bubalis) contributes a significant share of global milk production and is the major milk producing animal in several countries. Buffaloes are kept mostly by small-scale producers in developing countries, who raise one or two animals in mixed crop-livestock systems. Water buffaloes are classified into two subspecies: the river buffalo and the swamp buffalo. River buffaloes constitute approximately 70 percent of the world water buffalo population. River buffalo milk accounts for a substantial share of total milk production in India and Pakistan and is also important in the Near East. Swamp buffaloes are smaller and have lower milk yields than river buffaloes. They are present mainly in Eastern Asia and are primarily raised for draught power.

River buffaloes usually produce between 1500 and 4500 litres of milk per lactation. They have a significantly longer productive life than cattle, providing calves and milk until they are up to 20 years of age. The many factors that constrain commercial buffalo milk production include animals' late age at first calving, the seasonality of oestrus, and the long calving interval and dry period.

In recent decades, breeding programmes – especially in Bulgaria, China, Egypt, India and Pakistan – have attempted to improve the milk yield of river buffalo. Well-known specialized dairy buffalo breeds include Murrah, Nili-Ravi, Kundi, Surti, Jaffarabadi, Bhadawari and Mehsana.

Dairy buffalo facts

- The world population of water buffaloes is approximately 168 million head: more than 95% are in Asia; 2% are in Africa, particularly Egypt; 2% are in South America; and less than 1% are in Australia and Europe.
- The countries with the largest numbers of dairy buffaloes are India, Pakistan, China, Egypt and Nepal. In Pakistan, Egypt and Nepal there are more dairy buffaloes than dairy cows.
- Water buffaloes are the principle source of milk in South Asia.
- The largest water buffalo milk producers are India and Pakistan, where buffaloes produce more milk than cattle.

Water buffalo account for the second most widely available milk source in countries around the world. Within European countries, Italy accounts for 95% of all water buffalo, with 214,164 lactating buffalo (FAOSTAT, 2016) that are mainly reared in central and southern regions of Lazio, Campania, and Puglia (Borghese et al., 2000). The milk of this species accounts for over 50% of drinking milk in countries such as India, Pakistan, Egypt, and Nepal, whereas in Italy buffalo milk is used almost exclusively for mozzarella cheese production (Zicarelli, 2004).

Buffalo milk contains all the nutrients in higher proportions than cow milk. The compositional differences between buffalo and cow milk are reflected on their physico-chemical properties. Milk from buffalo is the preferred candidate for preparing milk and dairy products of western and traditional (indigenous) type and is nutritionally superior.

Almost all the products that are prepared from cow milk can be prepared from buffalo milk too with certain limitations owing to the differences in composition and physico-chemical properties but these differences have been fully exploited to the advantage of the producers, processors and consumers. It is no wonder that buffalo milk commands a premium price from collection centres linked to rural based farmers.

The percentage of fat harvested from buffalo milk is significantly higher when compared to cow milk. In the study performed by Asker et al. (1974), fat content of buffallo milks ranged from 6.9% to 8.5%. Varrichio et al. (2007) reported that fat content in buffalo milks averages 8.3% but can reach 15% under favourable conditions.

The emulsifying capacity of buffalo milk fat is better due to a higher proportion (50%) of butyric acid containing triglycerides compared to only 37% in cow milk. This is the reason for higher yield of butter and ghee prepared from buffalo milk.

Buffalo milk contains less cholesterol (total cholesterol 275 mg and free cholesterol 212mg per 100 g of fat) compared to cow milk (total cholesterol 330 mg and free cholesterol 280mg per 100 g of fat) and more tocopherol (334.21 µg per kg for buffalo and 312.3µg per kg of cow milk). Due to high peroxidase activity, buffalo milk can be preserved naturally for a longer period. Buffalo milk contains more calcium, a better calcium: phosphorous ratio and less sodium and potassium than in cow milk which makes it a better nutritional supplement for infants.

Buffalo milk is also one of the richest milks from a compositional point of view. Particularly, fat constitutes the main fraction of buffalo milk and is responsible for its high energetic and nutritive value.

Composition of buffalo milk

According to the definition of USDA (2011), water buffalo milk is the normal lacteal secretionpractically free of colostrum, obtained by the complete milking of one or more healthy water buffalo. Water buffalo milk shall be produced according to the sanitary standards of this ordinance. Quite a number of studies focused on cow milk, even if the milk produced by other
animals such as buffaloes are essential in human diet in different parts of the world. Buffalo milk is a totally natural product that can be consumed like any other milk.

It is one of the richest products from a compositional point of view and characterized by higher fat, total solids, proteins, caseins, lactose and ash contents than cow, goat, camel and human milk. Monitoring changes in composition of buffalo milk over years is important as an overall index for the combined effects of environmental and genetic factors. Zicarelli (2004a) recorded an increase in fat content of Italian buffalo milk from 7.3 to 8.3% and its protein content from 4.4 to 4.8% respectively from 1967 to 2000. Differences in the composition of buffalo milk in different localities reflect differences in breeds, management, feeding and environmental conditions.

The high milk solids of buffalo milk not only make it ideal for processing into superb dairy products but also contribute to significant energy savings in conducting that process. Buffalo milk yogurts and cheeses are natural thick set without recourse to adding addition milk proteins or gelling agents as with lesser milks. Dairies love to work with buffalo milk, which we all know makes the best mozzarella. The smooth texture and richness converts into a truly wonderful range of multiple award winning products.

Proteins: The protein content of buffalo milk is higher than in cow (Ragab *et al.,* 1958; Ganguli, 1973, Ahmad *et al.,* 2008).Of the total proteins of buffalo milk, ~80% are caseins and ~20% are whey proteins with traces of minor proteins (Laxminarayana and Dastur, 1968; Sirry *et al.,* 1984; Sahai, 1996). Whey proteins and minor proteins are even higher in colostrum than mature buffalo milk.

Caseins: Almost all casein of buffalo milk is present in the micellarform (Ganguli, 1973; Sabarwal and Ganguli, 1970a). Buffalo milk contains a negligible proportion of soluble casein (0.03 g.100mL-1) about 1 % of the total casein unlike cow milk (0.11 g.100mL-1) about 5 % of the total casein (Sabarwal and Ganguli, 1971).

Physico-chemical characteristics of caseins micelles: Casein micelles of buffalo have average diameter of 190 nm (Ahmad, 2010) which found about 10-20 nm bigger in size than that of cow agree with the findings of other authors (Ganguli, 1973; Sood *et al*., 1976;Sirry *et al.*1984; Sarswat, 1985).

The higher casein concentration in buffalo milk and almost 100% in colloidal form seems to have primary impact to increase the numbers of casein micelles and secondary impact on size. More casein concentration is possibly translated into more number of casein micelles in buffalo milk mL^{-1} as compared to number of casein micelles mL^{-1} of cow milk. Charges could be due to glycosylated parts present on κ-CN, the protein being present at the periphery of casein micelles which is similar as for cow milk (Ahmad, 2010). The lower hydration of buffalo milks' casein micelles as compared to cow milk could be due to their larger size and high concentration of colloidal calcium so it leaves less space for water molecules to inculcate. Some other authors (Kuchroo and Malik, 1976; Sabarwal and Ganguli, 1970a) also found the similar results. Further investigations are needed to observe the structural differences and compactness of casein micelles as compared to the casein micelles of other milks to better exploit the functional and nutritional characteristicsof individual caseins.

Opacity of the buffalo casein micelle is greater than that of the cow casein micelle (Sabarwal and Ganguli, 1970b). Buffalo casein contains lower proportions of sialic acid (2.0mg.g-1 casein), hexose (2.5mg) and hexosamine (1.8mg), but higher proportions of calcium (Sabarwal *et al.,* 1972), while heating of milk reduces sialic acid, hexose and hexosamine contents (Sabarwal and Ganguli, 1973). Electrophoretic separation of casein components showed 44, 53 and 3% for buffalo α_S-, β-, and κ-casein vs. 55, 39 and 6% for the cow milk casein fractions(Ganguli and Bhalerao, 1964). All three fractions of buffalo milk casein have slower mobility than cow milk casein. The proportions of α_{s1}-, α_{s2}-, -, β- and κ- caseins were 40, 6–9, 35 and 12%, respectively (Yamauchi *et al*., 1983). The N and P contents of buffalo αs1-casein are about 15 and 0.1%, respectively. Amino acid composition of buffalo and cow αs1-casein is similar. Buffalo κ-casein is heterogeneous with eight sub fractions, which are similar in P but different in carbohydrate contents. Amino acid composition of buffalo κ-casein is comparable to that of cow milk, but poorer in sialic acid. Overall 95% homology exists in the amino acids sequences of all casein classes between buffalo and cow milk.

Whey Protein: The proportions of whey proteins in buffalo milk are similar to those in cow milk, and the amino acid composition of buffalo β−lactoglobulin (β-Lg) is identical to that of cow milk(Mawal *et al.,* 1965) except that it does not exhibit genetic polymorphisms (Senand Sinha, 1961). The molecular weight of buffalo β−Lg is 38.5 kD. Buffalo and cow α−lactalbumin (α−LA) have the same crystalline form and similar nitrogen content. The molecular weight of buffalo α−LA is 16.2 kD, and no genetic polymorphisms have been observed (Malik and Bhatia, 1977). Buffaloα−LA has one major and three

minor fractions, but all are active in modifying the activity of glactosyltransferase in the synthesis of lactose (Sindhu and Singhal, 1988). The concentrations of immunoglobulins (Ig) are very high in buffalo colostrum (Kulkarni, 1981),and four classes have been identified (IgGa, IgA1, IgA2 and IgM). Lactoferrin content of buffalo milk is much higher than in cow milk (Sahai, 1996). Its content in buffalo colostrums is still higher (0.75 mg.mL-1). The molecular weight is 73.7– 74.0 kD.

Fat: Buffalo milk is nearly twice as rich in fat as compared to cow milk and the most important fraction responsible for its high energetic and nutritive value. Varrichio *et al.* (2007) reported the fact that the fat content has an average value of 8.3% but can also reach up to 15% under normal conditions. Tonhati *et al.,* (2011) found the fat yield means 90.1±24·6 g.kg-1. Medhammar *et al.* (2011) also found the interbreed differences of in total fat in buffalo, yak, mare and dromedary camel milks and as well in the mineral contents.

Fat related constituents: Fatty acid composition, however, in buffalo milkfat is different from that of cow milk fat (Ramamurthy and Narayanan, 1971; Joshi and Vyas, 1976; Arora *et al.*, 1986; Zicarelli, 2004b, Menard *et al.,* 2010).Some authors reported changes in the fatty acids composition of buffalo milk as a function ofbreed (Talpur *et al.,* 2007), lactatingstage (Arumughan and Narayanan, 1981), season (Talpur *et al.,* 2008; Asker *et al.,* 1978) and diet (Patiño*et al.,* 2008).The differences are present not only among species but also within species i.e. among breeds of buffaloes regarding fat and fatty acids concentration. Talpur *et al.* (2007) studied two major buffalo breeds of Pakistan i.e. Nili-Ravi and Kundi for fat. The milk fat of Kundi buffalo was found to contain significantly lower amount of saturated fatty acid contents than Nili-Ravi buffaloes (66.96 and 69.09g.100g-1),higher monounsaturated fatty acid contents (27.62 and 25.20 g.100g-1) and total *trans* fatty acids (3.48 vs. 2.48). In another study, Qureshi *et al.* (2010) found that Nili-Ravi dairy buffaloes produce milk almost similar to dairy cows regarding availability of cardio protective fatty acids, with the highest concentration of oleic acid (C18:1cis-9, 29.47 g/100 g). Buffaloes with moderate body condition yielded greater concentrations of these fatty acids followed by poor and highest ones.

Two hypercholestolemic fatty acids (C12:0 and C14:0) were associated with higher body condition. Proportions of C4, C16,C17, and C18 fatty acids (FA) are higher, but C6,C8, C10, C12, C14, and C14:1fatty acids are lower in buffalo than in cow milk fat. Soliman *et al.* (1979) gave an average total saturated and unsaturated fatty acid content of 71.7% and 28.3% respectively in Egyptian buffalo milk fat. The intra-molecular fatty acid distribution is similar to that of other species (Freeman *et al.,* 1965).Buffalo milk fat has a greater proportion of high melting triglycerides than that of cow milk

fat(9–12% and 5–6%, respectively) (Ramamurthy and Narayanan, 1974).The high melting triglycerides fraction contains less short-chain and unsaturated fatty acids.

High, medium and low molecular weight triglycerides in buffalo milk are 42%, 17%, and 41% of total, respectively (Arumughan and Narayanan, 1982). Colostrum and late lactation milk are rich in unsaturated but poor in saturated fatty acids (Anantakrishnan *et al.,*1946). Buffalo milk fat contains more tetraenoic and pentaenoic but less dienoic and trienoic fatty acids than cow milk fat (Ramamurthy and Narayanan, 1971).

Buffalo milk fat has a higher melting point, density, specific gravity and saponification value, but lower refractive index, acid and iodine values than cow milk fat, although they are affected by stage of lactation, season, feed and thermal oxidation (Angelo and Jain, 1982). Buffalo milk and ghee contain less free fatty acids than milk and ghee from cows (Pantulu and Ramamurthy, 1982; Lal and Narayanan, 1983).

Colostrum and mastitic milk contained more cholesterol than normal milk. Cholesterol content in fore-milk is higher than in stripping; also, it is higher in milk during the spring season. Esterified cholesterol, however, was higher in buffalo than in cow milk fat (64 and 48 mg.100g-1, respectively) (Bindal and Jain, 1973; Prasad and Pandita, 1987).The phospholipids content of milk is a function of fat content and size of fat globules. A significant correlation has been found between PL and fat content of buffalo milk. The phospholipid contents of buffalo milk is slightly higher (29.6mg.100mL-1) in summer time than in winter (24.7 mg.100mL 1). The phospholipid content ofbuffalo milk, butter and ghee per unit weight of fat is much lower than in cow milk fat (Baliga and Basu, 1956). Colostrum has more phospholipids, which becomes normal in 15 days. The phospholipid contents are maximum in January and minimum in July. The ratio of lecithin: cephalin: sphingomyelin is 48:40: 12 in cow milk and 40:48:12 in buffalo milk (Rawat, 1963).Buffalo milk contains gangliosides which are not present in cow milk (Berger *et al.,* 2005). A gangliosides fraction in buffalo milk show a GM1- specific binding to cholera a toxin subunit B. Also the lipholipic gangliosides of buffalo milk have anti-inflamatry activity (Colarow *et al.,* 2003).Milk Fat contains wide range of carbonyl compounds and their precursor keto-glycerides a part of the delicate flavor system of milk fat. Monocarbonyl content of buffalo milk fat is higher than that of cow milk

fat. Colostral fat contained 60-70% of total carbonyls in normal milk fat, increased rapidly during early lactation and then gradually therefore (Bhatand Rao, 1983).

Physico-chemical characteristics of fat globules: The fat globule in buffalo milk is coarse and bigger than in cow milk (1 ml buffalo milk contains about 2.7 million fat globules), with 60% having a size between 3.5 to 7.5 µm(Akhundov, 1958; Akhundov, 1959; Abd El-Hamid and Khader, 1989; Ahmad *et al.,* 2008).The average size of fat golubles in buffalo milk (5µm) is higher than cow, goat and sheep milk being 3.2, 2.6 and 3.0 µm respectively. El-Zeini *et al.* (2006) reported much average globule sizes (8.7µm) in buffalo milk as compared to 3.8, 3.8, 3.2 and 3.0 µm for cow, sheep, goats and camel milks. Higher percentage (20.34%) of large fat globules (16-18µm) has been found in buffalo milk but not in the milk of other ruminants. The respective parameters of buffalo milk fat globules are compactness, sphericity, surface roughness, length, width, orientation are 0.71, 0.59, 0.91, 58.34, 9.85, 4.67, 9.85, 4.15 and 107.46 respectively. The total concentration of saturated fatty acid of buffalo milk fat globule membrane varied from 66.2 to 78.3 and unsaturated fatty acids from 21.7 to 33.5% in agreement with result of milk fat globule membrane of buffalo milk from different breeds. The proteins: lipids ratios of isolated membranes vary from 3.2 to 4.7 but the total neutral and polar lipids are almost similar in different seasons (Sharma *et al.,* 1994; Sharma *et al.,* 1996).

Lactose: Lactose is a disaccharide made up of glucose and galactose bonded together in buffalo milk like other milks. Buffalo milk is richer source of lactose than cow, goat, sheep and camel milk so a good of source of energy for body activities particularly of brain and hormonal regulation. Before it can be used by the body, the bond must be broken by the enzyme lactase in the small intestine. People that have decreased activity of lactase in the small intestine may have problem of lactose digesting and this is referred to as lactose intolerance or malabsorption. Due to higher concentration, the chances of such problems are more by using buffalo milk but cases have not been noticed as for cow milk, may be due different repartition of lactose in the buffalo milk. Complex oligosaccharides constitute a large portion of lactose of milk and perform biological functions that are closely related to their structural conformation. They contribute to the growth of beneficial intestinal flora in the colon, postnatal stimulation of the immune system and provide defense against bacterial and viral infections by acting as competitive inhibitors for binding sites on the intestinal epithelial surface (Kunz *et al.,* 2000; Kunz and Rudloff, 2002). Varman and Sutherland (2001) have explained that lactose makes a major contribution to the colligative properties of milk, such as osmotic pressure, freezing point depression and boiling point elevation. Oligosaccharide distributions in human milk and colostrum and milk of domestic animals (cows, goats, sheep, and buffaloes) have also been studied by Mehra and Kelly (2006). The levels of oligosaccharides in cow, sheep and goat milk are much lower (Urashima*et al.,*

1997; Martinez-Ferez*et al.,* 2006), whereas comparable in buffalo milk. The low concentration of oligosaccharides in cow milk and colostrum has stalled their utilization as biologically active ingredients in the health care and food sector but it opens the door for milk and colostrum like of buffaloes having comparable oligosaccharides levels as in human milk. Much research interest is being shown in recent period on the potential of milk oligosaccharides in infant nutrition. A processed oligosaccharide mixture of buffalo milk induced significant stimulation of antibody, delayed type hypersensitivity response to sheep red blood cells in BALB/c mice and also stimulated nonspecific immune response of the animals in terms of macrophage migration index. Saksena *et al.* (1999) isolated a novel pentasaccharide from buffalo milk oligosaccharides containing a fraction with immune stimulant activity.

Currenly, there is only limited data and research findings on oligosaccharides in buffalo milk. Abd El-Fattah *et al.* (2012) observed that at calving, all components decreased gradually as the transition period advanced except lactose which conversely increased. Milk oligosaccharides are divided into neutral (don't contain any charged monosaccharides residues) and acidic (contain one or more residues of sialic acid that are negative charged) classes (Gopal and Gill, 2000). The galactose, *N*acetylgalactosamineand sialic acid contents of buffalo κ- CN fractions ranged from 0 to 4.3, 5.5 and 8.5 moles.mole-1 protein, respectively (Addeo *et al.,* 1977).

Aparna and Salimath (1995) reported the composition of oligosaccharides, and isolation and structural elucidation of disialyl lactose, from the colostrum of buffalo as three fractions with different concentration of glycopeptides 0.2-0.8%, 0.3-1.5%) and 2.2-2.8%. A sialoglycopeptide was isolated from buffalo colostrum in pure form which consists of fucose, galactose, mannose, *N*-acetyl glucosamine and *N*-acetyl neuraminic acid in the ratio 1:2:3:4:1, and aspartic acid, serine, threonine, proline and glutamic acid as the major amino acids. Glycine was identified as the *N*-terminal amino acid residue.

Physico-chemical properties of buffalo milk

Buffalo milk is very white and beautifully smooth. The pH of buffalo milk ranges from 6.57 to 6.84 and is not influenced by month, lactation number, or season of calving, but correlated with solid-not-fat and lactose contents (Minieri *et al*., 1965). Acidity varies from 0.05% to 0.20% (Dharmarajan *et al.,* 1950.), and its colostrum has greater acidity than mature milk. In fresh milk, lactic acid accounted for 25% of total acidity.

Acidity was correlated with fat and solid-not-fat percentage in buffalo milk but not in cow milk (Hofi *et al.,* 1966a). The freezing point of buffalo milk is in the range of – 0.552 to – 0.558° C (Hofi *et al.,*1966b), but boiling and souring decrease the freezing point, and vacuum treatment, cold storage, and the addition of water increase the freezing point. The maximum buffering index was 0.042 at pH 4.9–5.1 for buffalo milk and 0.035 at pH 5.1–5.2 for cow milk (Rao *et al.,*1955; Rao *et al.,* 1956). The refractive index of buffalo milk (at 40°C) varies from 1.346 to 1.353 compared to cow milk, which is 1.345 to 1.348, with proteins and lactose contributing most (Rangappa, 1947).Buffalo milk with 6.4% fat and 10.2% solid-not-fat had mean density of1.034 g.mL-1 at its freezing point (Roy and Chandra, 1978) with little difference between cow and buffalo milk, but separation of cream increased the density of buffalo milk (Abo- Elanga, 1966).Curd tension in buffalo milk (32–85 g) is nearly 1.5 times that of cow milk (28–54 g) and increases at the end of lactation (Rao *et al.,*1964), but heat treatment from pasteurization decreases it by 10–28%, boiling by 58%, sterilization by 87%, homogenization by 24–73%, and addition of sodium citrate or sodium hexameta- phosphate by up to 97% (Tambat and Srinivasan, 1979).

Functional elements of flaxseed

Flaxseed is one of the richest plant sources of the ω-3 fatty acid i.e. α-linolenic acid (ALA) (Gebauer et al. 2006; Tonon et al. 2011) and lignans (phytoestrogens) (Singh et al. 2011). The important flaxseed growing countries are Canada, China, United States, India and Ethiopia.

Various edible forms of flax are available in the food market - whole flaxseeds, milled flax, roasted flax and flax oil. According to its physico-chemical composition, flaxseed is a multicomponent system with bio-active plant substances such as oil, protein, dietary fiber, soluble polysaccharides, lignans, phenolic compounds, vitamins (A, C, F and E) and mineral (P, Mg, K, Na, Fe, Cu, Mn and Zn) (Bhatty 1995; Jheimbach and Port Royal 2009).

