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1. Introduction and aim

Milk and dairy products are considered important constituents of a balanced diet due to their numerous benefits on human health, even thanks to the microbiota associated to these fermented food products (Nagpal et al., 2012). Regarding this latter aspect, lactic acid bacteria are considered the main players in determining the positive health effects of fermented milks and related products, such as: antitumor activity, prevention of gastrointestinal infections, reduction of serum cholesterol levels and antimutagenic activity. Moreover, these microorganisms are involved in the development of characteristic flavour and aroma of the end-products by fermenting the raw materials (Shiby et al., 2013).

Also yeasts and moulds are important microbial populations, which can be found in dairy products (i.g.: *Geotrichum candidum, Debaryomyces hansenii, Penicillum, Aspergillus…*), where they play a key role in the development and enhancement of texture and flavour through the activity of some microbial extracellular enzymes in the food matrix (Fernandez et al., 2014).

Hence, it is logical to classify fermented milks as "functional foods" (Weaver, 2003; Kurien et al., 2005), which are foods with beneficial physiological effects on the human body.

This master thesis is centred on a particular acid-alcoholic fermented milk typically produced in Sardinia (Southern Italy) with goat or sheep milk, and commonly known as "*Gioddu*", or even "*Miciuratu*", "*Mezzoraddu*", or "*Latte ìschidu*" (literally meaning acidulous milk) by local people. In particular, the study was focussed on the identification of the bacterial components of the characteristic microbiota of this traditional milk-based beverage by using culture-dependent and culture-independent techniques.

1.1 Fermented fresh milk products

Milk is the most important foodstuff for a mammal, being the first food for the newborns. It provides nutrients and bioactive components, which stimulate growth and digestive maturation, favour the establishment of microflora, and the development of gut-associated lymphoid tissues (Ebringer et al., 2008). Moreover, it was recognized that milk from other mammals, such as cow, goat or sheep, was equally satisfying in meeting all the physiological demands of human beings in terms of energy and nutrients (Kroger et al., 1992).

In order to preserve the health benefits of milk, a typical technique used since ancient times is the fermentation. In fact, fermentation is one of the oldest methods for food processing, whose primary function is to extend the shelf life of the end products as well as to improve digestibility and enhance taste and flavour, thanks to the production of microbial metabolites (mainly lactic acid, ethyl alcohol, and other chemicals) by bacteria or yeasts.

According to the Codex Standard, fermented milk is "*a milk product obtained by fermentation of milk, which milk may have been manufactured from products obtained from milk with or without compositional modification as limited by the provision in Section 3.3, by the action of suitable microorganisms and resulting in reduction of pH with or without coagulation (iso-electric precipitation). These starter microorganisms shall be viable, active and abundant in the product to the date of minimum durability. If the product is heat-treated after fermentation the requirement for viable microorganisms does not apply*".

In more detail, fermented fresh milk products are milk products, which undergo fermentation but are not subjected to further ripening. This category of products can be divided into **traditional fermented fresh milk products** and **non-traditional fermented fresh milk products.** The first group is referred to those milk products, which have a substantial historical record, for example yogurt and kefir. The second group includes all those products, which have been developed as a result of deliberate modern scientific progress and they also may be the imitations of the traditional ones (Kurmann et al., 1992).

The exact origin of the manufacture of fermented milks is difficult to establish but it could be dated back to more than 10.000 years ago, when the way of life of humans changed from food gathering to food producing (Pederson, 1979). The geographical origin of these products was the Middle East and Balkans, though but they are currently manufactured in many countries, where the art of this ancient practice is combined to science (Tamime et al., 2002).

1.1.1 Health traits

Fermentation of milk has numerous positive effects. One of these is the ability to increase the preservation of the end products by the lowering of pH during the fermentation process but also to impact their organoleptic and physicochemical characteristics, thus contributing to the development of aroma and texture. Moreover, thanks