Lipids

Flaxseed is the richest plant source of the ω-3 fatty acid i.e. α-linolenic acid (Gebauer et al. 2006). Flaxseed oil is low in saturated fatty acids (9 %), moderate in monosaturated fatty acids (18 %), and rich in polyunsaturated fatty acid (73 %) (Cunnane et al. 1993). Of all lipids in flaxseed oil, α- linolenic acid is the major fatty acid ranging from 39.00 to 60.42 % followed by oleic, linoleic, palmitic and stearic acids, which provides an excellent ω-6:ω-3 fatty acid ratio of approximately 0.3:1 (Pellizzon et al. 2007). Although flaxseed oil is naturally high in anti-oxidant like tocopherols and beta-carotene, traditional flaxseed oil gets easily oxidized after being extracted and purified (Holstun and Zetocha 1994). The bioavailability of ALA is dependent on the type of flax ingested (ALA has greater bioavailability in oil than in milled seed, and has greater bioavailability in oil and milled seed than in whole seed) (Austria et al. 2008).

Health benefits

Flaxseed has potential health benefits besides the nutrition, due to mainly 3 reasons: first, due to its high content of ω-3 α-linolenic acid; Second, being rich in dietary soluble and insoluble fibers; and third, due to its high content of lignans, acting as anti-oxidants and phytoestrogens. ALA can be metabolized in the body into docosahexaenoic acid (DHA) (ω-3) and eicosapentaenoic acid (EPA) (ω-3). The health benefits of all ω-3 fatty acids (ALA, EPA and DHA) have been widely reported for several conditions including cardiovascular disease, hypertension, atherosclerosis, diabetes, cancer, arthritis, osteoporosis, autoimmune and neurological disorders (Simopoulos 2000; Gogus and Smith 2010). Flaxseed has also been reported to act as anti-arrhythmic (Ander et al. 2004), anti-

atherogenic (Dupasquier et al. 2006, 2007), and anti-inflammatory (Dupasquier et al. 2007) agent in addition to improving vascular function (Dupasquier et al. 2006).

Commercial utilization of flaxseeds in food products

Functional food revolution

Functional foods are those that provide a specific health benefit to the consumer over and above their nutritional value. Functional foods are relatively recent developments that meet a strengthening consumer demand for foods that enhance health and wellbeing. According to a new report by Global Industry Analysts, Inc. the global market for functional foods and drinks is projected to reach exceed $130 billion by the year 2015 (Global Industry Analysts 2010). The United States market dominates (>30 % of the total global market) and is showing a sustained growth of ~14 % per year, while its ~8 % per year across the world (Smithers 2008). In this large marketplace, the food industry is demanding economical, high-quality, novel and substantiated ingredients. In such a setting, flaxseed being rich in ω-3 fatty acids, lignans and fibers provide the industry with an excellent choice in developing the value-added food products. With the increasing rate of obesity and other chronic diseases in western societies, flax products are increasingly used as functional foods and nutraceuticals (Hasler et al. 2000; Lemay et al. 2002; Ogborn et al. 2002; Watkins et al. 2001). In recent years, as people have become more concerned about health, demand for flax in food and beverages, functional foods and dietary supplements has risen dramatically both in the U.S. and other countries. For example, Mintel's Global New Products Database (GNPD) reported that in 2005, 72 new products were launched in the United States that listed flax or flaxseed as an ingredient (Wilkes 2007). In the first 11 months of 2006, there were 75 new products launched enriched with flax or flaxseed (Wilkes 2007). Interest in flax and other ω-3-containing foods heightened further in May 2003 when the White House issued a letter to the U.S. Department of Agriculture (USDA) and the U.S. Food and Drug Administration (FDA) that asked them to promote the intake of ω-3 fatty acids in the diet (Wilkes 2007).

Flaxseeds for a new millennium

To achieve optimal nutrition through the intake of healthy foods, Food Science and Technology experts are creating a new framework for food-based dietary recommendations, principally in the areas of food physics, methods of food storage and preservation, nutrient restoration and fortification of foods, as well as the development of health-focused designer foods and functional foods (FAO/WHO 1996;

USDA 2010). Initiatives have been undertaken by the food industry to increase the level of ω-3 fatty acids, dietary fibers and anti-oxidants, etc. Flaxseed has drawn the attention of scientists, researchers and industry due to its ω-3 fatty acids and various health benefits. In the functional arena of 21st century, flaxseed's use is not just limited to its fibers but has been extended to its various and therapeutic attributes which make it a potent value added food ingredient. Although flaxseed oil unlike fish oil, does not contain EPA and DHA, but still it is gaining popularity in India and Western countries due to its high ALA content. A major hurdle with ω-3 rich fish oil is consumers' increased awareness of environmental contaminants [e.g., heavy metals and polychloro biphenyls (PCBs)] and bioaccumulation of these contaminants in fish. If FDA approves the flax to be labeled as a whole grain, the fortified food products variety will see enormous growth in future. Flax is a rich source of ALA (ω-3 fatty acid), dietary fibers, high quality proteins, antioxidants, and lignans, some of which offer synergistic health benefits. Flax contains almost no digestible or glycemic carbohydrates. In all respects, flax offers a model for whole grains or seeds and underscores the recognition given to the nutritional value of "whole grains", "whole seeds" and "whole foods".

It is evident that flaxseeds are the richest source of α-linolenic acid. It is also a considerable potential source of soluble fiber, antioxidants and high quality protein. Its long journey from being a medicine in ancient times to the health food source in 21st century has opened the doors for a large population. The role of flaxseed and ω-3 fatty acid in reducing the risks associated with cardiac and coronary disease, cancer (breast, colon, ovary and prostate) and other human health risk factors has been well known. When healthy heart is one of the most desired and highly demanded health benefits from functional foods; and where food industry's goal is to develop innovative solutions to address nutritional challenges, flaxseed is going to play a vital role for the same. Flaxseed can contribute in improving the availability of healthy food choices, specifically by improving the nutrient profile of foods through reductions in the salt, sugar and saturated fat content; and by increasing the content of ω-3 fatty acids and other bioactive compounds. With contribution from such factors, worldwide market for healthy heart foods is estimated to grow rapidly in the coming years. As a result, flaxseeds may be preferred ingredients of functional foods in future. There is no doubt that a change to an omega-3 rich and high fiber diet would be beneficial. Therefore the use of flaxseed in whole seed or ground form can be recommended as a dietary supplement. Modern techniques like high power ultrasound, micro-fluidization, spray granulation and nanoencapsulation will pave way for new approaches to the processing, stabilization and utilization of flaxseed oil. Further, enrichment of diets of the animals with

flax/flaxseed oil for production of ω-3 enriched eggs, milk, meat and other animal origin products could be another approach in utilizing flaxseeds.

MATERIALS & METHODS

Several authors observed the incorporation of PUFA in the diets has been carried out in several lactating species with the aim of improving the acidic profile of milk for direct human consumption or for dairy products. Among lipid sources, flaxseed has been successfully supplemented to cow (Caroprese et al., 2010; Cattani et al., 2014; Santillo et al., 2016), sheep (Zhang et al., 2006; Caroprese et al., 2011), and goat (Nudda et al., 2006, Luna et al., 2008; Caroprese et al., 2016), leading to a better n-3 PUFA profile in milk. Sunflower oil has been identified as a dietary fat supplement capable of reducing rumen protozoa for the duration of its utiliza-tion (Ivan et al., 2001).

To the best of our knowledge no studies have reported the role of fat supplementation of the diet in lactating buffalo cows. It would be useful to gain information on the effect of fat supplementation in terms of production and composition of buffalo milk. Therefore, the aim of the present research was to evaluate the effects of dietary flaxseed supplementation on the yield and quality of buffalo milk.

In particular, the fatty acid profile of milk from buffalo cows subjected to different diets was investigated.

Experimental design

The experiment was conducted in a dairy farm located in Foggia (Apulia region, Italy). The experiment included 48 Mediterranean buffalo cows during mid lactation (175 ± 22 DIM; ±SD); animals were homogeneous for age (52 ± 6 mo), BW (561 ± 15 kg), parity (2.08 ± 0.28), milk production (8.9 ± 0.80 kg/ day), milk fat (8.56 ± 0.9%), protein (4.73 ± 0.4%) content, and for fatty acids composition grouped as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and conjugated linoleic acids (CLA). buffalo cows receiving 3 flaxseed (**FS**) supplementation levels. Treatments were:

1) no flaxseed supplementation (**CO**);
2) low flaxseed supplementation (500 g/d; **FS500**);
3) moderate flaxseed supplementation (1,000g/d; **FS1000**).

Animals were assigned randomly to 1 of the 3 groups subjected to the different diets and received a diet based on concentrate mainly constituted by corn silage, wheat middlings, corn and soy flour, vetch and oat hay.

Chemical composition and milk forage units of the experimental diets are reported in Table 1. The chemical composition of diets was determined with standard procedures (AOAC, 1990). The flaxseed groups received the same diets of control group in which 500 (FS500) and 1,000 g/d (FS1000) of

concentrate was substituted with the same amount of whole flaxseed (Lin Tech, Tecnozoo srl, Torreselle di Piombino Dese, Italy).

The experiment lasted 7 wk; the first 2 wk were considered an adaptation period and the measurements were made during the last 5 wk. Buffalo cows were housed in cement paddocks with free access to water and were fed twice daily.

The total amount of flaxseed was given before the morning feeding to each buffalo cow of the FS groups; we checked that each animal consumed the total quantity of food and supplement given. Cows were milked mechanically twice daily at 06:00 and 18:00 h and milk production was recorded at each milking. Milk collection was done once a week on the same day throughout the experiment.

Individual milk samples were obtained by mixing milk from the morning and afternoon milkings in an amount proportional to milk yield. Individual milk samples were stored under refrigeration and transferred to laboratory for analyses.

Milk Analyses

Determination of Milk Composition and pH

Individual milk samples were collected and transported under refrigerated conditions to the laboratory for analyses. Before analyses an aliquot (30 ml) of milk was heated to 40°C, mixed gently and than analyzed for the determination of fat, protein, casein, lactose, and urea content using an infra-red spectrophotometer (MilkoScan FT 120; Foss Electric A/S, Hillerod, Denmark).

Milk samples were subjected to the determination of pH by pHametro 507 (Crison Spa tools, Carpi, Italy), using electrode for liquid. The pH highlights the "current" acidity (freshness state of milk), that is, the content in dissolved hydrogen ions. The milk has a pH close to neutrality and is a buffered solution, that is, for small additions of acids or bases, the pH does not vary. This is because there are acidic or basic functions that neutralize any free bases or acids added.

Energy-corrected milk (740 kcal) was calculated using the formula reported by Campanile et al. (1998). ([{fat (g/ kg) – 40 + protein (g/kg)-31} 0.01155]+1)milk yield.

Determination of Milk hygienic quality

Milk samples were analyzed for the determination of the level of somatic cells using an optofluorimeter (Fossomatic Minor, Foss-Electric A/S)

Determination of Milk fatty acids

Fatty acids extraction from milk samples was performed as described by Feng et al. (2004), with some modifications. Briefly, 30 mL of bulk milk were centrifuged at 17,800 . *g* for 45 min at 4°C. Then 1.0 g of fat was transferred into a microtube, left at room temperature for 30 min, and centrifuged at 19,300 x *g* for 40 min at 20°C.

Fatty acids methyl esters were then obtained as described in IDF (2002). One hundred milligrams of upper layer fat were placed into a 16- x 25-mm screw-cap Pyrex tube, into which 5 mL of hexane and 0.2 mL of methanolic KOH 2 *N* were added. The tube was vortexed, left to stand for 5 min in the dark, then 0.5 g of NaHSO4 . H2O were added. The hexane layer, containing the FAME, was placed into a GC vial; the vial was capped and placed at −20°C until GC analysis. The fatty acid composition of milk extracts was determined by capillary GC on an HP-88, 100-m x 0.25-mm x 0.20-μm capillary column (Agilent Technologies Inc., Santa Clara, CA) installed on an Agilent Technologies 6890N GC equipped with a flame ionization detector and a split injector. The initial oven temperature was 70°C, held for 4 min, subsequently increased to 175°C at a rate of 13°C/min, held for 27 min, then increased to 215°C at a rate of 4°C/min, and held for 45 min. Helium was used as the carrier gas and the column head pressure was 175 kPa. Both the injector and the detector were set at 250°C; the split ratio was 20:1. Fatty acids were identified by comparing their retention times with the fatty acid methyl standards (FIM-FAME-7-Mix, Matreya LLC, Pleasant Gap PA), added to C18:1 *trans*-11, C18:2 *cis*-9,*trans*-11, C18:2 *cis*-9, *cis*-11, C18:2 *trans*-9,*trans*-11, and C18:2 *trans*- 10,*cis*-12 (Matreya LLC, Pleasant Gap PA); peak areas were quantified using Agilent Chemstation software.

Atherogenic (**ArI**) and thrombogenic (**TI**) indexes were calculated according to Ulbricht and Southgate (1991) formula:

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

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)].

The Δ9-desaturation indexes were calculated according to Schennink et al. (2008) as follows:

C14 index =
[C14:1 *cis*-9/(C14:0 + C14:1 *cis*-9)] . 100;

C16 index =
[C16:1 *cis*-9/(C16:0 + C16:1 *cis*-9)] . 100; and

C18 index =
[C18:1 *cis*-9/(C18:0 + C18:1 *cis*-9)] . 100.

Statistical Analysis

All variables were tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Data on milk were processed using ANOVA for repeated measures (SAS Institute, 2011). The model included flaxseed supplementation level as non-repeated factors and time of sampling and its interactions as repeated factors. Individual animal variations within flaxseed supplementation level were used as error terms. When significant effects were found (at $P < 0.05$), the Tukey test was used as a post hoc test. The interaction of treatment and time was not significant for milk yield and composition, or for fatty acid content of milk; therefore, mean values for the mentioned parameters are presented.

RESULTS and DISCUSSION

Buffalo milk composition and hygienic quality

Low milk yield, poor reproductive performance (seasonal breeding behavior, anestrous, and longer calving interval) and low growth rate have been reported in buffaloes (Singh and Mehra, 1990; Qureshi et al., 2002; Sahoo et al., 2004; Wynn et al., 2009). Especially in many parts of Asia, where buffalo is widely spread, irregular and inadequate availability of quality feedstuffs and their utilization are the main causes of poor performance of buffaloes. Many efforts have been made in the last few decades to improve the nutrients supply and utilization in buffaloes with varying degree of success. Sarwar et al. (2009) reported a detailedreview of nutritional strategies tested in buffalo rearing system in order to improve animals performance. Research have been reported on available feed resources such as cereal straws and corncobs, ensiling fodders, use of industrial and agriculture by-products, dietary addition of fermentation modifiers, vitamins, and usage of ruminally protected dietary protein and fat sources that have shown significant potential to improve both growth and milk yield in buffaloes. Use of metabolic and fermentation modifiers, manipulations of rumen microorganisms in buffaloes could also play a significant role in improving its productivity.

Although buffalo milk is totally destined to cheese production in Mediterranean region, the high percentage of fat in such milk is of particular interest for the potential role of carrier of beneficial fatty acids in human nutrition. Previous research on buffalo cows aimed to improve CLA content through dietary manipulation (Tyagi et al., 2007). These authors supplied linoleic acid and linolenic acid as a substrate for rumen biohydrogenation using both grains containing more linoleic acid and pasture and green fodders predominantly containing linolenic acid. The experiment concluded that a near1:1 ratio of omega-3 and omega-6 fatty acids and more than a two fold increase in CLA content in buffalo milk and its products can be achieved by feeding buffaloes on a high proportion Berseem fodder without affecting sensory parameters of milk and milk products.

In the present study fat supplementation was performed using whole flaxseed, with the aim to improve the nutritional quality and especially the fatty acid profile of buffalo milk.

Yield and composition of milk from buffalo cows subjected to different feeding regimens are presented in Table 2. In our study, fat content was not influenced by the dietary treatments. The extent to which dietary lipid is protected from microbial attack in the rumen may be an important factor influencing the extent of change in milk fat percent and milk fat yield. In general, protected lipid supplements tend to promote increased milk fat percentage and yield of fat (Storry, 1981; Storry et al., 1980; Ashes et al.,

1992) however, the effect of feeding lipid sources which are not protected from microbial digestion in the rumen is less predictable.

The effect of flaxseed supplementation is controversial, although few studies have been conducted on buffalo cows, El-Aziz et al. (2012) found that crushed flaxseed supplementation led to higher milk fat than control diet. In contrast, no effect was reported for whole flaxseed supplementation on Holstein (Petit, 2002) and Simmental cows (Santillo et al., 2016). The effect of flaxseed on milk fat may be due to the different processing of supplemental flaxseed.

A number of studies have been conducted to determine the influence of feeding full fat flaxseed on milk composition. In one study flaxseed was included in the diet of lactating Holstein cows at levels ranging from 50 g kg^{-1} to 150 g kg^{-1} of the total diet (DM basis) as the intact seed or after dry rolling (Kennelly and Khorasani, 1992; Khorasani and Kennelly, 1994). Cows readily consumed the unprocessed flaxseed and feed intake at all levels of flaxseed supplementation was similar to that observed for cows fed the control diet. Milk yield (range 26.2 to 27.4 kg day^{-1} and fat percentage range 3.4 to 3.6%) were not influenced by treatment. Milk protein percentage declined linearly (3.21% for control animals compared with 3.13% for cows fed 150 g kg^{-1} flaxseed) with increasing level of flaxseed inclusion in the diet. Milk yield was not affected by flaxseed supplementation. Previous research on buffalo cows supplemented with crushed flaxseed (221 or 442 g/animal per day) showed a significant increase of 10 to 18% milk yield with respect to the control diet (El-Aziz et al., 2012). Production was expressed as ECM and this parameter was influenced by flaxseed ($P < 0.05$) which was higher in milk from non supplemented flaxseed diets. It is reported that all the main species that comprise the ruminal cellulolytic flora, i.e., *F. succinogenes*, *Ruminococcus spp*. and the anaerobic fungi, appear vulnerable to inhibition by PUFA. Therefore, dietary PUFA supplementation has to take care that fibre digestion is not compromised.

Protein and casein content of milk was affected by flaxseed supplementation ($P < 0.05$ and $P < 0.01$, respectively); both protein and casein was lower in flaxseed-supplemented diets.

Levels of urea in milk was not different among experimental groups, with mean value of 0.03 ± 0.008% in accordance with levels reported for buffalo milk (Campanile et al., 1998); the present results showed no differences in the efficiency of utilization of dietary nitrogen among treatments. A diet containing more rumen degradable protein than required by the microorganisms in the rumen, protein is degraded into ammonia that after absorbed in the rumen is metabolized to urea by the liver (Franzolin, 2010).

Health and functionality of mammary gland apparatus were preliminary evaluated through the level of somatic cells in milk. Somatic cells, indeed, are leukocytes and epithelial cells normally found in milk. The former derive from the blood and the latter are endogenous to the mammary gland. Leukocytes play an important role in the mammary gland defense against invading bacteria through the teat canal or through different sites of infection.

Somatic cell count in the present trial was not affected by treatments evidencing a good hygienic quality of milk, according to European Union Directives (46/92 and 71/94) that set a limit of 400,000 cells/mL for SCC in buffalo milk when the milk was used for products made with raw milk. In comparison with bovine milk, European Community Regulations set the range between 200.000 and 400.000 cells /ml, even if a mammary gland quarter is considered normal when it produces milk with less than 300,000 cells/ml.

Lactose content and pH of milk were not affected by treatments and fell within the range reported for buffalo milk (Fox, 2003), confirming the absence of IMI in the animals involved in the trial.

Buffalo milk fatty acid profile

Unsaturated fatty acids, particularly a-linolenic acid (LNA; cis-9,cis-12,cis-15–18:3) and linoleic acid (LA; cis-9,cis-12–18:2), are abundant in grass and other ruminant feedstuffs, yet are present at low concentrations in meat and milk. Indeed, ruminal microorganisms hydrogenate PUFA present in forages and thereby restrict the availability of health-promoting PUFA in food product from ruminant species. As the consumption of dairy products and ruminant meats is often associated with an increased incidence of coronary heart disease in man, the transformation of unsaturated fatty acids to saturated fatty acids, or biohydrogenation, in ruminants represents a major human health issue.

In the present experiment the dietary supplementation of flaxseed was able to influence the fatty acid profile of milk from buffalo cows.

Fatty acid composition of individual milk from buffalo cows subjected to different feeding regimens is reported in Table 3. The fatty acids in milk were grouped according to the length of the carbon chain in short chain fatty acids comprising fatty acids from C4:0 to C12:1 (SCFA), medium-chain fatty acids with C14:0 to C16:1 (MCFA) and long-chain fatty acids with a carbon chain greater than C18:0 (LCFA). Fatty acids grouped in short- ($P < 0.01$), medium- ($P < 0.001$), and long-chains ($P < 0.001$) were affected by flaxseed supplementation.

Short-chain fatty acids, in particular C8:0 and C10:0, were the lowest in milk from buffalo fed the highest level of flaxseed supplementation. Medium-chain fatty acids were the lowest in FS1000, intermediate in FS500. Myristic and palmitic acids were the most represented among medium-chain fatty acids; however, the supplementation with 1,000 g of whole flaxseed was able to decrease lauric and palmitic acids by about 10 and 20%, respectively, compared to control milk. Kennelly (1996) reported that the supplementation of flaxseed in the diet caused a reduction in the concentration of short-chain (C4 to Cl2) fatty acids as well as Cl6 while the concentration of 18-carbon fatty acids increased. In that study the change in the concentration of fatty acids in milk was proportional to the level of supplementatio of flaxseed in the diet: at the highest level of flaxseed supplementation (150 g kg^{-1} of diet DM) the concentration of short-, medium- and long-chain fatty acids were 81%, 71% and 146% respectively, of control values. The reduction in short- and medium-chain fatty acids has been attributed to a reduction in ruminal production of acetate and butyrate (precursors for de novo synthesis of these fatty acids in the mammary gland) as well as a dilution or direct inhibitory effect of dietary long-chain fatty acids on de novo synthesis (Grummer, 1991).

The C14:0 is mainly synthesized *de novo* by the mammary gland and C14:1 is synthesized from the desaturation of C14:0 (Corl et al., 2001), whereas the mammary gland produces half the palmitic acid (Mansbridge and Blake, 1997). Furthermore, ruminal protozoa are an important source of lipids for the host animal (Or-Rashid et al., 2007) and have a higher concentration of C16:0 than bacteria (Varadyova et al., 2008). The protozoa have also been considered to be candidates for biohydrogenation because of their high content of CLA and VA, although no truly protozoal (i.e., not due to associated bacteria) biohydrogenation activity has been demonstrated (Devillard et al. 2006).

The lower percentages of C16:0 in FS groups may be regarded as an outcome of the fat source and level on the ruminal ability to produce fatty acids. It was reported that supplementation with sunflower oil for sheep decreased the concentration of C16:0 in the rumen content (Toral et al., 2009). Moreover, supplementation of rice bran as a source of linoleic acid in cow led to lower concentration of milk C16:0 than control, probably due to a decreased ruminal protozoa (Castano et al., 2014).

Long-chain fatty acids were the highest in FS1000, intermediate in FS500, and the lowest in milk from buffalo receiving no flaxseed supplementation. Flaxseed supplementation influenced significantly the percentage of stearic, vaccenic, oleic, and linolenic acids, showing that the level of linolenic acid and of the intermediates of its biohydrogenation in milk reflected the level of dietary FS supplementation. In particular, vaccenic acid is produced in the rumen and then absorbed by the gut to be transported in the

mammary gland, where it is used for endogenous synthesis of rumenic acid through stearoyl-CoA desaturase activity (Luna et al., 2008).

In Graph 1 is reported total CLA in milk from buffalo cows subjected to different feeding regimens. Total CLA content showed the same trend of long chain fatty acids, with an increase of about 7% in FS500 and of 22% in FS1000 than the control. El-Aziz et al. (2012) reported an elevation by about 50% in the level of milk CLA in buffalo cows fed crushed flaxseed rather than control diet. In previous experiments on Simmental and Holstein-Friesian cow milk, a slight but not significant increase was reported for CLA in milk of whole flaxseed supplementation (1,000 g/d) with respect to the control (Cattani et al., 2014; Santillo et al., 2016); however, in Italian Friesian cows administration of 1,200 g/d per cow of whole flaxseed resulted in a significant increase of CLA content in milk (Caroprese et al., 2010). The different behavior of CLA content in different species and breeds fed whole flaxseed may be ascribed to the different gene expression and activity of stearoyl-CoA desaturase in the mammary gland. Indeed, compared with the homologous genes in cattle, sheep, and goat, the river buffalo stearoyl CoA desaturase is characterized by a higher genetic variability (Pauciullo et al., 2010).

Talpur et al. (2008) reported substantial variation in milk fatty acids composition especially CLA content across season: summer milk was characterized by a better profile with less saturated fatty acids, more unsaturated and CLA proportions in buffalo milk compared to rest of the year. The result was due to naturally grown fresh grass during summer affecting the biohydrogenation pathways.

Accordingly also in PDO Mozzarella di Bufala Campana the composition of triacylglycerols and FAs is similar to that of the parent milk with significant differences in the concentration along with different seasons of the year. Romano et al (2011) found that the level of the most common CLA isomer, cis-9, trans-11, ranged from 0.74% to 0.83%. and autumn cheese had more CLA than winter cheese because grassfed water buffalos produce milk that is naturally higher in CLA content. Further more the same authors highlighted that buffalo milk and cheese production chain should focus on feed type, inherent fat and process parameters that might decrease the native content of CLA in milk.

A different way to increase CLA content in buffalo milk was reported by Van Nieuwenhove et al. (2007); the authors evaluated the ability of some dairy bacteria to produce CLA in buffalo milk supplemented with free LA. The most tolerant strain to LA was Lactobacillus casei, while Lactobacillus rhamnosus produced the maximum level of CLA at high LA concentrations (800 μg ml^{-1}). The strains tested were able to grow in buffalo milk, showing the highest CLA formation near stationary phase. CLA production varied among strain, but all showed the higher percentage of

conversion at low LA concentration. Previous studies reported that many milk compounds, like proteins, could neutralize the negative effects of fatty acids on bacterial metabolism (Boyaval et al. 1995; Kim and Liu 2002). This process could explain bacterial growth in buffalo milk even at high concentrations of LA and the higher CLA production.

Saturated fatty acids, MUFA, PUFA, ArI and TI indexes, and desaturase indexes of milk from buffalo cows subjected to different feeding regimens are reported in Table 4. Saturated fatty acids, MUFA, PUFA were influenced only by flaxseed supplementation ($P < 0.001$). Saturated fatty acids and MUFA were the lowest and the highest in FS1000, respectively.

Polyunsaturated fatty acids were the highest in milk from buffalo receiving flaxseed supplementation compared with the control milk. Atherogenic index, TI, and n-6/n-3 were always the lowest in FS1000 groups, and TI and n-6/n-3 were intermediate in milk from animals receiving FS500.

Kennelly (1996) reported that the concentrations of monounsaturated and polyunsaturated fatty acids increased linearly while the concentration of saturated fatty acids declined with increasing levels of dietary flaxseed. Finally, it was reported that flaxseed feeding produced relatively small changes in the concentration of C18:3 in milk, indicating that extensive biohydrogenation of o-linolenic acid occurred in the rumen.

Present study confirmed the possibility to produce products of acceptable quality containing elevated levels of C18:3 and CLA. However, these products may have a shorter shelf life and require that modifications be made to standard procedures for manufacture of butter and cheese (McDonald and Scott, 1977) especially when the degree of unsaturation of milk fat is substantially increased.

The nutritional indexes ArI, TI, and n-6/n-3 were influenced by flaxseed supplementation The modifications of nutritional indexes in milk showed the role of flaxseed supplementation on the health properties of buffalo milk. Flaxseed supplementation evidenced a reduction of desaturase indexes respect to the control group. Although the concentrations of C14:1, C16:1, and C18:1 in milk increased with the inclusion of flaxseed in the diet but the desaturase indexes did not follow the same trend, suggesting that the increase of these UFA was not related to an increase in Δ9- desaturase activity. The same behavior was observed also in sheep supplemented with grape seed and linseed alone or in combination respect to a control diet (Correddu et al., 2016).

CONCLUSION

Fatty acids present in milk fat are derived either directly or indirectly from the diet. There are four main sources of fatty acids in ruminant diets, and they differ in the type and levels of fatty acids which they contribute to the diet: forages, oils and oilseeds, fish oil, fat supplements. The form in which the oil is presented to the rumen can have an effect on the fatty acid composition of milk fat; thus it is important to offer a higher degree of protection from hydrogenation occurring in the rumen.

Although the composition of milk from ruminant species is influenced by the type and level of fat in the diet, the relationship is modified by the effect of the digestive tract. This effect could be massive in the rumen where extensive metabolism of lipid occurs before digestion and absorption in the small intestine.

The modest efficiencies with which fatty acids regarded as important in human nutrition have been transferred from the diet to milk fat, raise important questions regarding the dietary sources investigated to date. In buffalo, limited research have been conducted to investigate the effect of dietary supplementation on fatty acid profile of milk.

Flaxseed supplementation was tested in buffalo cows feeding for the production of milk destined to cheese production. Milk production calculated as energy corrected milk was influenced by flaxseed, and this parameter was higher in milk from non supplemented flaxseed diets. Protein and casein content of milk was also affected by flaxseed supplementation, being both protein and casein lower in flaxseed-supplemented diets.

The main result concerned the fatty acid pattern of buffalo milk, demonstrating that flaxseed supplementation was successful in modifying the nutritional quality of fatty acid profile. Fatty acids grouped in short-, medium-, and long-chain FA were affected by flaxseed supplementation. In particular flaxseed supplementation influenced significantly the percentage of stearic, vaccenic, oleic, and linolenic acids with a reduction of saturated FA and an increase of linolenic acid and the intermediates of its biohydrogenation in milk. Furthermore, total CLA content evidenced an increase of about 7% in FS500 and of 22% in FS1000 than control milk. Nutritional value of the acidic profile in buffalo milk is influenced by dietary flaxseed supplementation, and its improvement reflects the level of flaxseed supplementation. Consumer interest is increasingly oriented to the improvement of the nutritional quality of food of animal origin especially concerning the the level of unsaturated fat in their diet. Buffalo milk with elevated levels of oleic, linoleic and linolenic acids may capture sufficient market share to make it commercially viable. Probably, the greatest challenge to the commercial

production of buffalo milk containing elevated levels of unsaturated fatty acids may concern the collection and processing system which should be targeted to the quality of milk produced.

TABLES

Table 1. Ingredients, chemical composition and *Milk Forage Unit* (MkFU) of the experimental diets (% on DM basis)

	Diets[1]		
Parameter	CO	FS500	FS1000
Ingredients			
Corn silage	35.06	34.63	33.23
Wheat middlings	10.40	9.86	7.61
Straw	13.40	13.29	13.50
Vetch and oat hay	15.43	15.42	15.51
Soy	15.61	15.36	14.19
Corn	10.41	8.62	10.44
Flaxseed	-	2.77	5.45
Chemical composition			
Ether extract	2.69	3.84	4.90
Crude protein	14.89	14.93	14.95
NSC	33.03	31.89	32.45
ADF	25.53	25.83	25.92
NDF	45.61	44.12	44.20
ADL	3.95	4.16	4.37
MkFU	0.87	0.89	0.88

NSC= non structural carbohydrates; ADF=acid detergent fiber; NDF=neutral detergent fiber; ADL = acid detergent lignin.
[1] Co- control diet with no flaxseed supplementation; FS500- low flaxseed supplementation (500 g); FS1000- moderate flaxseed supplementation (1000 g).

Table 2. Yield and composition of milk from buffalo cows subjected to different feeding regimens.

	Diets [1]				
	CO	FS500	FS1000		Effect, P
				SEM	Flaxseed
Milk yield, kg/die	9.14	8.51	7.75	0.49	NS
ECM yield, kg/die	16.19[b]	12.42[a]	13.12[a]	1.05	*
Fat, %	9.91	8.77	10.06	0.29	NS
Protein, %	4.97[b]	4.74[a]	4.68[a]	0.08	*
Casein, %	3.89[b]	3.71[a]	3.67[a]	0.06	**
Lactose, %	4.79	4.75	4.63	0.05	NS
pH	6.72	6.83	6.72	0.02	NS
Log_{10} SCC	5.33	5.42	5.14	0.06	NS

[1] Co- control diet with no flaxseed supplementation; FS500- low flaxseed supplementation (500 g); FS1000- moderate flaxseed supplementation (1000 g).
*$P < 0.05$, **$P < 0.01$.
[a-c] Values followed by different letters differ significantly at $P < 0.05$.

Table 3. Fatty acid composition (% of FAME) of milk from buffalo cows subjected to different feeding regimens.

	Diets [1]				
	CO	FS500	FS1000	SEM	Effect, P Flaxseed
Short-chain	13.06^{b}	13.24^{b}	11.63^{a}	0.4	**
C8:0	1.37^{b}	1.44^{b}	1.11^{a}	0.03	**
C10:0	2.41^{b}	2.49^{b}	1.91^{a}	0.08	*
Medium-chain	51.44^{c}	48.61^{b}	42.61^{a}	0.57	***
C14:0	11.95^{b}	12.23^{b}	10.78^{a}	0.2	***
C14:1	0.98^{b}	0.65^{a}	0.83^{a}	0.09	**
C16:0	33.9^{c}	30.79^{b}	27.39^{a}	0.6	***
C16:1	1.7^{b}	1.53^{ab}	1.32^{a}	0.1	***
Long-chain	32.75^{a}	35.97^{b}	43.18^{c}	0.84	***
C18:0	8.27^{b}	9.18^{c}	12.2^{d}	0.26	***
C18:1,*trans*11	3.74^{ab}	4.27^{bc}	4.88^{d}	0.15	***
C18:1,*cis*9	16.3^{a}	17.79^{b}	21.05^{c}	0.66	***

C18:3n3	0.27[a]	0.42[b]	0.58[d]	0.03	***
Total CLA	1.17[a]	1.23[b]	1.45[c]	0.04	***

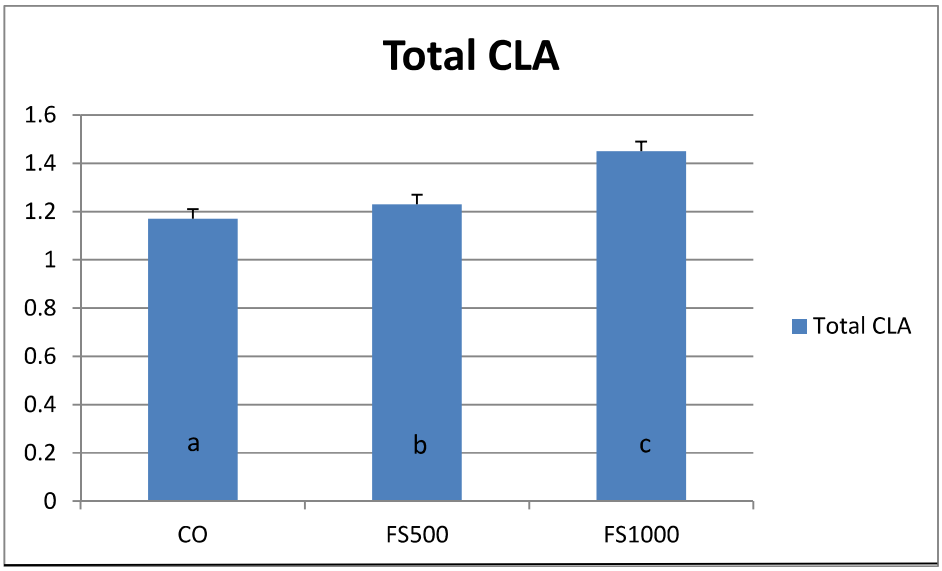
[1] Co- control diet with no flaxseed supplementation; FS500- low flaxseed supplementation (500 g); FS1000- moderate flaxseed supplementation (1000 g).
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
[a-c] Values followed by different letters differ significantly at $P < 0.05$.

Table 4. SFA, MUFA, PUFA, nutritional indices and desaturation indices of milk from buffalo cows subjected to different feeding regimens.

	Diets[1]				
	Co	FS500	FS1000	SEM	Effect, P
SFA	73.4[b]	71.2[b]	67.3[a]	0.78	***
MUFA	23.1[a]	24.9[a]	28.5[b]	0.73	***
PUFA	3.8[a]	4.14[b]	4.4[b]	0.14	***
AI	3.3[b]	2.9[b]	2.3[a]	0.13	***
TI	3.8[c]	3.4[b]	2.8[a]	0.14	***
n6/n3	9.3[c]	7.1[b]	5.7[a]	0.25	***
Δ^9 C14	7.7[ab]	7.3[a]	7.5[a]	0.31	*
Δ^9 C16	5.6[a]	4.8[a]	4.6[a]	0.23	***
Δ^9 C18	67.3[a]	66.4[a]	62.2[a]	1.14	***

[1] Co- control diet with no flaxseed supplementation; FS500- low flaxseed supplementation (500 g); FS1000- moderate flaxseed supplementation (1000 g).
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
[a-c] Values followed by different letters differ significantly at $P < 0.05$.
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)].
The Δ9-desaturation indexes:
C14 index =[C14:1 *cis*-9/(C14:0 + C14:1 *cis*-9)] . 100;
C16 index =[C16:1 *cis*-9/(C16:0 + C16:1 *cis*-9)] . 100;
C18 index =[C18:1 *cis*-9/(C18:0 + C18:1 *cis*-9)] . 100.

Graph 1. Total CLA in milk from buffalo cows subjected to different feeding regimens.



Co- control diet with no flaxseed supplementation;
FS500- low flaxseed supplementation (500 g);
FS1000- moderate flaxseed supplementation (1000 g).

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SECOND TRIAL

CONTENTS

ABSTRACT

The objective of this second trial of PhD thesis is to evaluate the sensory profile and consumers' liking of functional ovine cheese containing probiotic cultures, also observing the action of different strains of probiotic, added to rennet paste for cheesemaking, to assess their ability to produce conjugated linoleic acid (CLA) from free linoleic acid (LA) and Free Amino Acids (FAA), able to positively influence the nutritional and organoletic quality of the cheese. Probiotic are defined as "live microorganisms that, when administred in adeguate amounts, confer a health benefit on the host" (Food and Agricuture Organization of United Nations; World Health Organization-FAO/WHO, 2001).

The sensory perception of a food is a very important product characteristic; it is a complex process, which is influenced by many factors, such as content of flavour compounds, the texture, and appearance of the product, but also several characteristics of the individual consuming the product in a certain environment.

Aroma development in cheese products is influenced by metabolic activities of cheese bacteria, glycolysis, lipolysis and proteolysis. Flavor compounds in cheese arise from the action of enzymes, from the rennet (substitute), the milk, the (secondary) starter and non-starter bacteria, together with non-enzymatic conversions (Walstra et al., 1993; Kosikowski et al., 1997; Skeie et al., 2000). In cheese making, selected starter cultures are of main importance for controlling flavor formation. In order to respond to the increasing demand for products with improved aroma characteristics, the use of bacterial strains for cheese ripening is considered a very promising method.

Ovine cheese was made from ewe's milk by animals reared in extensive conditions; cheesemaking trials were performed by using rennet paste containing probiotic cells. The experimental test was conducted in a cattle farm where animals (Gentile di Puglia sheep) were reared with a semiextensive system, that provides for the integration of the food ration with concentrated in autumn and winter months.

The experimental cheeses are produced using three different rennet pastes lamb rennet in traditional lamb pasta (cheese thus obtained was called cheese control-C), rennet paste lamb containing a mix of *Bifidobacterium lactis* and *Bifidobacterium* longum (BB), and cheese manufactured using lamb rennet paste containing *Lactobacillus acidophilus* (LA).Three experimental cheesemaking were performed for each type of rennet. The rennet had a content of chymosin: pepsin of at 80:20.

The probiotic micro-organisms have been incorporated in the rennet paste 24h before each job in a concentration of 10^9 cfu / g of rennet. The average cell count of probiotic bacteria in the experimental cheese was found to be 7,4x10^7ufc / g and 7,1x10^7ufc / g in LA and BB cheeses, respectively, at the end of the vesting period.

The cell counts show that the production of cheese from sheep's milk pecorino probiotic Gentile di Puglia was conducted successfully. In the literature it is reported that the minimum dose of probiotics able to really play a beneficial effect on the health of the host is of 10^8-10^9 cfu / ml which corresponds to the assumption of 100-g of product containing 10^6-10^7 cfu / ml per day (Lourens-Hattingh, 2001).

Ovine cheese containing probiotic strains highlighted a more intense proteolysis and a greater level of short chain free fatty acids and conjugated linoleic acid due to the metabolic activity of the adjunct microflora. The sensorial profile of ovine cheese showed lower humidity and gumminess in cheeses containing probiotics as a consequence of differences in the maturing process; furthermore, probiotic cheeses scored higher ratings for salty and pungent attributes. An interaction effect of probiotic, gender, and age of the consumers was detected in the perceived and the expected liking. The higher rate of expected liking in all experimental cheeses is attributed to the information given, regarding not only the presence of probiotic strains but also the farming conditions and cheesemaking technology.

Keywords: probiotic; ovine cheese; sensory profile; cheese liking

INTRODUCTION

Today, food is not merely viewed as a vehicle for essential nutrients to ensure proper growth and development, but as a route to optimal wellness. Functional foods are those which contain some health-promoting components that go beyond the traditional nutrients; one way in which foods can be modified to become functional is by adding probiotics (Castro et al.2015). Several health benefits are claimed for foods containing probiotic microorganisms, especially lactobacilli and bifidobacteria (Candela et al., 2010). The characteristics and habitat of most Lactobacillus species are well-known. Some of the species of this genus have a long history of apparent safe use in industrial and agricultural applications. Members of the *Lactobacillus* genus are daily consumed in large quantities in a variety of fermented foods by people of all ages, ethnic groups and health status with apparently no ill-effects.

The development of probiotic cheeses implies knowledge of the processing steps as well as a level of influence (positive or negative) on the survival of microorganisms, sensory acceptance, chemical stability, and microbiological conditions throughout shelf life (Granato et al.2010).

The manufacture of probiotic cheese should have minimum changes when compared to traditional products (Cruz et al.2009), and must provide an adequate probiotic cell load upon cheese consumption. Cheeses could offer certain advantages as a delivery system of live probiotics to the gastrointestinal tract, having a higher pH than fermented milk and a high fat content that may protect the organisms during passage through the gastrointestinal tract Stanton et al.1998). The potential of different typologies of ovine cheese, e.g., semi-hard cheese and *pasta filata* stretched curd as a functional food delivering different probiotic bacteria has been previously reported (Santillo et al. 2008; Albenzio et al.2013) Those reports showed that probiotics yielded a complex outfit of proteolytic and lipolytic enzymes able to influence not only cheese microbiology but also the maturing process. Furthermore, probiotics in ovine cheese were involved in the production of molecules such as essential amino acids (Santillo et al. 2007), bioactive peptides (Albenzio et al.2015), and polyunsaturated fatty acids and conjugated linoleic acid (CLA) (Santillo et al. 2009).

Lipolysis is an important biochemical event occurring during cheese ripening that leads to the formation of FFA, which are precursors of compounds that are volatile and contribute to flavor (Collins et al., 2003). Conjugated linoleic acid (CLA) is a term that refers to a mixture of positional and geometric isomers of linoleic acid (LA) in which double bonds are conjugated. It has received great attention for several beneficial health properties; for example CLA has been reported to prevent carcinogenesis (Ha et al. 1990; Ip et al. 1991) and atherosclerosis (Lee et al. 1994; Nicolosi et al. 1997), modulate immune response (Hayek et al. 1999) and reduce body fat (Park et al. 1997).

Another important process is proteolysis that directly contributes to cheese flavours by releasing peptides and amino acids. Amino acids (AA) are substrates for transamination, dehydrogenation, decarboxylation and reduction, producing a wide variety of flavour compounds.

The commercial success of any functional food, especially those containing probiotic strains, ultimately depends on taste, appearance, price, and health claim appeal to consumers (Granato et al.2010). Thus, in the development of probiotic ovine cheese, the sensory evaluation by consumers is a crucial and essential step that rules product innovation. Furthermore, the information about the characteristics of the innovative dairy product must be provided to the consumers, it being a factor able to increase consumers' awareness and willingness to choose the new food. Napolitano *et al.* (Napolitano et al.2010), reported that expectation induced by the information can affect the quality perception and oriented the consumers in their choice. The application of sensory methodology allows researchers and developers to obtain important results on the formulated product with respect to its descriptive sensory profile and acceptance on the consumer market (Cruz et al.2010). Developing a sensory profile of the product helps to identify the principal sensory features of functional products, their negative and positive attributes compared to the conventional product. The results from a descriptive analysis test also provides a basis for determining those sensory attributes that are important to acceptance. Some authors (Barcenas et al. 2007; Lavanchy et al. 1999); provided a very interesting guide for the sensory evaluation of ewe's milk cheese with a trained panel. However, it is crucial to remember that a trained panel must not measure liking, acceptance, or preference. Once panelists are trained to identify and quantify attributes in products (or grades and defects as in product judging), they are no longer typical consumers (Cruz et al.2010).

Probiotics are bacteria, generally lactobacilli and bifidobacteria, associated with a plethora of health promoting properties. It is suggested that the concentration of viable probiotics in the product should reach 10^7 CFU g^{-1} or mL^{-1} at the time of consumption (Reid 2008). In addition, certain probiotic bacteria have demonstrated to produce conjugated linoleic acid (CLA), which has gained considerable attention because of its anticarcinogenic property, among others (Coakley et al.2003).

Cheese is one of the most diffused vehicles to hold efficiently probiotic bacteria, and thus introduce them into the human diet (Saxelin et al.2003). For a long time the cow's milk cheeses were mostly studied for the incorporation of probiotics (Bergamini et al.2010a; Gomes et al.2011; Ong et al.2007; Souza and Saad 2009). However, today the supplementation of ovine and caprine cheeses with probiotic bacteria is a usual practice as can be seen in the widespread literature about this topic

(Albenzio et al.2013; dosSantos et al.2012; Özeretal.2009; Santillo et al. 2012; Santillo and Albenzio 2008).

This trend is due to the increasing interest in giving added value to this food that already has benefits inherent to its nutritional and organoleptic properties. However, the ovine cheeses are gaining popularity and acceptance on the regional market. Therefore, the development of a probiotic cheese from ewe's milk results in an interesting alternative for diversifying the existing products.

For this reason, the aim of this study was to evaluate the survival of a probiotic bacteria incorporated in ovine cheese and its effect on the final product characteristics such as the sensory profile and consumers' liking, chemical parameters, proteolysis, lipolysis, CLA content and volatile profiles.

PECORA GENTILE DI PUGLIA

Sheep milk and cheese production in the Mediterranean area

European sheep production, in the Mediterranean areas represent important economic, environmental and sociological issues.

About 46% of the world sheep milk originates from the Mediterranean area. The most important countries in terms of the number of dairy sheep and goats are Greece, Italy, Spain, France and Turkey in Europe, and Algeria, Egypt and Libya in North Africa (FAOSTAT, 2013). The productive cycle of Mediterranean sheep is very closely linked to the Mediterranean climate which is characterized by a mild winter, with rainfall occurring in autumn and spring, and a very dry summer.

The production of whole fresh sheep milk in the world accounts for approximately 1.4% of the global milk yield (9,584 MTON/year as average for 2007-2011; FAOSTAT, 2013). In many countries, sheep milk and milk products (e.g., yogurt and cheese) are produced using unspecialized breeds and are consumed locally, whereas in many Mediterranean (e.g., Italy, Spain, France, Portugal, Greece, Turkey, Israel) and East European countries (especially Bulgaria and Romania) sheep milk is often produced by specialized dairy breeds, and sheep milk products, especially cheese, are commercialized internationally.

Dairy sheep and goat breeding in Europe is most common around the Mediterranean basin, particularly in France, Greece, Italy, and Spain. The milk of sheep and goat is mainly reserved for cheese making that is usually conducted at farm level or in small local dairies. A large part of the production, principally for sheep milk cheese, is protected by the European Community (EC) regulations: the Protected Designation of Origin (PDO) and the Protected Geographic Indication (PGI). These designations constitute an element for the protection of the biodiversity, i.e. territory, animals, microbes, practices, and production systems.

In Italy, the main use for sheep milk is for cheese making. Its exploitation as such is more important in Sardinia, Tuscany, Lazio, and Sicily. The transformation of sheep milk into cheese is carried out mainly at industrial levels, although a significant amount of this milk is processed in small dairies by the breeders themselves. The manufacture of many Italian sheep milk cheeses is regulated by a Protected Designation of Origin (PDO) which identifies the designation of a product of which the production, processing and preparation has to take place in a specific geographical area and has to be characterized by a recognised and approved assessment. These designations constitute an element for the protection of the biodiversity, i.e. territory, animals, microbes, practices, and production systems. Microbiological features and diversity of sheep milk compared to the cow's milk give peculiar

attributes to the cheeses. In several cases, rennet paste, produced from the abomasa of the suckling lambs or kids is used, strongly influencing chemical and sensory characteristics of the cheeses. In this review the technological, microbiological, and phyisico-chemical aspects of the PDO and PGI Italian sheep dairy products are reported.

Taking into account the high quality of sheep milk, characterized by low allergenic activity and high concentration of nutraceutical compounds, and the relatively high price of sheep cheese, there is an interesting potential worldwide market for this industry, with a growing interest in various countries such as U.S., Brazil, and China.

Pecorinos are traditional, creamery, hard, drum-shaped cheeses. They come in a variety of flavours determined by their age. Aged Pecorinos referred to as 'stagionato' are hard and crumbly in texture with buttery and nutty flavours. Young or 'semi-stagionato' and 'fresco' Pecorinos feature a softer texture with mild, creamy flavours. A good Pecorino will have smooth, hard rind that is pale straw to dark brown in colour. The rind will vary in colour, depending on the age of the cheese, and may include a protecting coating of lard or oil. Its compact interior is white to pale yellow in colour, with irregular, small eyes. Pecorino is a preferred cheese in many pasta cheeses and an obvious choice in Italian regions where the cheese is produced.

Pecorino cheese is the true ambassador of Italian exports. It is up by a superb 23% – seven times more than the average increase of the country's exports, which remains stable at +3.5%.

The top buyers are the Americans (+28%), followed by the English (+22%) and the French (+16%). Good performance has also been seen in Asia where Japanese demand is growing at 9% and Chinese demand has boomed, increasing by as much as 500%.

This highly appreciated product is mainly exported from Sardinia, a region where 3.2 of the peninsula's 6.2 million sheep graze, with Sicily following at some distance with its 770,000 animals, and then Lazio (630,000) and, finally, Tuscany (420,000).

Around 400,000 tonnes of sheep milk is produced in Italy per year and a good 67,000 tonnes of sheep cheese. For Pecorino Romano PDO alone, production amounts to 25,000 tonnes, of which 60% is exported.

Expo 2015 has undoubtedly played an important role in the growth – or 'rediscovery' let's say – that Italy's Pecorino is enjoying. Amongst good choices and not-so-good choices, the fact is that Expo 2015 let the world know about the Italian food and agriculture model during its six months in Milan.

According to data from Coldiretti, approximately 2,000 young people have chosen to try their hand at managing flocks of sheep, confirming the state of grace of the Italian agri-food sector, which is the second largest sector in the country's economy. Many want to continue their parents' business, but there is also a considerable number of novice entrepreneurs who are driven by the desire to find alternative employment that brings them into contact with nature.

Last year the Italian agri-food sector came close to 36 billion euros, registering an increase of +7%. Made in Italy products have a strong consumer appeal due to their quality and genuineness, therefore **the problem of counterfeit products** is more serious today than ever before. What is more, there is often a lack of serious law enforcement efforts. In the United States, for example, more than 20.5 million kilos of Pecorino Romano are produced each year which do not specify the geographical origin and some of which, paradoxically, **are not even made from sheep milk**.

A boom in young sheep farmers and 200,000 more sheep in five years

There are almost seven and a half million sheep in Italy, just under **200,000 more compared to five years ago**. According to recent estimates by the European Commission, the number of sheep in Italy is on the rise again. A dossier was recently prepared by Coldiretti on the occasion of **Sheep Day** in which thousands of sheep farmers who breed all types of sheep gathered together at the **University Sports Centre (CUS) in Aquila**, twenty years after the birth of **Dolly the sheep** (May 1996). It is no secret that the **boom in demand for cheese from abroad** has been the driving force behind this revival in sheep farming, as well as innovations that have revolutionised livestock breeding such as cosmetics, fashion, construction, schools, environmental maintenance, pet therapy and even new products, such as sheep milk ice cream and anti-cholesterol Pecorino cheese. The business climate is more positive; at the moment **young farmers** are not so much worried by the crisis but rather by bureaucratic delays and inefficiency and attacks by wild animals, such as wild boar and wolves, which have multiplied in the countryside. In addition, ancient breeds, of which at least 38 have been saved from extinction, are now reappearing. They include the rustic **Sardinian sheep**, the **Sopravissana** sheep which is well-known for its high quality wool, the **Comisana** sheep with its characteristic red head, the **Massese** sheep with its unusual black coat, the **Appenninica**, **Merinizzata** and **Barbaresca Siciliana** breeds and also the **Bergamasca**, which is suitable for transhumance and is the largest sheep breed in the world. Fortunately this wealth of biodiversity can particularly be found in underprivileged areas of Italy, thereby bringing added value to them. (Alimenta, 2016)

Exports of Italian pecorino cheese

Throughout 2015 the sales performance of pecorino cheese abroad have shown good performance with + 3.8 % in volume and + 16.5 % in value compared to 2014. The US market continues to lead as the first buyer of Italian Style, with a 63% share in volume and a higher total turnover of 100 million euro (+ 19.6 % compared to 2014) .

Even in the EU area , the 2015 data indicate positive changes over the previous year (+ 8 % in quantity; + 16 % in value) . While maintaining the primacy in terms of quantities, mailings to Germany (-0.5 %) , compared with a significant growth of direct volumes were reduced slightly in France (+ 11.6 %).

Data are reported in table1

Export of Italian pecorino (1) by country of destination

January-December	2014 (ton)	2015 (ton)	var.% 15/14	2014 (.000 €)	2015 (.000 €)	var.% 15/14
Export tot.	16.624	17.251	3,8%	139.109	162.043	16,5%
- United States of America	10.423	10.809	3,7%	84.861	101.492	19,6%
- Germany	1.406	1.400	-0,5%	14.133	14.891	5,4%
- France	1.031	1.151	11,6%	7.813	9.123	16,8%
- United Kingdom	713	709	-0,6%	5.747	6.810	18,5%
- Netherland	339	434	27,9%	3.202	4.426	38,2%
- Other Countries	2.712	2.749	1,4%	23.352	25.302	8,4%

Table 1 *(1)* customs code *04069063 Pecorino/Fiore Sardo*
source: ISMEA data ISTAT

Sales of pecorino cheese abroad have continue to increase in 2014 , reaching a record level in eleven months of 125.7 million euro (+ 11 % compared to the period January-November 2013), even if the performance is extraordinary by exclusively attributable to higher export average values (8.34 euro / kg, up 15% over last year) .

In fact, in the period January-November 2014 , export volumes fell (-3.5 %) , mainly due to the limited availability of the product and the resulting halt of the mailings to key community partners (especially Germany and France) and the slowdown in shipments to the US market (+ 1.6 % in volume) .

The United States is still the main outlet of the national production of pecorino and in the first eleven months of 2014 approximately 63% of output volumes from Italy has been absorbed by the market " Stars and Stripes " , generating a revenue growth in the next + 22 % .

The US market remains the first outlet for the Italian Style, with a market share in excess of 60% value and a turnover last year reached almost 85 million euro due to a sustained increase (+ 25% compared to 2013) of export average values (average unit price for exports amounted to 8.14 EUR / kg in 2014 compared to 6.76 EUR / kg in 2013). (Ismea, 2016)

In 2015 the recovery of consumption and the supremacy of the dollar have encouraged, in general, the US demand for imported cheeses and, in particular, has further increased the demand for pecorino.

In just nine months, Italy has sent to the United States well 8,486 tons of cheese for a value of over 73 million euro, an increase of 20% and 42% in comparison with the same period last year. Italy, while confirming its leadership in the segment of grated pecorino, is gaining market shares "stars and stripes" especially in the segment of sheep to the meal, taking advantage of the price competitiveness compared to the Iberian products.

Functional Foods: Concepts and Outlook

With an increase in humam life expectancy, and the exponential growth of health care costs, society needs to overcome new challenges through the development of new scientific knowledge and technologies that result in important modifications in people's life style (Kwak and Jukes 2001). This tendency and advances in food science/technology are providing the food industry with increasingly effective techniques to control and improve the physical structure and the chemical composition of food products, creating functional foods that provide attributes beyond nourishing properties (Behrens et al. 2001).

The health/nutrition paradigm has changed significantly during the past 2 decades. Today, food is not merely viewed as a vehicle for essential nutrients to ensure proper growth and development, but as a route to optimal wellness. This "food as medicine" paradigm will continue to be driven by several key factors, including increased consumer interest in controlling their own health; demographics, including increases in the elder and ethnic subpopulations; escalating health care costs; a highly competitive food market with small profit margins; advances in technology, such as biotechnology, nanotechnology, nutrigenomics, and changes in food regulations and evidence-based science linking diet to reduction in nontransmissible chronic disease risks (American Dietetic Association 2009).

Functional foods are those that contain one or more compound that provide important or limited functions in the organism, promoting welfare and health, or for reduction in the risk and protection of hypertension, diabetes, cancer, osteoporosis, and heart diseases (Arihara et al. 2004). These foods present a potential to promote health through mechanisms not foreseen in conventional nutrition, with the need to be pointed out that these effects restrict them to the promotion of well-being and health by maximizing physiological functions of a person and not for the cure of illnesses (Sanders 1998; Roberfroid 2000). Functional foods generally contain one or more beneficial compounds such as prebiotic, probiotic, antioxidant polyphenols and sterols, carotenoids, and others (Shah 2001; Andlauer and Fürst 2002; Granato et al. 2010a).

Modifying foods through biotechnology to improve their nutritional value and health attributes may also bring new functional foods to the marketplace, such as increased ω-3 fatty acids or absence of *trans* fat, although the topic remains controversial.

A medical food represents a food that is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation. Examples of medical foods include oral supplements in the form of phenylketonuria formulas free of phenylalanine, and diabetic, renal, and liver formulations.

Lastly, foods for special dietary use have a particular use for which a food purports or is represented to be used, including but not limited to the following: supplying a special dietary need that exists by reason of a physical, physiological, pathological, or other condition; supplying a vitamin,mineral, or other ingredient for use by humans to supplement the diet by increasing the total dietary intake; supplying a special dietary need by reason of being a food for use as the sole item of the diet. Examples of such foods include infant foods, hypoallergenic foods such as gluten-free foods and lactose-free foods, and foods offered for reducing weight.

According to Hamilton-Miller and other authors (1999), the food industry has to satisfy the demands of the consumer to succeed in promoting the consumption of functional probiotic products.

Viana and others (2008) conducted a study to evaluate the perception and the attitudes toward probiotic foods of the population in the city of Rio de Janeiro, Brazil. In general, the study found that the population was confused with respect to probiotic foods and the benefits arising from their consumption. Therefore, the food industry should focus on an elementary easy-to-understand

educational program using accessible language to increase the awareness related to these products and of the health benefits probiotic products may confer, if consumed along with a balanced diet.

Probiotics as Functional Foods: Definitions

Among the foods whose alleged health claims have been widely promoted in the media during the past few years and that present multidimensional studies for technological and industrial uses, the ones with probiotic strains stand out (Lourens-Hattingh and Viljoen 2001). The dairy sector, which is strongly linked to probiotics, is the largest functional food market accounting for nearly 33% of the broad market, while cereal products have just over 22% (Leatherhead Food International 2006).Moreover, in recent years, per capita consumption of yogurt has increased drastically because many do consumers associate yogurt with good health (Hekmat and Reid 2006).

Although the concept of probiotics was introduced in the early 20th century, the term was not coined until the 1960s. The definition of the term has evolved through the years. According to Fooks and other authors (1999), the word *probiotic* derives from 2 Greeks words that mean *for life*. This term was 1st used to describe a microbial substance that stimulated the growth of other microorganisms (Lilley and Stillwell 1965) or tissue extracts that promoted microbial growth (Sperty 1971), but it did not receive general acceptance. Parker (1974) was the 1st author to use the word probiotic in the context of animal supplementation and it was defined as organisms and substances that contributed to the balance of the intestinal flora. Fuller (1989) defined probiotics as food supplements containing live microorganisms that affect the host in a healthy way, balancing the intestinal flora. Many other definitions of the term probiotic have been published since (Sanders 2003); however, the most widely accepted definition is that "probiotics are live microorganisms, administrated in certain quantities that confer health benefits to the host" (FAO/WHO 2001).

Although various strains of lactic acid bacteria have been described as probiotic, relatively few meet the standards of the United Nations of having clinical trial documentation, and many are too sensitive to intense acidity and presence of bile salts in the human gastrointestinal tract, so they die en route to the gut (Hekmat and Reid 2006). The majority of probiotic products available in the marketplace contain species of *Lactobacillus* and *Bifidobacterium*, which are the main genera of Gram-positive bacteria currently characterized as probiotics (FAO/WHO 2001). Different species of probiotic microorganisms have been employed in the food industry, such as: *Lactobacillus acidophilus, L. casei, L. johnsonii, L. rhamnosus, L. thermophilus, L. reuteri, L. delbrueckii* subsp. *bulgaricus,*

Bifidobacterium bifidum, B. longum, B. brevis, B. infantis, and *B. animalis* (Knorr 1998). *Lactobacillus. delbrueckii* spp. *bulgaricus,* and *Streptococcus thermophilus* are found in a number of preparations such as traditional yogurts, frozen yogurts, and in desserts in some places (Senok 2009).

There are some ideal properties of the probiotic strains that would benefit human health and could be used in the probiotics industry. These include resistance to acid and bile; attachment to the human gut epithelial cells; colonization in the human intestine; production of antimicrobial substances, including bacteriocins; good growth characteristics and beneficial effects on the human health. One of the most important characteristics of a probiotic strain is that it must be nonpathogenic and generally regarded as safe (GRAS). Probiotics must also present some desirable characteristics, such as maintenance of viability during processing and storage, ease of application in products, and resistance to the physicochemical processing of the food (Prado et al. 2008).

These bacteria should not be pathogenic, toxic, mutagenic, or carcinogenic in the host organism, must be antagonistic to pathogens and be genetically stable without a plasmid transfer mechanism, especially concerning antibiotic resistance; they must survive during digestion and possess the ability to adhere and colonize the gut mucosa, promoting immuno-stimulation without inflammatory effects (Saarela et al. 2000). It is important to report that these bacteria should be present in a dairy food to a minimum level of 10^6 CFU/g or the daily intake should be about 10^8 CFU/g, with the aim to compensate for the possible reduction in the number of the probiotic microorganisms during the passage through the gut (Shah 2007).

Some Health Effects of Probiotic Microorganisms

It is believed in academia that probiotic microorganisms can improve health and, through media and marketing, the population must become more aware of this. To supply growing demand, the food industry has been developing new probiotic products.

Several health benefits are attributed to the ingestion of probiotic-containing foods, some of them have been proven scientifically (Figure 1) and others still require further studies in humans.

Moreover, probiotic bacteria have been shown to stimulate nonspecific host resistance to microbial pathogens (Perdigòn et al.1986; Perdigòn et al. 1998), and to modulate the host's immune responses to potentially harmful antigens with a potential to down-regulate hypersensitivity reactions.

There is no doubt that dairy products are the main vehicle for probiotic supplementation.

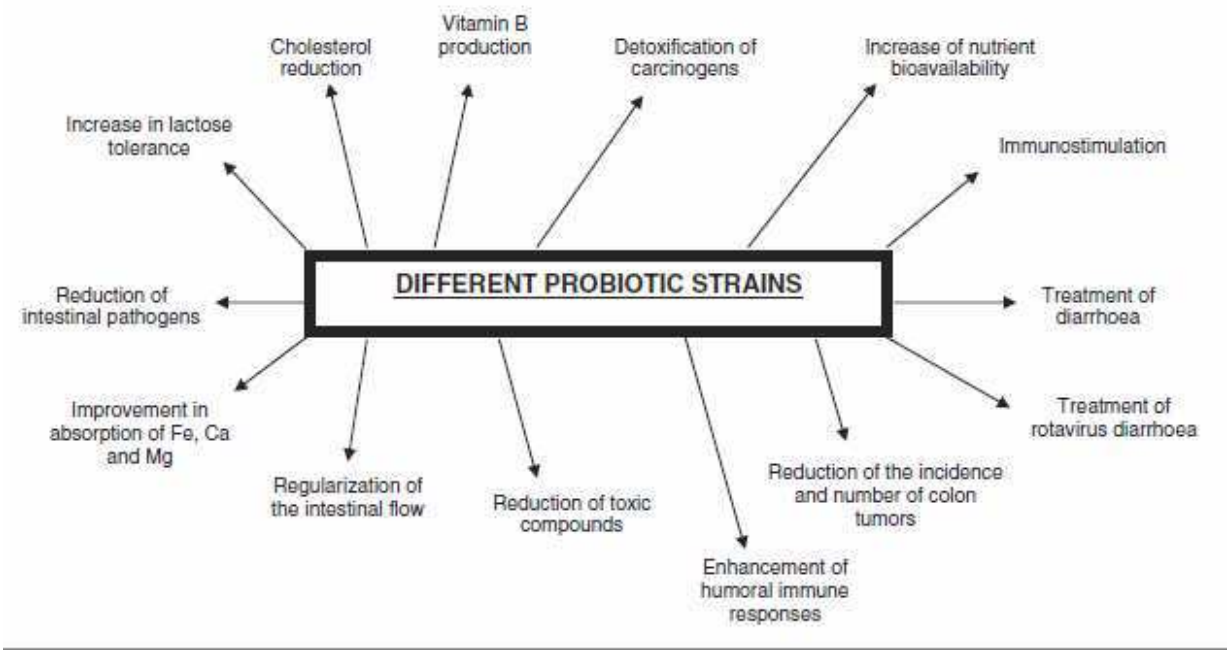

Figure 1- Some documented physiological benefit of foods contening probiotic bactiria

Traditional pecorino cheese and health cheese

A lot of sheep milk is transformed into cheese, milk quality is evaluated mainly in terms of its technological and coagulation properties, which depend primarily on fat and protein contents and on the somatic cell count (SCC). However, the increasing attention of consumers to the nutritional and health aspects of food has recently shifted the focus of dairy sheep producers towards the achievement of an appropriate milk lipid composition. Furthermore, more and more consumers are demanding dairy products with a special flavor associated with the territory where the animals live.

Pecorino is the name given to all Italian cheeses made from sheep's milk.

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Pecorino manufacture is still performed using traditional protocols and artisanal procedures, such as

the use of rennet paste obtained from the abomasa of suckling lambs or kids. Rennet paste contains a wide spectrum of enzymes that play a role not only in the coagulating phase but also on the lipolytic and proteolytic processes occurring during cheese ripening.

The incorporation of probiotic bacteria in rennet paste represents a strategy to obtain typical cheeses with functional characteristics without any modification in Pecorino cheese production protocol (Santillo and Albenzio, 2008; Santillo et al., 2009; Albenzio et al.,2010). Moreover, the same authors verified that the use of probiotic rennet did not affect adversely the composition, texture, and sensory features of the cheese.

These results could encourage producers to offer typical products while also adapting to new market requests for healthy dairy products. Indeed, the current consumer market of dairy products requires a continuous effort in terms of quality and innovation.

Consumer perception of food products is a very complex phenomenon that is influenced by a wide range of characteristics.

Organic cheese productions are often associated with traditional cheese making practices used in small-scale enterprises. Napolitano *et al.* (2010) studied the effect of information about organic production on *Pecorino* cheese liking and consumer WTP.

They found similar liking scores given in blind conditions (*i.e.* without information on the product) for organic *Pecorino* cheese and conventional *Pecorino* cheese. However, expected liking scores (without tasting and based on information only) were higher for organic than for conventional *Pecorino* cheese and actual liking of the organic product moved in the direction of expectancy. The authors concluded that the information about organic farming could be a major determinant of cheese liking. Consumers, in addition, showed a willingness to pay organic *Pecorino* cheese higher (4.20±0.13 EUR/100 g) than the local retail price for conventional *Pecorino* (1.90 EUR/100 g) and even for organic cheese (3.00 EUR/100 g). Therefore, the information about production process of traditional cheese may provide a potential tool for product differentiation, particularly for small-scale enterprises producing cheese according to traditional practices.

Many factors, such as distinctive consumer attitudes and beliefs and different family income levels, may have affected the diverse response of consumers from different countries to traditional cheese.

Innovation of traditional cheese

In order to increase the efficiency of production processes, traditional foods, and traditional cheese in particular, can be subjected to innovations. Acceptance of innovation in traditional cheese is strongly dependent on type of product and type of innovation. Some suggested areas for potential improvement of traditional food products are their safety, healthiness and convenience. The addition of functional ingredients, such as probiotic bacteria may be accepted if tangible benefits are exposed to the consumer. An example of innovation in traditional cheese may be represented by the incorporation of probiotic strains (*Bifidobacterium longum* and *Bifidobacterium lactis* or *Lactobacillus acidophilus*) into the cheese matrix of *Scamorza* cheese obtained from ovine milk (Albenzio *et al*., 2013).

FUNCTIONAL DAIRY PROBIOTIC FOOD DEVELOPMENT: *Trends, Concepts, and Products*

The main role of food is providing enough nutrients to meet metabolic requirements in human body, while giving the consumer a satisfaction feeling and well-being (Homayouni, 2008a). Beyond meeting nutrition needs, food may have different physiological functions and may play detrimental or beneficial roles in some diseases (Koletzko et al., 1998).

Functional foods were developed in order to promote a well-being state, improving health, and reducing the diseases risk. "Functional food" means; special foods which have preventional and/or curing effects beyond its nutritional (Homayouni, 2008a). There is a wide rage of functional foods that were developed recently and many of them are being produced in all over the world including probiotic, prebiotic and symbiotic foods as well as foods enriched with antioxidants, isoflavons, phytosterols, anthosyanins and fat-reduced, sugar-reduced or salt-reduced foods. Among these foods, probiotic functional foods are the first choice to exert positive effects on the human health. Probiotic functional foods were divided into dairy probiotic foods and non-dairy probiotic foods. Some of dairy probiotic foods including probiotic ice cream, frozen fermented dairy deserts, probiotic cheese, bioyoghurt, drinking yoghurt, kefir, Freeze-dried yoghurt and spray dried milk powder have been employed as possible delivery vehicles for probiotic bacteria (Haynes and Playne, 2002; Homayouni et al., 2008b; Homayouni et al., 2012; Ejtahed et al., 2011; Ejtahed et al., 2012; Mirzaei et al., 2012 Kailasapathy and Rybka, 1997; Ravula and Shah, 1998; Stanton et al., 2001). Probiotics are distinct as live micro-organisms which, when administered in sufficient amounts present a health benefit on the

host (Food and Agriculture Organization of United Nations; World Health Organization - FAO/WHO, 2002; Homayouni, 2009). In recent years probiotic bacteria have increasingly been incorporated into dairy foods as dietary adjuncts. *Lactobacillus* and *Bifidobacterium* are the most common probiotic bacterial cells that were used in the production of fermented and non-fermented dairy products.

Consumption of probiotic bacteria via dairy food products is an ideal way to re-establish the intestinal micro-floral balance. It must conform to certain requirements for a dairy food product to be considered as a valuable alternative for delivery of probiotic bacteria in one hand and for variety of probiotic cultures to use as a dietary adjunct and to exert a positive influence in the other hand. The culture must be native of the human gastrointestinal tract, having the ability to ferment prebiotics, survives passage through the stomach and small bowel in adequate numbers, be capable of colonizing in site of action, and have beneficial effects on human health. In order to survive, the strain must be resistant to acidic conditions (gastric pH 1-4), alkaline conditions (bile salts present in the small bowel), enzymes present in the intestine (lysozyme) and toxic metabolites produced during digestion (Homayouni et al., 2008d).For example in traditional yoghurt production, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were used as starter culture. These bacteria do not belong to the indigenous intestinal flora, are not bile-acid resistant and do not survive passage through the gut. So, the traditional yoghurt culture is not to be considering as probiotic.

Development of functional dairy foods

Innovation is today's business demand and development of a new functional food is an expensive process and is very important for both food companies and consumers. Regulations should encourage food companies to follow functional food development.

Development of dairy probiotic products requires detailed knowledge of both products and customers. It needs to manage customer knowledge effectively (Walzem, 2004; Jousse, 2008). Fundamental risks can affect the development of new functional food products and may leads to fail the development process. Development of new functional food products is very challenging and it has to complete the consumer's expectations for palatable and healthy products (Fogliano and Vitaglione, 2005; Granato et al., 2010; Shah, 2007). So, the development and commerce of functional food products is rather complex, expensive, and uncertain. Key points regarding for a successful functional food product development are consumer demands, technological conditions, and legislative regulatory background.

However, consumer's knowledge of the health effects of specific ingredients can affect the acceptance of specific functional food. Therefore, functional ingredients that are in consumers mind for a long period of time, such as minerals, fiber, and vitamins, achieve considerably higher rates of consumer acceptance than new products, such as foods enriched with probiotics, prebiotics, flavonoids, carotenoids, and conjugated linilenic acid (CLA). Several ways to make a functional food product is to eliminating an allergenic protein, lactose, phenylanine and etc from the natural food product; by fortification with a micronutrient; by adding antioxidants, probiotics or prebiotics); by replacing a component, or by increasing bioavailability or stability of a component known to produce a functional effect or to reduce the disease-risk potential of the food (Roberfroid, 2000; Siro, et al., 2008; Granato, et al., 2010). Field of functional probiotic foods requires the cooperation of food technologists, nutritionists, medical doctors, and food chemists in order to obtain innovative products. In this way, these foods may be able to adjust physiological parameters related to health status or disease prevention in human. So, the design and development of functional probiotic foods is a scientific work (Hasler, 1998; Walzem, 2004; Fogliano and Vitaglione, 2005) which is an expensive and multistage process that takes into account many factors, such as sensory acceptance, physical and microbial stability, price, and chemical and other intrinsic functional properties to be successful in the marketplace. Moreover, consumer attitude toward the functional probiotic product also needs to be understood and taken into consideration.

Consumer attitude toward functional dairy foods

The development of functional probiotic foods is increasing, as their market increases day by day, although the consumer's information about these foods is increasing without relation to gender, age, and educational or economic levels of the consumers. The therapeutical effect of a functional probiotic food may depend on the consumer's characteristics and the type of carrier and enrichment considered. For instance, yoghurt is most preferred by its enrichment with calcium and fiber. Ingredients such as vitamins and minerals applied in fortification of functional foods are widely recognized and accepted by consumers, but new functional ingredients such as probiotics and prebiotics are not common to them. So, there is a need for increasing the consumer knowledge with respect to these new special ingredients (Hillian, 2000; Luckow and Delahunty, 2004; Ares and Gambaro, 2007; Vianna et al., 2008). The sensory properties of prebiotic functional foods in comparison with conventional products can lead to different acceptance level. Oligofructose provides some suitable sensory properties such as

rounder mouth feel, reduced aftertaste, and slight sweetness to the products. These properties are responsible for high score values for taste, creaminess, and overall acceptability of functional food products. The first important marker in choosing a functional food is flavor, and health consideration is in the second order. If the ingredients added give unpleasant flavors to the product, consumers are not interested in consume such functional probiotic food even if this results in health advantages. This means that flavor is correlated to intrinsic sensory properties of the product such as overall acceptability. In general, as functional products consumption increases, the acceptance of such products may increase, even if the sensory profiles are different from conventional products. When functional ingredients such as probiotics are added to dairy foods, consumers must be aware of probiotics health benefits in order to recognize the functional probiotic foods as being more beneficial than the conventional ones. Functional probiotic food industry should communicate with consumer in a clear way and this is one of the most important aspects for success (Tepper and Trail, 1998; Matilla-Sandholm et al., 1999; Roberfroid, 2000; Tuorila and Cardello, 2002; Nicolay, 2003; Vieira, 2003; Homayouni, 2008a).

MATERIALS & METHODS

Experimental design

Gentile di Puglia ewe's milk was used for Pecorino cheese production. Three Pecorino cheesemaking trials were performed in duplicate at industrial plant scale according to the protocol reported by Santillo and Albenzio(2008) using the 3 different experimental rennet pastes. Experimental cheeses were denoted: cheese manufactured using lamb rennet paste without probiotic (C), cheese manufactured using lamb rennet paste containing a mix of *Bifidobacterium lactis* and *Bifidobacterium longum* (BB), and cheese manufactured using lamb rennet paste containing *Lactobacillus acidophilus* (LA).

Rennet paste was produced according to the following protocol: briefly, abomasa were extracted from suckling lambs and the perivisceral fat was removed; the abomasa were stored in salt at 6 °C and 70% relative humidity for 3 months and then ground to obtain a paste.

The ripened rennet paste was inoculated with fresh probiotic cells to obtain a final concentration of 10^9 cfu/g of rennet one day before ovine cheese manufacturing. Three experimental cheesemaking trials were performed for each type of lamb rennet paste. Ewe's milk from morning and afternoon milking was collected for two consecutive days, stored at 4 °C, and processed on the third day for cheese production.

Three experimental cheesemaking trials were performed for each type of lamb rennet paste. Each cheesemaking trial was performed with the same batch of milk. Briefly, raw ewes' milk was thermized (65 °C for 5 min) and then cooled to 38 °C. Rennet paste was added (30 mL of an aqueous solution 60% *w*/*v*) to obtain coagulation in 30 min, then the curd was cut to grain size. After molding and pressing, the curds were held at 42 °C for 5 h, then salted in brine for 12 h (20% *w*/*v* NaCl) and ripened for 45 day (12 °C; R.H. 90%). Cheese weight was about 1.5 kg. At 45 day of ripening, counts of probiotic bacteria in cheese were 7.4 × 107 cfu/g and 7.1 × 107 cfu/g of cheese for *L. acidophilus* and the mix of bifidobacteria, respectively. After 45 day of ripening, experimental cheeses were stored under vacuum at 4 °C. Cheeses were sampled and analyzed in duplicate at 45day of ripening.

Protocol for the production of Pecorino foggiano cheese are reported in figure 2.

Bacteria production

Lyophilized cultures of *Bifidobacterium lactis, Bifidobacterium longum* and *Lactobacillus acidophilus* were first propagated in De Man, Rogosa, Sharpe (MRS) broth (Merck, Darmstadt, Germany), under anaerobiosis at 37°C for 24 h. Cells were harvested by centrifugation at 4000 rpm for 10 min, washed

twice with sterile distilled water, and then inoculated in rennet paste at a concentration of 11 $\log_{10}$ cfu/g of rennet paste. The paste containing probiotics was held for 24 h at 4°C before cheese production. Cells were observed and identified by microscopic examination.

Rennet Paste Production

The clotting of milk by rennet is a key passage in cheese making that, markedly, could affect the characteristics of produced cheese. Nowadays different types of rennet are available. They differ both on their origin, animal, vegetable, microbial and recombinant from genetically modified microorganism, and their physical state, liquid, powder or paste.

The most used rennet derives from the abomasa (fourth stomach or vell) of unweaned calves. It is available as liquid or powder form.

In Mediterranean countries, where sheep and goat breeding is largely diffused, is common the use of lamb or kid rennet paste.

The abomasa for rennet paste production were extracted from suckling lambs (42 d of age): perivisceral fat was removed and the abomasa were stored in salt at 6°C and 70% relative humidity. After 3 mo, the ripened abomasa were ground to obtain a paste, which was kept in a dark glass jar at 4°C. Lamb rennet paste was characterized by a clotting activity of 110 international milk clotting U/g, chymosin-to-pepsin ratio of 70:30, and a lipolytic activity of 11.33 international lipolytic U/g determined according to Santillo et al. (2007, 2009).

In figure 1 are reported protocol for the production of rennet pasta.

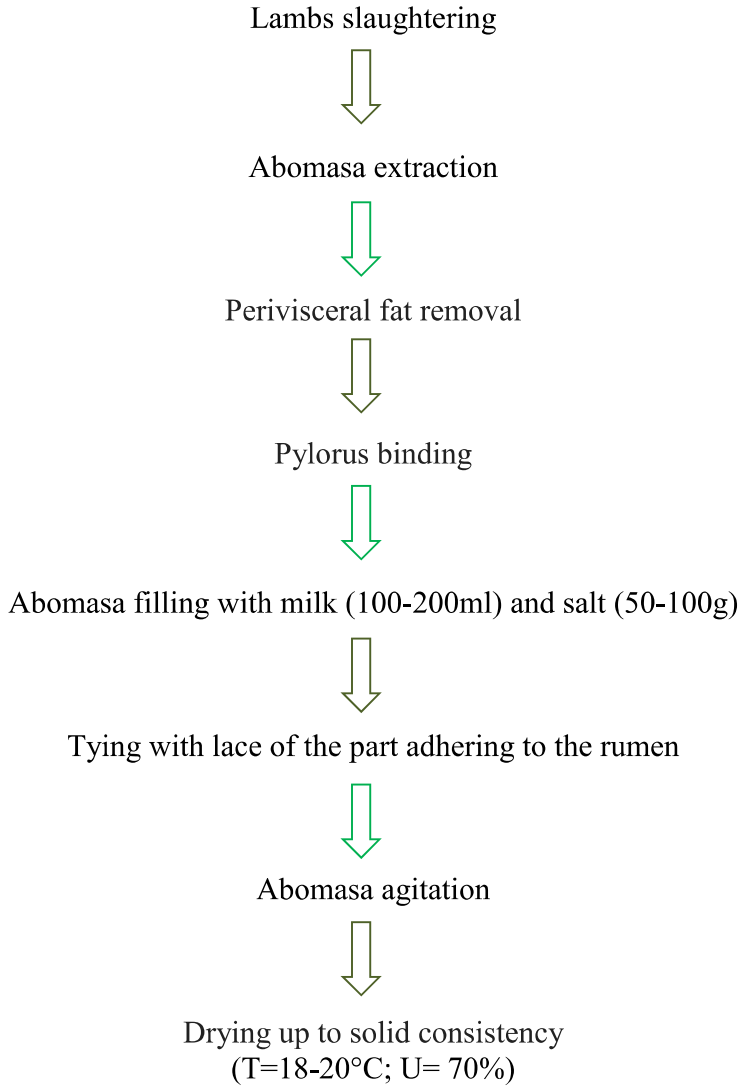


Figure 1. Protocol for the production of rennet pasta.

Definition of the Shelf Life of Rennet

Rennet samples, prepared as described above, were stored at 4°C and analyzed to assess the viable count of probiotic and naturally occurring microflora. Total viable count (30°C for 24–48 h; plate count agar), lactic acid bacteria (**LAB**; 30°C for 48–72 h under anaerobic conditions on MRS agar), *L. acidophilus* on acidified MRS bifidobacteria on cMRS.

Milk-Clotting Activity and Coagulum Characteristics

Determination of total milk-clotting activity was carried out according to the IDF (2006, standard 199). The total milk-clotting activity of the test sample was calculated by interpolation relative to a bovine rennet with an enzyme composition of 75:25 (chymosin: pepsin) and a known milk-clotting activity. Results are reported as international milk-clotting units (**IMCU**) per gram of rennet paste.

Chymosin and Pepsin Activities in the Rennet Pastes

Aqueous rennet extracts (20%, wt/vol) were dialyzed for 3 h against 0.02 *M* piperazine buffer at pH 5.3 through a dialysis tubing cellulose membrane(D9277, Sigma-Aldrich, Milano, Italy). Samples of the same rennet extract, both dialyzed and nondialyzed, were used to determine chymosin and pepsin activities according to the IDF (1997, standard 157a). Enzyme activity is reported as rennet units (RU); 1RUis defined as the amount of enzyme contained in 1 mL of an enzyme preparation, which clots 10 mL of a reconstituted skim milk in 100 s at 30°C.

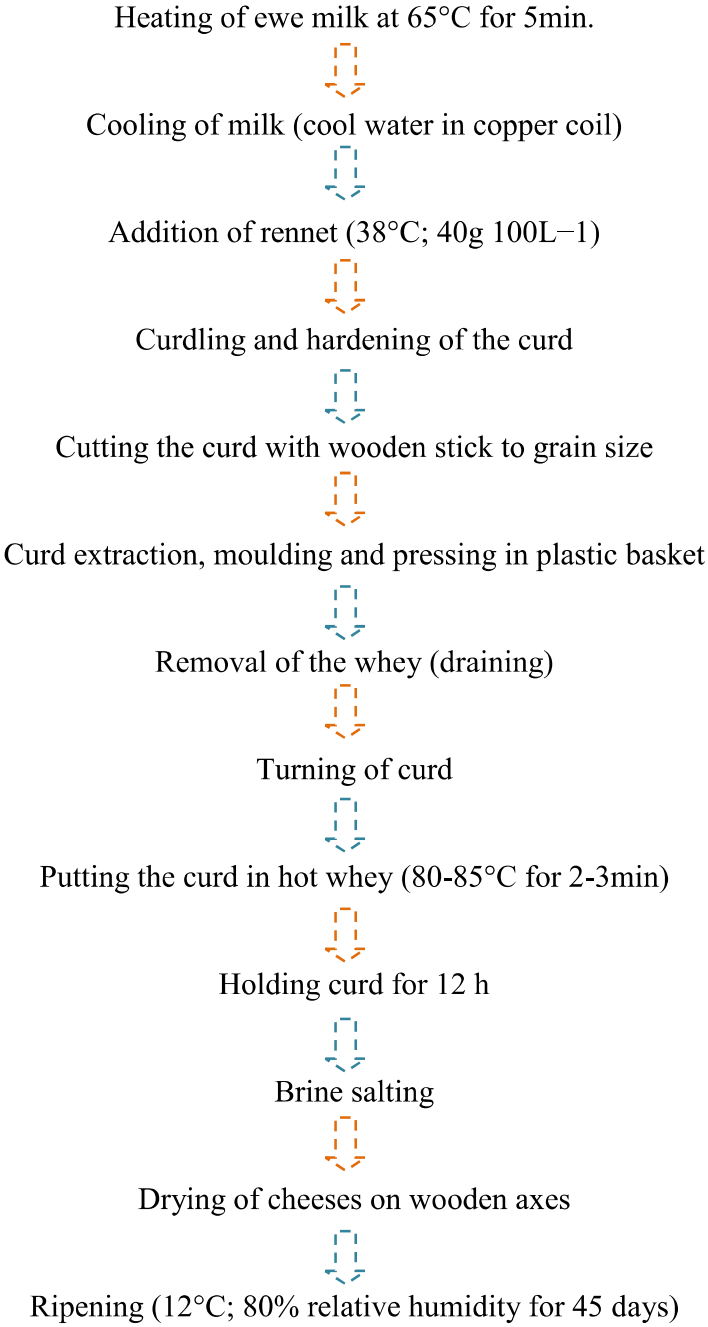


Figure 2. Protocol for the production of Pecorino foggiano cheese.

Analyses of Ewe Milk

Samples of Gentile di Puglia ewe bulk milk used for cheesemaking were analyzed for fat, protein, and lactose content (MilkoScan FT 120; Foss Electric A/S, Hillerød, Denmark), and SCC (Fossomatic Minor, DK-3400; Foss-Electric A/S). Milk renneting characteristics (clotting time, rate of clot formation, and clot firmness after 30 min) were measured using a Formagraph (Foss Electric A/S).

Analyses of Cheese

Chemical Composition and Microbiology of Cheese

The dry matter content, NaCl, and pH of cheeses were determined according to the International Dairy Federation (IDF, 1986, 1988, 1989, respectively). Total nitrogen, NPN, non-casein nitrogen, and phosphotungstic acid-soluble nitrogen were determined as described by Gripon et al. (1975) and water-soluble nitrogen (**WSN**) was measured as described by Stadhouders (1960). The total nitrogen minus WSN gave the casein nitrogen; WSN minus NPN gave the proteose- peptone (**PP**) fraction (Prieto, et al., 2002). Samples of 20 g of cheese recovered both from the outer and inner part of cheese were diluted with 180 mL of saline solution (0.9% NaCl) in a stomacher bag and blended for 1 min with a Stomacher Lab Blender 400 (International PBI S.p.A., Milan, Italy). Decimal dilutions of cheese homogenates were performed and microbiological counts were carried out.

The media and the conditions used for the enumerationswere as follows: plate count agar, incubated at 7°C for 7 to 10 d or at 30°C for 48 h for psychrotrophic and mesophilic bacteria, respectively; MRS agar, modified by adding 0.17g/L of cycloheximide (Sigma-Aldrich S.r.l.), incubated at 30°C and 44°C for 4 d under anaerobic conditions for the evaluation of mesophilic and thermophilic lactobacilli; M17 agar, added to 0.17 g/L of cycloheximide, incubated at 30 and 44°C for 4 d under anaerobic conditions for the evaluation of mesophilic and thermophilic lactococci; acidified MRS agar, adjusted to pH 5.0 using 1.0 *N* HCl and incubated at 37°C for 48 to 72 h for the evaluation of *L. acidophilus* (counts were confirmed by microscopic examination); cMRS agar (MRS + cysteine), added to NPLN solution (nalidixic acid, 750 g/L; paromomycin sulfate, 10 mg/L; neomycin sulfate, 5 mg/L; lithium chloride, 150 mg/L; the supplements were from Sigma-Aldrich S.r.l.; Vinderola and Reinheimer, 1999), incubated at 37°C for 48 to 72 h for the evaluation of bifidobacteria; violet red bile glucose agar, incubated at 37°C for 18 to 24 h for *Enterobacteriaceae*; *Pseudomonas* agar base, modified by adding *Pseudomonas* cetrimide, Fucidin, and cephalotin (CFC)-selective supplement after autoclaving at

121°C for 15 min, incubated at 30°C for 48 h, for *Pseudomonas* spp.; and Baird-Parker medium, added to egg yolk tellurite emulsion, incubated at 37°C for 48 h for staphylococci. All of the media and the supplements used were from Oxoid S.p.A. The analyses were performed in duplicate and cell counts were confirmed by microscopic examination. Starting from 60 d of ripening, the beads were no longer visually detectable in cheese.

Electrophoretic Analysis of Cheese

The pH 4.6-insoluble N fractions of Pecorino cheese were analyzed by urea-PAGE using a Protean II xi vertical slab gel unit (Bio-Rad Laboratories Ltd., Watford, UK). The stacking and resolving gel system was prepared as described by Andrews (1983). The gels were stained according to the method of Blakesley and Boezi (1977) with Coomassie Brilliant Blue G250. The destained gels were acquired by means of the Gel Doc EQ system (Bio-Rad Laboratories Ltd.) using a white light conversion screen and analyzed with Quantity One software (Bio-Rad Laboratories Ltd.) to determine the signal intensity (optical density) of the defined bands. Bands were identified by comparison with urea-PAGE electrophoretograms obtained under comparable conditions (Trujillo et al., 2000; Bustamante et al., 2003; Santillo et al., 2007). Given 100% the sum of the intensity of the defined bands in a lane, the relative quantity of each band was determined as the percentage of the signal intensity of the defined bands in a lane.

Determination of free amino acids in cheese samples

Individual amino acids were analysed in freeze-dried water-soluble extracts of the cheeses. For the construction of calibration curves 17 amino acids (Agilent PN 5061-3331) plus 4 supplemental amino acids (Agilent technologies, Waldbronn, Germany, PN 5062-2478), were combined at various concentrations with fixed amounts of internal standards. The internal standards (norvaline and sarcosine) were part of the supplemental amino acids kit. Amino acids were pre-column-derivatised with o-phthalaldehyde-3mercaptopropionic acid (OPA) reagent (Agilent technologies, Waldbronn, Germany, PN 5061-3335) and fluorenylmethyl chloroformate (FMOC) reagent (Agilent technologies, Waldbronn, Germany, PN 5061-3337). The derivatised FAA were separated, identified and quantified by reversedphase HPLC (Agilent 1100, equipped with a binary pump G1312A, automatic sampler G1313A, degassing system and column oven thermostatised at 40 1C) on a ZORBAX Eclipse AAA column (4.6mm_150mm, 3.5mm, Agilent PN 9634000-9029). The mobile phases were: (A) 40 nM

NaH2PO4 pH 7.8; (B) acetonitrile: methanol: water (45:45:10, v/v/v). Quantitation was done using the area under each peak with the Agilent software ChemStation. Detection was performed on a Agilent diode-array detector (DAD) G1315B and a fluorescence detector (FLD) G1321A.

Determination of FFA in Cheese.

Volatile FFA in cheese were extracted with diethyl ether: hexane (1:1, vol/vol), after grinding with sodium sulfate and addition of 2.5 *M* sulfuric acid (Ha and Lindsay, 1990). Free fatty acids were isolated using an aminopropyl column as adsorbent; the desorption of the FFA was carried out with 2% formic acid in diethyl ether (de Jong and Badings, 1990). The underivatized FFA were separated directly by capillary gas chromatography (Varian 3800, Varian, Milan, Italy) using a capillary column (CP 8853, WCOT fused silica 60 m, 0.32 mm, 0.25 μm, Varian). Operating conditions were a helium flow rate of 1.3 mL/min, a flame-ionization detector at 250°C, a split/ splitless injector at 250°C with a split ratio 1:10 and an injection volume of 1 μL. The temperature program of the column was 5 min at 65°C, increased at rate of 4°C/ min to a final temperature of 220°C, and then held for 20 min. The individual fatty acids peaks were identified by comparison of retention times with those of known mixture of standard fatty acids (Sigma-Aldrich Chemie, Steinheim, Germany).

Determination of CLA in Cheese.

Pure CLAisomers were purchased as fatty acid methyl esters from Matreya Inc. (Pleasant Gap, PA). Acetonitrile and *n*hexane were for HPLC use (J.T. Baker, Deventer, the Netherlands). Total lipids from cheeses were extracted using a published procedure (Mills, 1959). Briefly, 5 g of cheese, 0.5 g of potassium oxalate (Fluka Chemie GmbH, Buchs, Switzerland) and 25 mL of ethyl alcohol (J.T. Baker) were homogenized for 2 min. The homogenized mixtures were poured into a 100-mL centrifuge tube with 10 mL of diethyl ether and 10 mL of petroleum ether (J.T. Baker) and were centrifuged at 469 × *g* for 7 min at room temperature. The lower phase was re-extracted 2 more times with 10 mL of diethyl ether and petroleum ether (1:1, vol/vol). The combined organic phases were transferred into 500-mL separatory funnel, where 100 mL of distilled water and 7.5 mL of saturated sodium chloride were added. The organic extract was washed 2 more times with 25 mL of distilled water, any emulsion formed was broken up by the addition of saturated sodium chloride (2–5 mL), and it was allowed to stand for 30 min. The combined organic layer was dried over anhydrous Na2SO4 and the organic

solvent was removed by rotator evaporator at 38°C; the total lipids were determined gravimetrically. A portion of total lipids (50 mg) was methylated by NaOCH3 (Sigma-Aldrich Chemie) at 50°C, was extracted with *n*-hexane, dried over anhydrous Na2SO4, and analyzed directly by HPLC.

The high-performance liquid chromatograph (Thermo Separation Products, Piscataway, NJ) was equipped with a membrane degasser (LDC Analytical, Riviera Beach, FL), a 10-µL injection loop, and an UV diode array detector (Thermo Separation Products); 3 analytical silver-impregnated columns were fitted in series (ChromSpher 5 Lipids, 4.6 mm i.d. × 250 mm stainless steel, Varian Medical Equipment Inc.). The mobile phase was 0.5% diethyl ether anhydrous (J.T. Baker), 0.1% acetonitrile (J.T. Baker) in *n*-hexane and operated isocratically; the solvent flow was 1.0 mL/min.

The CLA isomers were measured at 234 nm (Sehat et al., 1998).

Cheese flavour

Flavour of cheese is determined by its taste and aroma and results from the correct balance and concentration of numerous sapid and aromatic compounds perceived during cheese consumption. Proteolysis and lipolysis are of great importance in the development of cheese flavour and are ruled by the residual milk clotting enzyme, milk proteinase and lipase, proteolytic and lipolytic enzymes from starter and non starter bacteria, and lipases associated to certain coagulants (Collins et al., 2003; Visser, 1993). However, excessive proteolysis and lipolysis could result in off-flavour because high concentrations of bitter peptides and volatile FFA, respectively, influence cheese flavour either directly or as precursors of other compounds (Broadbent et al., 2002; Pinho et al 2004). The aminoacids liberated in the cheese matrix during secondary proteolysis undergo further catabolic reactions which involve decarboxilation, deamination, transammination, desulfuration leading to the production of compounds such as amines, acids, and thiols. It is accepted that FFA, especially short chain FFA, have a direct impact on cheese flavour, FFAs also act as precursor molecules which lead to the production of other flavour compounds, such as methylketones, esters, fatty acids lactones and alcohls (McSweeney and Sousa, 2000; Tziboula-Clarke, 2003). Diet is a main factor affecting the odour of fresh milk because odorous substances may be transferred to the milk directly to inhaled air into the blood and from there to the milk and by direct absorption from the digestive tract; and via rumen gases to the blood and milk. Moio et al., 1996 identified two sesquiterpenes in milk and cheese produced from sheep fed on a natural pasture being these contstituent of significance for their role in determining milk and cheese flavour and a chemical markers of the milk used to make cheese. Luna et al. (2005) reported

that the organoleptic characteristics of cheeses made from CLA-enriched milk, from ewes fed linseed supplements, did not differ from control cheeses. Processing milk high SCC is associated with an increase in the proteolysis rate and a modification of cheese proteolytic pattern (Coulon et al., 2004). The contribution of indigenous proteinases in the development of cheese flavour have a possible negative implication for the accumulation of bitter peptides which are gradually formed by further degradation of γ-CN compounds (Visser, 1993). Revilla et al. (2007) reported a lower overall acceptance of ewe hard cheese made with high SCC milk compared to medium and low SCC finding that the former was judge as weakly bonded, very grainly, and crumbly. On the contrary, Pirisi et al., (1996, 2000) did not found significant differences in sensory characteristics and lipolysis comparing ewe cheeses made from milk with low and high somatic cell count. Accordling, also in goat milk Jaubert et al., (1996) and Morgan and Gaspard (1999) found a minor effect of SCC on the goatish flavour that is instead mainly influenced by cheesemaking technology, in particular ripening methods. Lamb rennet paste contains lipolytic enzymes which initiate free fatty acid formation (Bustamante et al., 2000; Santillo et al., 2007b; Virto et al., 2003) thus giving the cheeses a sharp, piquant aroma; in particular butyric acid contributes to the cheesy lipolyzed aroma (Pinho et al., 2004). Agboola et al., (2004) investigated the formation of bitter peptides, defined as peptides with a molecular mass of 165-6500 g/mol, in semi hard ovine cheese: the results showed that cheese made with *Rhizomucor miehei* developed more bitter peptides compared to calf rennet. In general, the coagulant also influences the development of bitterness by an excessively high activity which depends on its level and retention in cheese curd; in addition the presence of certain starter i.e. lactococci have a propensity to cause bitterness (Fox et al., 2000).

In ovine and caprine cheese the addition of starter and probiotic cultures (Albenzio et al., 2001, 2010; Corbo et al., 2001; Kalavrouzioti et al., 2005; Santillo and Albenzio, 2008; Santillo et al., 2007a, 2009a) has been associated with an increased proteolysis and lipolysis. These cheeses were tested for sensory attributes by a panel of non trained consumers. The result showed an absence of perceived sensory attributes in ovine cheese whereas flavour and general acceptance of caprine cheese had higher scores than the control cheese.

High pressure treatment of goat milk destined to cheese production (Saldo et al., 2003) had no negligible changes on the volatile composition therefore pressure can be regarded as a safe technology not producing unexpected compounds in milk and cheese.

Determination of Textural Parameters in Cheese.

Samples for cheese texture analysis were obtained by cutting a 1-cm-thick slice from the central diameter of the cheese after 45 day of ripening. Then, 6 rectangular parallelepipeds, 1 × 1 cm thick and 2 cm long, were obtained from the slide. The cheese samples were left at 20°C for 10 min before testing. Texture profile analysis was evaluated with an Instron 4301 tensile tester (Instron Ltd., High Wycombe, UK), using a modified compression device that avoids transversal elongation of the samples. Each sample underwent 2 cycles of 80% compression; force × time data were used to calculate the following parameters: hardness, cohesiveness, springiness, gumminess, and chewiness. A typical texture profile of cheese obtained using the Instron equipment is interpreted to obtain the parameters reported in the study. Hardness is the height of the second peak (H2) in the first bite; cohesiveness is the ratio of area on second bite to that of the first bite (A2/A1). Gumminess is the hardness × cohesiveness × 100 (A1 × (A2/A1) × 100); Elasticity is the difference between distance B and distance C (C is the same measurement made using an inelastic material); chewiness is the hardness × cohesiveness × springiness (A1 × (A2/A1) × (B - C)).

Sensory Analysis

Subjects were recruited among students and personnel of the University of Foggia. The consumer panel consisted of 80 subjects; the panel was homogeneous on the basis of age (20–45 years) and gender. In addition subjects were selected using predetermined screening criteria based on consumption of dairy products made from ewe's milk and their awareness of probiotic food products; subjects were asked to give an example of a food containing probiotics.

The sensory analysis of ovine cheese was carried out on 45 day ripened cheese using the descriptive model of Coppola *et al.*(1990) with a few modifications (De Angelis et al. 2008).

Before the sensory analysis, the untrained panel was preliminarily briefed on the use of the sensory attributes on a 9-point intensity scale (0–9) of the scorecard. The scorecard contained descriptive attributes according to Santillo *et al.* (2012) adapted for ovine cheese, namely 2 for odor/flavor (overall intensity, acid, rancid), 8 for taste (overall intensity, salty, acid, pungent, bitter, sweet, mold, rancid), color (overall intensity, uniformity), appearance, and texture (overall uniformity, moisture, chalky, rubbery, grainy). Cheeses were taken out of the refrigerator 1 h before serving. Each sample was assigned a 3-digit random number, and cheese slices (1.5 mm thick) from the 3 replications of the same batch were mixed randomly so that all replications from the same batch were presented an equal

number of times. A glass of water and unsalted crispy bread were provided and consumers were instructed to take a small bite of bread and a sip of water after each cheese tasting. Duplicate trays of samples were presented to the panel at 10-min intervals.

Determination of Functional Ovine Cheese Liking

The perceived, expected, and actual liking was measured in accordance with the protocol proposed by Napolitano *et al.* (2010). The protocol provided for three sessions: in the first session subjects were asked to taste the ovine cheeses and rate their liking in blind conditions without receiving any information on the production protocol of cheese (perceived liking). In the second session the subjects received the information and were asked to rate their liking on the basis only of the information given (expected liking). In the third session consumers were given the cheeses along with the information and expressed their liking (actual liking). Consumers rated their liking in a 9-point hedonic scale anchored with "like extremely" and "dislike extremely" and with a neutral center point of "neither like nor dislike".

In sessions 2 and 3 the following information concerning ovine cheese production was given to the consumers.

Ovine cheese-cheese made using milk from ewes reared in extensive conditions based on grazing on natural pasture. The production of cheese involved the use of whole milk heated to a temperature of 65 °C for few minutes and the use of rennet paste as coagulant. Rennet paste is a type of coagulant used for typical dairy productions; it is a white paste obtained from the abomasa of suckling lambs. The cheese is salted in brine and then subjected to aging at controlled temperature and humidity for up to 45 days.

Functional ovine cheese containing *Lactobacillus acidophilus*-cheese made using milk from ewes reared in extensive conditions based on grazing on natural pasture. The production of cheese involved the use of whole milk heated to a temperature of 65 °C for few minutes and the use of rennet paste as coagulant. Rennet paste is a type of coagulant used for typical dairy productions; it is a white paste obtained from the abomasa of suckling lambs. The cheese is salted in brine and then subjected to aging at controlled temperature and humidity for up to 45 days. The rennet paste contains live cells of *Lactobacillus acidophilus* recognized as probiotic able to exert beneficial effects on human health. The cheese contains high level of probiotic cells in accordance with the current guidelines for food products.

Functional ovine cheese containing bifidobacteria-cheese made using milk from ewes reared in extensive conditions based on grazing on natural pasture. The production of cheese involved the use of whole milk heated to a temperature of 65 °C for few minutes and the use of rennet paste as coagulant. Rennet paste is a type of coagulant used for typical dairy productions; it is a white paste obtained from the abomasa of suckling lambs. The cheese is salted in brine and then subjected to aging at controlled temperature and humidity for up to 45 days. The rennet paste contains live cells of *Bifidobacterium longum* and *Bifidobacterium lactis* recognized as probiotic able to exert beneficial effects on human health. The cheese contains high level of probiotic cells in accordance with the current guidelines for food products.

Statistical Analysis

All the variables were tested for a normal distribution using the Shapiro-Wilk test [23]. Data were analyzed by ANOVA using the GLM procedure of SAS [24], and the effect of probiotic strain was tested on chemical and sensory attributes of ovine cheese. The effect of probiotic, age, and gender of the panelist, and the interaction of these variables, was studied for the perceived, expected and actual liking of the ovine cheese. When significant effects were found ($p < 0.05$), Student's *t*-test was used to locate significant differences between means.

RESULTS AND DISCUSSION

Gentile di Puglia Ewe raw and thermised Milk

The experimentation has concerned the production, the harvest and the transformation of Gentile di Puglia ewe's milk. Sheep were subjected to mechanical milking and bulk milk was stored for 2 days at refrigeration temperature before experimental cheesemaking. In table 1 is reported the main chemical composition and pH value of sheep milk of raw mass at the end of the storage period and the same milk processed for thermisation before the cheese making.

The milk of this breed shows high levels of fat, protein and casein that make it particularly suited to the dairy processing for obtaining a high nutritional quality products. In this experiment it was observed that the massal sheep milk, at the end of the refrigerated storage period, has an adequate nutritional composition and suitable pH values.

In Table 2 are reported the data of reological parameters and the somatic cell count of the raw bulk milk at the end of the storage period and of the same milk subjected to thermisation treatment before cheesemaking. As regards the somatic cells, the determination of this parameter in bovine milk is widely used as index of mastitis. However, somatic cell count also represents for the sheep and goat milk a valuable aid for defining the health status of the breast. It was reported that in sheep milk there is an increase in the total bacterial count in milk samples when the somatic cell content is greater than 500,000 cells / ml and a worsening of the attitude of the milk for cheesemaking when the content of somatic cells it is greater than 700,000 cells / ml (Sevi et al., 1999).

The good quality of raw milk and the correct milk storage conditions are likely to have limited the activity of endogenous and exogenous enzymes associated with somatic cells and the microbial flora. It's well known, in fact, that a broad spectrum enzyme is associated with leukocyte populations of milk and these enzymes may operate in the course of the milk storage contributing to the degradation of fat and protein; the integrity of the casein fraction, in particular, is important in ensuring the quality of dairy milk (Albenzio et al., 2009).

The nutritional characteristics, in particular the content of casein and milk fat, in association with suitable hygienic characteristics of milk are responsible for the technological quality of the measured milk through the reological parameters clotting time, speed of formation of the clot and curd firmness at 30 minutes. In the present experiment the coagulation of milk parameters, in fact, are within the range given for sheep's milk characterized by a somatic cell count less than 500,000 somatic cells / ml of milk (Albenzio et al., 2011).

Although the compositional and hygienic characteristics of milk were suitable for processing without a heat recovery treatment, processing of milk thermisation was conducted to further reduce and contain the natural microflora of the milk before the addition of rennet. The time / temperature combination selected in this work has guaranteed that the nutritional characteristics of the starting raw milk remained almost unaltered but, at the same time has allowed the development of probiotics added in the boiler through the coagulant, minimizing competition for nutritive substrates by the natural microflora of the milk.

Microbial Characteristics of Lamb Rennet Paste

The critical threshold of viable probiotic is 6 log cfu/g, as this level of population is the minimum required for probiotic action in the small intestine (Rosburg et al.2010); thus, this level was set as the critical breakpoint for the beads.

The model fit the data satisfactorily, as shown by the regression coefficient. *Lactobacillus acidophilus* and the combination of *B. longum* and *B. lactis* experienced a different death kinetic, as one could infer from the shape parameter: *L. acidophilus* highlighted a downward curve, characterized by an initial phase when cell count did not decrease or did it slowly. On the other hand, *B. longum* and *B. lactis* stood for an upward kinetic [i.e., a trend characterized by an initial fast decrease of cell count, followed by a tail effect (i.e., a residual level of the target for a prolonged storage time)]. The first reduction time for primary growth/death model indicates the time to attain a reduction of cell count of 1 log cfu/g and was higher for *L. acidophilus* than for the mix of bifidobacteria. Combining the primary fitting parameters and using a critical threshold for cell count of 6 log cfu/g, shelf life was evaluated, resulting in 6.05 and 1.70 d for lactobacilli and bifidobacteria, respectively. After the time points evaluated, cells in the beads continued to live and were released in the rennet or in the cheese during cheese making. The use of a reparameterized version of the Weibull equation allowed the evaluation of cell death time, corresponding to 18.20 and 39.44 d for lactobacilli and bifidobacteria, respectively. The evaluation of the shape parameter (parameter *p* of the Weibull equation), as well as the fitting procedure to attain first reduction time, shoulder length, and death time values, highlighted some important details on the different death kinetic experienced by *L.acidophilus* and bifidobacteria. For *L.acidophilus*, cells experienced a death kinetic characterized by at least 2 phases: a shoulder length and an exponential death phase. The practical implications of this kind of curve were very strong: cell counts of *L. acidophilus*, in the rennet retained their viability for 4 to 5 d, without any significant decrease.

Thereafter, a fast reduction was observed, thus suggesting that the effect of environment (e.g., rennet) became significant after a prolonged exposure time. On the other hand, the combination of *B. longum* and *B. lactis* experienced a kinetic characterized by an initial death slope, then followed by a tail, thus highlighting that, for these microorganisms, the adverse effect influenced the viability of the target microorganisms just after the release from beads. Then, cells probably acquired a kind of resistance, resulting in the tail effect.

Cell counts of the different microorganisms (both the naturally occurring microflora and that released by beads) just after cheese making and during ripening are reported in Table 3. Cheese microflora is generally composed of genera belonging to the group of LAB, along with some spoiling or hygiene indicator microorganisms (*Pseudomonas* spp., staphylococci, *Enterobacteriaceae*, and pathogenic bacteria). Cheeses analyzed throughout this research showed *Enterobacteriaceae*, *Pseudomonas* spp., and staphylococci at an undetectable level for the entire running time, thus suggesting good quality of the raw materials as well as good processing. Concerning the naturally occurring microflora, LAB represent the main group able to colonize dairy products. Thermophilic LAB strains are generally used as starter cultures in many kind of products; on the other hand, mesophilic lactobacilli and lactococci prevail in natural fermentations (Di Cagno et al., 2010). Due to the fact that cheese making was conducted without whey cultures, the naturally occurring microflora of cheese was mainly composed of mesophilic lactic acid bacteria, ca. 4 log cfu/g at the beginning and then increasing significantly throughout storage; this result was also confirmed by total viable count. Thermophilic LAB were at an undetectable level for the entire running time (data not shown). The levels of *L.acidophilus* and bifidobacteria, confirmed also by microscopic examination, were at low levels (ca. 2 log cfu/g), then increased significantly within the storage, due to release from beads; after 60 d, cell numbers of *L.acidophilus* and bifidobacteriawere 7.52 and 6.84 log cfu/g, respectively. Throughout storage, bifidobacteria experienced a decrease, reaching a level of 5.44 log cfu/g.

Chemical composition of probiotic ovine cheese

The experimental cheese was made from Gentile di Puglia ewe's milk in a traditional protocol making use of rennet of lamb as a coagulant. Rennet is among the factors that most affect the typical characteristics of the cheese and rennet, lamb or kid, it differs significantly from the one liquid and powder form, usually veal, for the presence of proteolytic and lipolytic enzymes in able to drive the maturation process. In particular, the rennet from suckling lamb is characterized by high content of

chymosin and pre-gastric lipase involved, respectively, in the primary proteolysis process and in the process of lipolysis.

The experimental cheese was produced using traditional rennet paste (cheese C), lamb rennet paste containing living cells of Lactobacillus acidophilus (LA cheese), and lamb rennet paste containing a mix of live cells of Bifidobacterium lactis and Bifidobacterium longum (BB cheese).

The chemical composition of the pecorino cheese produced from Gentile di Puglia ewe's milk , at 45 days of ripening, is reported in Table 4. The moisture content results to be comparable in experimental cheeses at the end of the studied ripening period. The fat content appears to be lower in the control cheese compared to cheese made with rennet paste containing probiotic bacteria. No differences were recorded for the salt content in the cheese, which has an average value of 2.5% in cheeses at 45 days of ripening.

The dry matter content of the cheese depends mainly on the temperature and the relative humidity of the ripening room; while the content of salt depends on the concentration of the brine and from dwell time of the forms in brine. The lack of differences for these parameters highlights both the standardization of the cheese production technology and the lack of influence of rennet, containing or not containing live cells of probiotic bacteria, on the moisture and salt content of cheese. It is reported that the cheeses made with rennet paste show higher humidity values compared to cheeses produced with vegetable rennet (Nunez et al., 1991; Freitas and Malcata, 1996). The humidity values of pecorino cheese to 45 days of ripening, appear to be lower than those reported for cheese products from ewe's milk using artisanal rennet lamb pasta (Irigoyen et al., 2002).

The casein content in the experimental cheeses is found to be higher in the control cheese compared to the BB and LA cheeses. The casein content in cheese is widely used to monitor the primary proteolytic processes, being the casein the substrate of the action of ripening agents. It's well known that the rennet plays an important role in the process of hydrolysis of the casein releasing primary proteolysis products, which represent the substrate for enzymes associated with the microorganisms (Pirisi et al., 2007). The enzymatic activity of the rennet paste act differently depending on the casein fractions involved: α-CN, undergoes a higher proteolysis compared to fractions of β-CN, being more susceptible to chymosin activities, already in the coagulation phases and, subsequently, in the seasoning.

The pH value results to be lower in cheese containing probiotics compared to the control cheese in agreement with previous experiments (Albenzio et al., 2010). The reduction of pH is normally an event associated with the ripening of cheese, in particular in the first weeks of ripening. The effect of

different types of rennet on the pH value of the cheese at 45 days of ripening could be attributed to greater push acidifying operated by the probiotic bacteria in BB and LA cheeses.

Proteolysis is the major biochemical process that occurs during cheese ripening; it determines substantive changes of the structure of the cheese, through the degradation of the protein matrix, and the formation of flavour through the release of peptides, free amino acids and their degradation products. In particular, the primary proteolysis that occurs in the early stages of cheese making, concerns the intact casein and is mainly carried out by the activity of coagulant; subsequently the emitted high molecular weight peptides undergo a further hydrolysis due to endogenous protease and microbial enzymes. The values relating to certain nitrogen fraction of the pecorino cheese produced using different experimental lamb rennet paste, at 45 days of ripening, are reported in Table 5.

In particular, the value of non-casein nitrogen expressed on total nitrogen it appears to be higher in LA cheese compared to C and BB cheeses; on the contrary non-protein nitrogen and phosphotungstic acid-soluble nitrogen fraction on total nitrogen appear to be higher in the cheese BB. The latter fraction represents, with good approximation, the amount of free amino acids present in the cheese matrix.

Data concering the nitrogen soluble fraction in water of pecorino cheese produced using different experimental lamb rennet pastes, at 45 days of curing, are shown in Graph 1. The fraction of water-soluble nitrogen appears to be the lowest in cheese control, intermediate in cheese with rennet product containing L. acidophilus and the highest in the cheese containing the bifidobacteria mix. This data allows to evaluate the performance of proteolysis in the cheese and is, in fact, defined cheese ripening coefficient; it is interesting to note that, in this experiment, the presence of probiotic bacteria added to rennet can enhance and accelerate the process of proteolysis during seasoning. This observation is confirmed by the study of free amino acids in cheese; Graph 2 shows the level of total free amino acids in pecorino cheese produced using different experimental lamb rennet pastes, at 45 days of ripening. The results concerning this parameter show, in fact, the highest concentration of free amino acids in the BB cheese (7424.56 mg / g cheese), an intermediate concentration in the LA cheese (6440.86 mg / g cheese) and a lower concentration in the control cheese (3598.55 mg / g of cheese). The amino acid spectrum study allows a qualitative evaluation of the proteolysis highlighting differences that influence both the nutritional quality and the organoleptic qualities of the cheese. The composition of free amino acids of the pecorino cheese produced using different experimental rennet, at 45 days of ripening, is reported in Table 6.

The results obtained in this trial show that the free amino acids glutamic acid, serine, glycine, arginine, tryptophan, isoleucine show higher values in the cheese containing bifidobacteria; The free amino acids valine, phenylalanine record higher values in the cheese containing *L. acidophilus*. Finally, the aspartate records higher values in the control cheese.

Proteolysis contributes directly to determine the flavor and aroma due to the formation of free amino acids and peptides for the presence of secondary catabolic actions on protein macromolecules such as transamination, oxidative deamination, decarboxylation, the desulphurisation, the catabolism of aromatic amino acids and reactions of amino acids with other compounds.

The composition of the amino acid fraction and the relative proportion of the amino acids is fundamental to the development of the flavor (Broome et al., 1990, Molina et al., 1999). However, an increase of free amino acids concentration in the cheese does not necessarily lead to an increase in the intensity of the flavour (Christensen et al., 1995). Fox and Wallace (1997) suggest that the flavour and the concentration of free amino acids are not always related, in fact different cheeses (Cheddar, Gouda, Edam) have different flavor although the concentration and the proportion in free amino acids is very similar. The amino acids released in the cheese matrix during the hydrolytic processes borne by the casein fraction represents an index of processes trend. They are, moreover, mainly responsible for the aroma development of the cheese because the aromatic amino acids (phenylalanine, tyrosine, tryptophan) and those of branched chain (leucine, isoleucine, valine) are precursors of aromatic compounds.

From a nutritional point of view, it is interesting to underline the high concentrations of valine, methionine and threonine, since they represent essential amino acids for man. Furthermore, it has been reported that these amino acids are involved in leukocyte and immunoglobulin synthesis (Voet & Voet, 1990 Defa et al., 1999) and that the hydrophobic amino acids such as, tryptophan, phenylalanine, tyrosine, and leucine is associated with a bitter flavour (Roudot-Alargon, 1996).

Some authors (Martinez-Cuesta et A.L., 2001) reported that some strains of Lactobacillus spp., used as a non-starter lactic acid bacteria cultures in cheese production, possess an enzyme system f with significant debittering activities.

The electro-phoretogram of the pH 4.6-soluble nitrogen fraction in cheese produced using different experimental rennet, at 45 days of ripening, is shown in Figure 1. The electrophoresis conducted with urea-PAGE technique has revealed the casein fractions of cheese and the products resulting from their degradation that, for the high molecular weight, remain in the nitrogen insoluble fraction. In all the

cheeses, there is a greater degradation of the fraction relative to α-casein with the formation of a high number of bands corresponding to peptides with lower molecular weight and localized close to the positive electrode. The increased degradation of α-casein compared to the β-casein could be attributed to the composition of the rennet and in particular to a high amount of chymosin contained in rennet obtained from infants lambs. In many kind of cheese the α_s-casein is highly hydrolyzed, contrary to the β-casein which remains essentially unchanged; some authors note in α_s-casein the presence of 6-8 bonds susceptible to chymosin action (Mulvihill and Fox, 1976). It was observed that in cheese ripening some fraction from α-casein degradation are hydrolyzed into peptides with lower molecular weight from chymosin and Cell Envelope proteinases (CEPs) of lactococchi (Sousa et al., 2001). With regard to β-casein, in the cheeses analyzed in this population it appears to be less degraded compared to α-casein, according to other authors (Hayaloglu et al., 2004). The most responsible agent for the degradation of β-casein fraction is plasmin (Kelly et al, 2006), an endogenous proteolytic enzyme responsible for the liberation of γ- caseins, shown at the top of the electro-phoretogram. Furthermore, the electrophoretic analysis shows that bands attributable to α-casein and β-casein result to be less degraded in the control cheese compared to cheese containing probiotic bacteria, according with the higher concentration of the nitrogen fraction casein recorded in experimental cheeses .

The electro-phoretogram of the pH 4.6 soluble nitrogen fraction in cheese produced using different experimental lamb rennet pastes at 45 days of ripening, is shown in Figure 2. The electrophoretic analysis shows that the cheese containing *bifidus bacteria* have a different profile than other experimental cheeses. In particular, there is a higher number of bands in the upper part of the gel; on the contrary, in the nearest part of the positive electrode gel, there's a lower number of bands characterized by a lower intensity. The differences recorded in the electrophoretic analysis are attributable to the action of probiotic bacteria employed, that are able to influence the proteolytic pattern.

Lipolysis of triglycerides during cheese ripening allows the release of free fatty acids in the cheese, which contribute to the formation of taste and flavor. The proteolysis agents are attributable to endogenous lipase of milk and those made by rennet, and also to lipolytic enzymes associated with lactic Among the short chain fatty acids, butyric acid is found to be higher in cheese containing probiotics compared to control cheese. Free fatty acids levels in experimental cheeses at 45 days of ripening are reported in Table 7. The content of isomers of conjugated linoleic acid (CLA) has shown high levels in cheese containing probiotics compared to control cheese. Some authors report the *L.*

acidophilus ability of isomerizing linoleic acid into CLA, as a detoxification mechanism over linoleic acid (Alonso, 2003). The beneficial effects of CLA on human health has been amply demonstrated; these molecules, in fact, perform, in the human organism, biological functions far beyond their energetic role. A wide literature has highlighted the multiple beneficial effects of CLA on human health that can be useful for cardiovascular disease prevention and the development of adipose tissue, diseases such as diabetes and some cancers, such as breast cancer, skin , liver and colon and the effect on the immune system.

Textural parameters and sensorial attributes of ovine cheese

Texture of cheese can be defined as a sensory and composite attribute resulting from a combination of physical properties, perceived by the senses of sight, touch and hearing. The mechanical properties of the cheese are related to the composition, structure and strength of the attractions between the structural elements that compose it.

Textural parameters of experimental cheeses at 45 day of ripening are reported in Table 8. Cohesiveness and gumminess were affected by the addition of probiotic cells, being higher in BB cheese, intermediate in LA, and lower in C cheese.

Sensorial attributes of ovine cheese at 45 day of ripening are reported in Table 9. Among appearance attributes, differences emerged for humidity and gumminess, which turned out to be the lowest in both cheeses containing probiotics whereas graininess was the highest in LA cheese. No differences were reported for uniformity and chalky appearance. All the attributes ascribed to color and odor were not affected by the experimental treatment. Regarding taste attributes, salty scored the highest value in BB and pungent was higher in both probiotic cheeses. The overall taste intensity and acid, bitter, sweet, mold, and rancid attributes were not affected by treatment.

The consumers' liking of pecorino cheese produced using different experimental rennet, at 45 days of ripening, is reported in Graph 3. It is interesting to observe that there are no particular differences in the preference attributed by consumers to the experimental cheeses. This result is particularly encouraging because it allows to state that the use of probiotic bacteria is not responsible for aroma and flavor defects and does not make the cheese different from the one produced with traditional rennet.

The rating given by the consumer panel during the three hedonic tests was not affected by the use of probiotic strains in ovine cheese production, scoring a mean value of 7.01 ± 0.2, 7.64 ± 0.25, and 7.34 ± 0.3 for the perceived, the expected, and the actual liking, respectively. The rating of ovine cheese

liking is presented in Table 10. Cheese liking was not affected both by probiotic addition and consumers' group, whereas an interaction effect of probiotic, gender, and age of consumers was detected in the perceived and the expected liking. A higher rate was expressed for the perceived liking of the BB and LA cheeses by female consumers over 30 years. On the contrary, females less than 30 years old scored lower rates for the probiotic cheeses. BB cheese received an intermediate score from males less than 30 years old, whereas LA cheese received intermediate scores from males over 30 years old. For expected liking, higher rates were found in female over 30 years for all the experimental cheeses, whereas LA cheese scored higher rates in males over 30 years. The actual liking of experimental cheeses was not influenced by age and gender. All experimental cheeses obtained a judgment from "moderately pleasant" to "very pleasant".

CONCLUSION

Cheese ripening involves different biochemical pathways, such as proteolysis and lipolysis ascribed to endogenous and exogenous factors, mainly enzymes yielded by rennet and microflora. Proteolysis is the main process in cheese ripening; determining changes in the texture due to the breakdown of the protein network, and in flavor formation through the release of peptides, free amino acids, and catabolic products (Albenzio et al.2010). Cheese containing a mix of *Bifidobacterium longum* and *Bifidobacterium lactis,* and cheese containing *Lactobacillus acidophilus* highlighted a more intense proteolysis than cheese without probiotic as evidenced by a greater hydrolysis of the intact casein fractions. The disruption of the casein matrix led to a major accumulation of nitrogen fractions comprising low molecular weight peptides and free amino acids. However, differences emerged among probiotic cheeses, with cheese containing bifidobacteria showing a higher proteolytic potential. Previous studies reported that ovine cheese containing a mix of *B. longum* and *B. lactis* showed greater caseins hydrolysis, evidencing that probiotic strains have a different impact on the proteolysis of cheese (Stanton et al.1998, Albenzio et al.2010). BB cheese showed a more complex FAA profile as an outcome of the proteinase and peptidase activities, evidencing the real contribution of adjunct microflora to cheese ripening (Corbo et al.2001, Santillo et al.2012). Lipolysis is another important biochemical event that leads to the formation of FFAs which also contribute to cheese flavour with volatile compounds (Caporaso et al.2015). Lipases associated with lactic acid microflora are acknowledged to be selective for short chain fatty acid release (De la Fuente et al. 1993). The greater level of C4:0 in probiotic cheese could be attributed to the metabolic activity of the adjunct microflora; in particular, the ability of *L. acidophilus* to convert linoleic acid in to CLA is a consequence of the detoxification mechanism enacted by this probiotic strain. CLA enrichment led to an ameliorated composition of the fat fraction of ovine cheese; indeed the involvement of these polyunsaturated fatty acids has been reported in the prevention of many human diseases (Williams 2000).

Cheese texture may be defined as a composite sensory attribute resulting from a combination of physical properties perceived by the senses of sight, touch, and hearing (Pinho et al.2004). Cheese texture could be measured indirectly by using instrumental rheological techniques and is related to the composition, structure, and strength of the attractions between the structural elements of the cheese. The behavior of the texture profile revealed that cheeses containing bifidobacteria were less brittle, in accordance with Buriti *et al.*, probably as a consequence of the greater proteolysis associated with the cheese maturing process.

Evaluation of sensory attributes permits the definition of the perceived profile and overall acceptability by consumers of dairy products with innovative characteristics. The analysis of the sensorial profile of the cheeses permits the identification of specific attributes that could be preference drivers and the evaluation of the impact of health information on consumer preference, expectation, and choice. Higher scores for humidity and gumminess were in accordance with the minor proteolytic process observed in the control cheese; cheese without probiotic was judged as the cheese with an overall lower intensity for taste attributes. Regarding taste attributes, the score for the Pungent attribute doubled in probiotic cheese with respect to the control cheese as an outcome of the major accumulation of C4:0. Butyric acid is one the major odorants in Cheddar and Camembert (Yvon and Rijnen 2001), and is also an important component of Feta cheese, contributing greatly to its flavor and piquant taste (Georgala et al.2005). The salty taste, together with the pungent attribute, led to higher overall intensity perceived upon consumption of cheese containing bifidobacteria compared to control cheese. It is worth noting that the drivers of cheese acceptability are different depending on the consumer's gender; a previous study on Caciocavallo cheese reported that salty and piquant attributes were mostly enjoyed by male rather than female panelists (Santillo et al.2012).

Some food attributes are directly perceived as price and sensory properties; others must be communication objects as healthy and/or ethical aspects. Indeed, the perception of a healthier product (e.g., cheese containing high levels of probiotic microorganisms) could increase the actual acceptability of cheese (Napolitano et al. 2007). The absence of differences among cheeses in the rating of ovine cheese liking is an encouraging result because the adjunct of probiotic cultures in cheese did not lead to the development of aroma and flavor defects that permit consumers to distinguish between a traditional and an innovative cheese. Overall, the manufacture of probiotic cheese should have minimum changes when compared to traditional products (Granato et al.2010). The addition of bifidobacteria in Gouda cheese (Gomes et al.1995) and in Cottage cheese

(Blanchette et al.1996) led to negative effects on cheese flavor, reducing the acceptability of probiotic cheese with respect to a traditional one. In Cheddar cheese, Ong *et al.* reported that cheese acceptance depended on the probiotic microorganism used in cheesemaking, evidencing the advisability of consumer acceptance testing whenever selected probiotic strains are used in the cheesemaking process.

The perceived liking of ovine cheese determined in blind conditions represented the baseline for the evaluation of the impact of information on consumers' expectation; in particular, grouping the panel on the base of gender and age allowed for the evaluation of information on different segments of

consumers. The expected liking was different from the perceived liking expressed in blind conditions, indicating that a negative disconfirmation occurred: higher rates of expected liking highlighted that the products are worse than expected although the overall rate referred to an acceptable product with a score over 6.5. In general, the higher rates of expected liking in all experimental cheeses was attributed to the information given to consumers concerning not only the presence of probiotic strains in cheese but also the farming conditions of the ewes, and the particular cheesemaking technology. It can be argued that consumers are prone to consider the farming conditions and the production of ovine cheese using traditional protocols as an added value to the quality of ovine cheese. Napolitano *et al.* reported that information about organic farming can be a major determinant of cheese liking when comparing organic and conventional cheese. When the cluster of females over 30 years was considered, a higher weight was ascribed to the information related to the healthy effect of cheese when compared to male consumers and females less than 30 years of age. The higher expected liking for LA cheese in males over 30 years old could be ascribed to the fact that *L. acidophilus* is one of the most commonly used probiotic strains used in dairy foods. Probiotic products seemed to create a higher expectation in females over 30 years, probably due to an elevated consciousness of the benefits of healthy food. Indeed, a choice experiment on semi-hard cheese highlighted that female, rather than male participants, were affected by health information on their diet choices, evidencing clear diet-health awareness (Øvrum et al. 2012). When actual liking was measured, scores moved towards the expectation liking rate, evidencing that assimilation of the information given contributed to the increase of ovine cheese liking.

Probiotic cheese combines nutritional and functional properties and is promising for the expansion of dairy products from ovine milk. The experimental cheeses containing probiotics were judged as the cheeses with an overall higher intensity for taste attributes in accordance with the more intense proteolysis and a greater level of short chain free fatty acids and conjugated linoleic acid. The results from the present research also highlight that the design and development of functional ovine cheese represents a technological innovation that needs to be supplied with adequate information in order to be able to orient consumers in their food choice. Nowadays, consumers are aware of the great impact of nutrition on health and reward ovine cheese as a food product associated with a sustainable production system. Special attention must be paid to the sensory profile of probiotic ovine cheese in order to provide information on the cheese attributes able to influence consumers' liking.

TABLES ,
GRAPHS & FIGURES

Table 1. Chemical composition and pH of raw and thermised milk of Gentile di Puglia sheep

Parameter	Rm	Sem	Tm	Sem
pH	6.69	0.03	6.57	0.03
Fat, %	7.98	0.056	7.11	0.03
Protein, %	6.28	0.042	6.31	0.03
Lactose, %	4.48	0.042	4.51	0.07
Casein, %	4.96	0.007	5.02	0.07

Rm = raw milk
Tm = thermised milk

Table 2. Reological paramethers and somatic cells content in raw and thermised milk of Gentile di Puglia sheep

Parameter	Rm	Sem	Tm	Sem
r, min	8.49	0.06	9.44	0.20
k20, min	1.17	0.03	1.29	0.01
a30, mm	62.60	0.03	50.12	0.18
somatic cells(SCC), log_{10} cells/ml	5.24	0.03	5.27	0.05

Rm = raw milk
Tm = thermised milk
r = rennet clotting time
k20 = curd firming time
a30 = curd firmness

Table 3. Cell load (log10 cfu/g) of the principal microbial groups and *Lactobacillus acidophilus* and bifidobacteria in RP, RP-L, and RP-B cheese[1]

	Ripening time, d			
Item	**0**	**30**	**60**	**120**
Mesophilic bacteria				
RP	$5.45^{a,A}$	$6.79^{b,A}$	$7.26^{b,A}$	$7.08^{b,A}$
RP-L	$5.34^{a,A}$	$6.86^{b,A}$	$7.48^{c,A}$	$6.72^{b,AB}$
RP-B	$5.22^{a,A}$	$6.78^{c,A}$	$7.36^{d,A}$	$6.08^{b,B}$
Mesophilic lactobacilli				
RP	$4.23^{a,A}$	$7.58^{b,A}$	$7.15^{b,A}$	$6.92^{b,A}$
RP-L	$4.51^{a,A}$	$7.49^{c,A}$	$7.68^{c,B}$	$7.26^{bc,A}$
RP-B	$4.55^{a,A}$	$7.32^{b,A}$	$7.32^{b,AB}$	$6.16^{c,B}$
Mesophilic lactococci				
RP	$4.12^{a,A}$	$6.69^{b,A}$	$7.11^{b,A}$	$7.12^{b,A}$
RP-L	$4.33^{a,A}$	$6.39^{bc,A}$	$7.63^{d,AB}$	$6.88^{c,A}$
RP-B	$4.44^{a,A}$	$5.36^{b,B}$	$7.24^{c,A}$	$8.30^{d,B}$
L. acidophilus				
RP	—[2]	—	—	—
RP-L	2.11^{a}	7.57^{c}	7.52^{c}	6.92^{bc}
RP-B	—	—	—	—
Bifidobacteria				
RP	—	—	—	—
RP-L	—	—	—	—
RP-B	2.45^{a}	6.89^{c}	6.84^{c}	5.44^{b}

[a–d]Means within a row with different superscript lowercase letters differ ($P < 0.05$).
[A,B]For each time of analysis and medium, different superscript uppercase letters indicate significant differences ($P < 0.05$) among the samples.
[1]RP = rennet paste; RP-L = rennet paste containing encapsulated *L. acidophilus*; RP-B = rennet paste containing encapsulated *Bifidobacterium longum* and *Bifidobacterium lactis*.
[2]Below the detection limit (1 log cfu/g).

Table 4. Moisture, salt, ether extract and pH of pecorino cheese from Gentile di Puglia sheep milk to 45 days of ripening

Parameter	C	BB	LA	SEM	Effects
Moisture, %	34.05	33.43	34.88	0.19	NS
Ether extract, %	26.70^{a}	31.80^{b}	29.96^{a}	0.7	***
NaCl/U, %	2.78	2.11	2.82	0.03	NS
pH	5.26^{b}	5.02^{a}	5.03^{a}	0.05	**
Casein, %	29.46^{b}	23.85^{a}	24.32^{a}	0.4	**

Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*;
SEM = standard error;
** $p < 0.01$; *** $p < 0.001$;
NS = Not Significant

Table 5. Nitrogen Fractions in pecorino cheese from Gentile di Puglia sheep milk to 45 days of ripening

Parameter	C	BB	LA	SEM	Effect,P
Non casein nitrogen(NCN) / Total nitrogen(TN), %	14.31a	15.21a	19.36b	0.34	**
Non protein nitogen(NPN) / Total nitrogen(TN), %	8.79a	12.36b	10.15a	0.46	*
Phosphotungstic acid-soluble nitrogen(PASN) / Total nitrogen(TN), %	6.41a	10.99b	7.41a	0.64	*

Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*;
SEM = standard error;
* $p < 0.05$; ** $p < 0.01$;

Table 6. Free amino acids in cheese from Gentile di Puglia sheep milk to 45 days of ripening

Free amino acids, µg/g of chesse	C	BB	LA	SEM	Effect, P
Glutamic acid	29.90a	237.39b	14.44c	10.58	*
Asparagine	64.49b	42.85a	0.01nd	1.67	***
Serina	0.01a	251.89b	0.36a	0.9	***
Glutamine	47.61a	100.22b	178.33c	15.97	*
Histidine	32.91a	100.01b	88.99b	8.31	*
Glycine	78.32b	149.50c	50.77a	6.68	**
Threonine	229.46a	397.80b	228.62a	17.48	**
Arginine	39.02a	156.72b	49.44a	4.28	***
Alanine	13.28a	63.03b	13.16a	4.34	**
Tyrosine	16.03a	32.22b	17.74a	2.59	*
Cysteine	72.75a	335.98b	71.87a	23.37	**
Valine	440.80a	911.02b	1323.90c	63.01	**
Methionine	301.11	289.08	441.95	43.20	NS
Tryptophan	43.24a	342.68c	218.74b	12.47	**
Phenylalanine	422.14b	1251.39c	1828.53c	97.62	**
Isoleucine	81.50a	482.77b	96.14a	23.46	**
Leucine	48.70a	298.21c	145.03b	21.11	**
Lysine	1562.50	1761.75	1614.92	39.12	NS
Hydroxyproline	51.60a	107.20b	45.93a	8.30	**
Proline	23.15a	62.81b	12.04	6.78	*

Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*;
SEM = standard error;
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;
NS = Not Significant

Table 7. Free fatty acids in cheese from Gentile di Puglia sheep milk to 45 days of ripening

Fat acids, %	BB	C	LA	SEM	Effect,P
C 4:0	24.15b	14.25a	19.66b	1.18	*
C 6:0	5.13	4.48	6.5	0.78	NS
C 8:0	7.13	5.97	5.93	0.61	NS
C 10:0	12.01	11.85	14.57	1.07	NS
C 12:0	6.4a	7.03ab	8.43b	0.36	*
C 14:0	8.3	9.91	9.92	0.62	NS
C 16:0	15.65a	20.02b	21.33b	0.89	*
C 18:0	4.33a	3.98a	6.45b	0.14	**
C 18:1,t9	1.43	2.24	1.37	0.18	NS
C 18:1,c9	5.19	6.48	5.43	0.31	NS
C 18:2	1.79	2.05	2.23	0.25	NS
C 18:3,n3	1.29	1.53	1.33	0.1	NS
CLA	1.98b	0.88a	2.09b	0.07	*

Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*;
SEM = standard error;
* $p < 0.05$; ** $p < 0.01$;
NS = Not Significant

Table 8. Rheological parameters of probiotic ovine cheese at 45 day of ripening (*n* = 18).

Parameter	C	BB	LA	SEM	Effect,P Probiotic
Hardness, N	25.6	29.4	26.0	2.1	NS
Cohesiveness	0.1 [a]	0.2 [b]	0.2 [a,b]	0.1	*
Gumminess,N	0.4 [a]	0.9 [b]	0.6 [a,b]	0.1	*
Chewiness,Nxm	4.5	5.8	4.5	1.0	NS
Elasticity, mm	7.9	7.9	7.9	0.8	NS

Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*;
SEM = standard error;
* $p < 0.05$;
NS = not significant;
a,b Mean values followed with different superscripts differ significantly.

Table 9. Sensorial attributes of probiotic ovine cheese at 45 day of ripening

Parameter		Cheese			SEM	Effect, p
		C	BB	LA		Probiotic
Appearance						
	Uniformity	5.1	5.4	5.1	0.3	NS
	Humidity	6.4 [b]	4.8 [a]	5.0 [a]	0.3	***
	Chalky	3.8	4.0	3.7	0.4	NS
	Gumminess	5.1 [b]	4.0 [a]	3.8 [a]	0.3	**
	Graininess	2.7 [a]	2.8 [a]	2.9 [b]	0.3	NS
Color						
	Uniformity	6.3	5.9	5.8	0.2	NS
	Intensity	6.1	5.9	5.6	0.3	NS
Odor						
	Intensity	6.1	5.7	5.6	0.3	NS
	Acid	3.4	3.4	3.1	0.3	NS
	Rancid	1.8	1.7	1.8	0.3	NS
Taste						
	Intensity	5.9 [a]	6.8 [b]	6.1 [a,b]	0.3	*
	Salty	4.3 [a]	5.7 [b]	4.8 [a]	0.3	***
	Acid	2.7	3.3	3.2	0.3	NS
	Pungent	2.5 [a]	4.6 [b]	4.1 [b]	0.3	***
	Bitter	2.7	2.5	2.8	0.3	NS
	Sweet	3.5	2.7	3.1	0.3	NS
	Mould	1.1	0.9	1.2	0.3	NS
	Rancid	1.0	1.3	1.9	0.4	NS

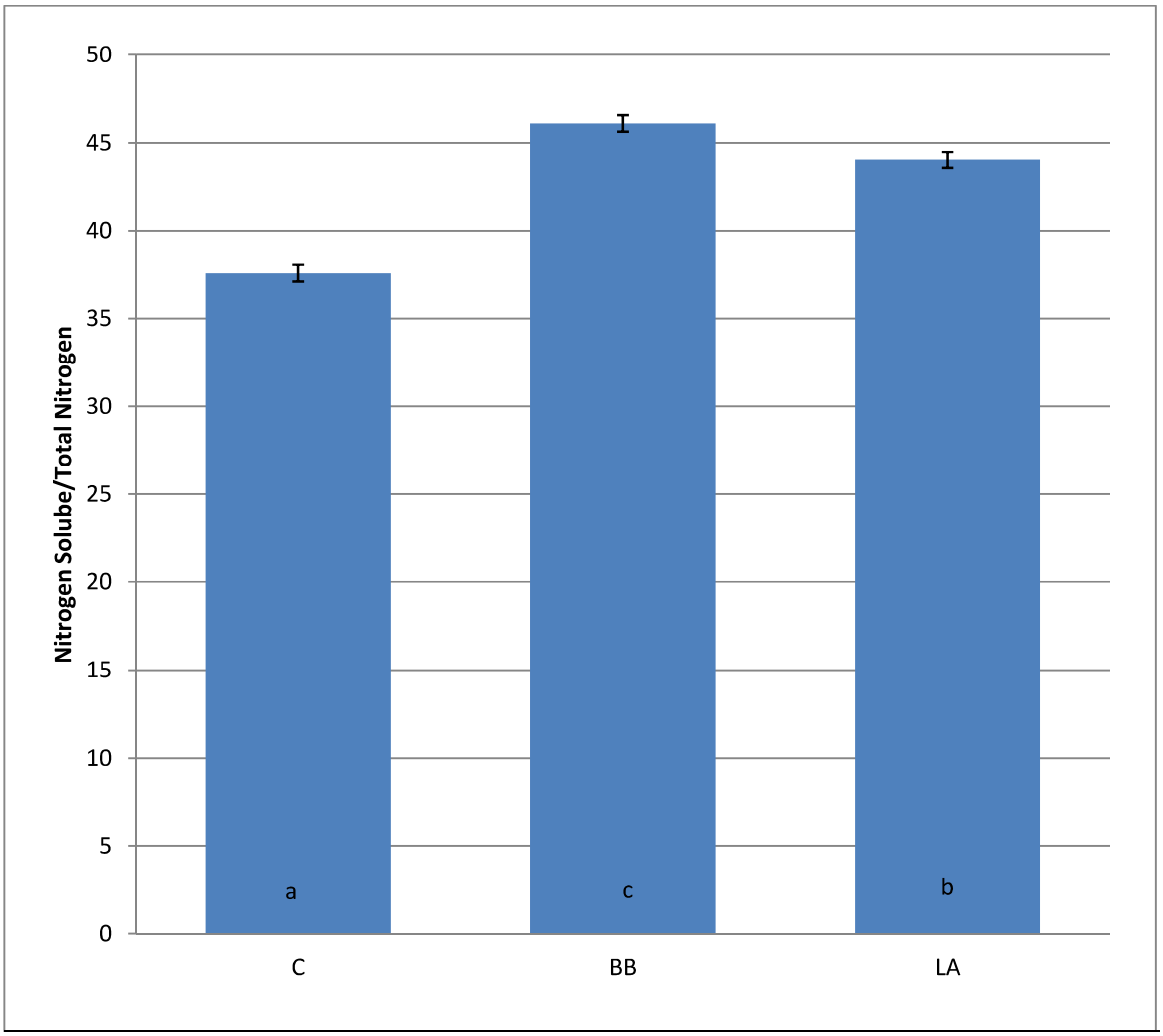
Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*;
SEM = standard error;
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;
NS = not significant;
a,b Mean values followed with different superscripts differ significantly

Table 10. Rating of probiotic ovine cheese liking

Parameter	Gender and Age	Cheese C	Cheese LA	Cheese BB	SEM	Effect, p Probiotic	Effect, p Gender x Gender	Effect, p Probiotic x x Age
Percevied liking								
	M < 30 (n = 19)	6.5 [a]	7.1 [a,b]	6.6[a]				
	M > 30 (n = 21)	6.5 [a]	6.5 [a]	7.1 [a,b]				
					0.2	NS	NS	*
	F < 30 (n = 21)	7.1 [a,b]	6.5 [a]	6.6 [a]				
	F > 30 (n = 19)	6.6 [a]	7.3 [b]	7.3 [b]				
Expected liking								
	M < 30 (n=19)	7.5 [a]	7.3 [a]	7.7[b]				
	M > 30 (n=21)	7.7 [a]	7.6 [a]	7.8 [b]				
					0.2	NS	NS	*
	F < 30 (n =21)	7.5 [a]	7.1 [a]	7.1 [a]				
	F > 30 (n =19)	7.8 [b]	8.2 [b]	8.1 [b]				
Actual liking								
	M < 30 (n= 19)	7.1	7.5	7.3				
	M > 30 (n= 21)	7.2	7.5	7.1				
					0.4	NS	NS	NS
	F < 30 (n = 21)	7.2	7.1	7.1				
	F > 30 (n = 19)	7.7	7.6	7.5				

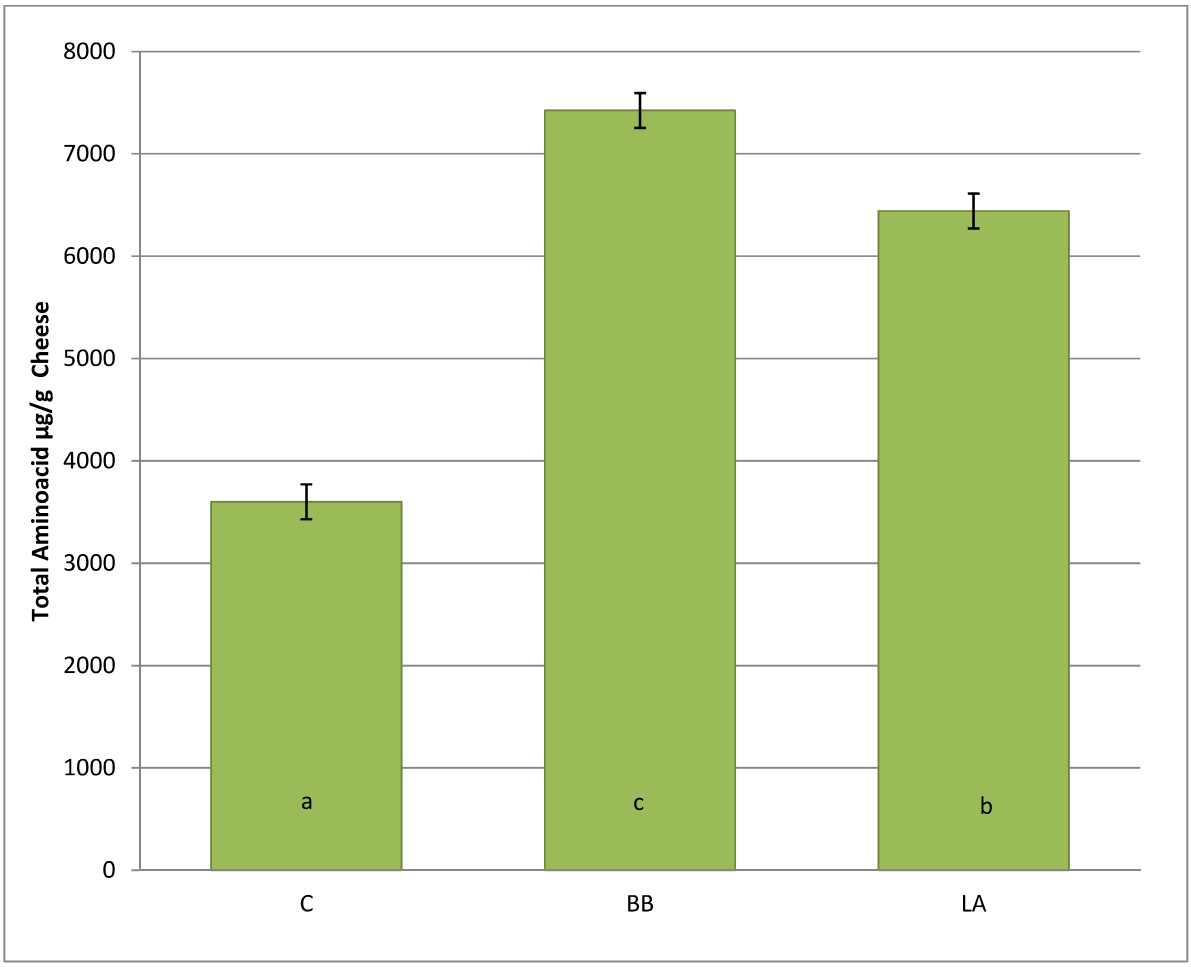
Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*;
SEM = standard error;
* $p < 0.05$; NS = not significant;
a,b Mean values followed with different superscripts differ significantly

Graph 1. Nitrogen soluble fraction at pH 4.6 in pecorino cheese from Gentile di Puglia sheep milk to 45 days of ripening



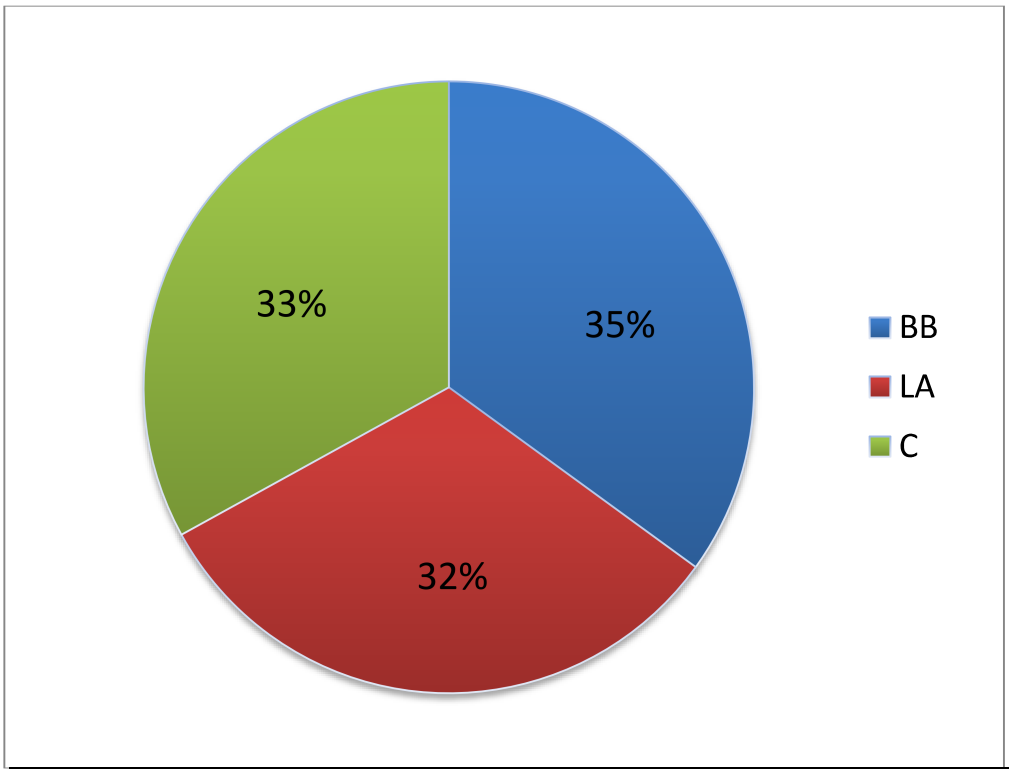
Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*

Graph 2. Total free amino acids in pecorino cheese from Gentile di Puglia sheep milk to 45 days of ripening



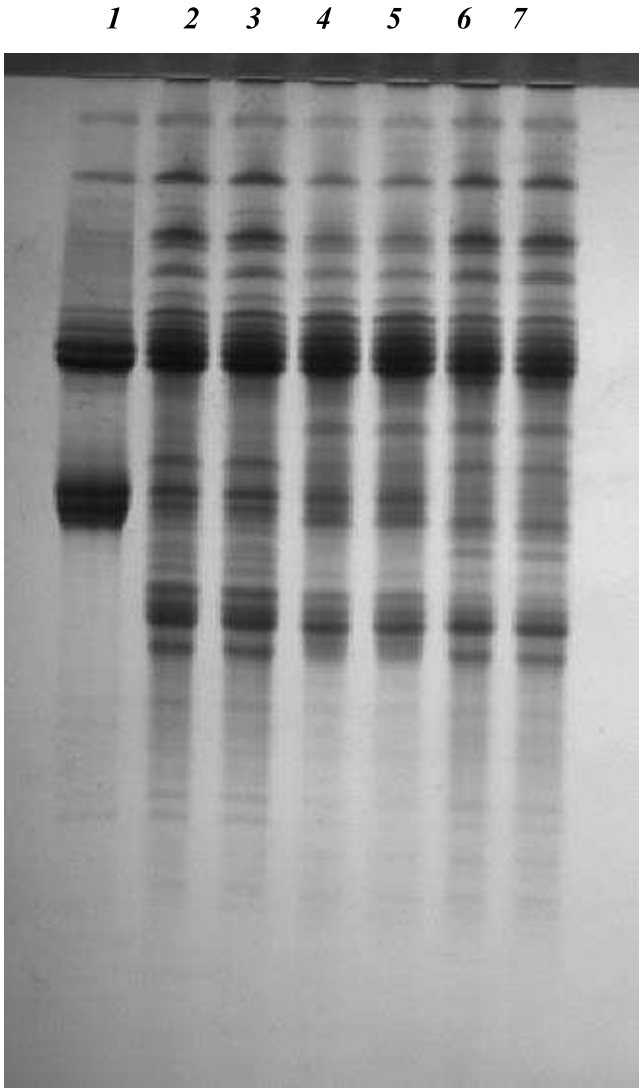
Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*

Graph 3. Consumer's liking for the different types of cheese from Gentile di Puglia sheep milk.



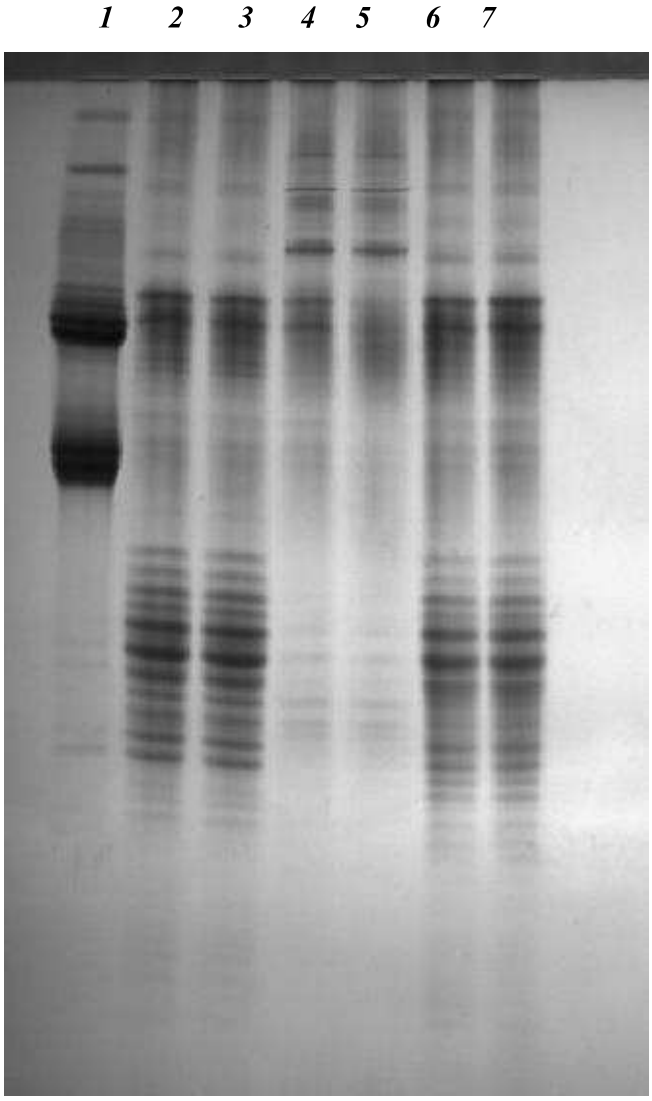
Cheese: C = cheese made with traditional lamb rennet paste;
BB = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*;
LA = cheese made with lamb rennet paste containing *L. acidophilus*

Figure 1. Electro-phoretogram at pH 4,6 - insoluble N fraction of pecorino cheese from Gentile di Puglia sheep milk at 45 of ripeninig



Line 1= sodium caseinate standard;
lines 2-3= cheese made with traditional lamb rennet paste; (cheese: C);
lines 4-5 = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*; (cheese BB);
lines 6-7= cheese made with lamb rennet paste containing *L. acidophilus* (cheese LA).

Figure 2. Electro-phoretogram at pH 4,6 - soluble N fraction of pecorino cheese from Gentile di Puglia sheep milk at 45 of ripeninig



Line 1= sodium caseinate standard;
lines 2-3= cheese made with traditional lamb rennet paste; (cheese: C);
lines 4-5 = cheese made with lamb rennet paste containing a mix of *B. longum* and *B. lactis*; (cheese BB);
lines 6-7= cheese made with lamb rennet paste containing *L. acidophilus* (cheese LA).

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Accettato per la pubblicazione in Italian Journal of Animal Science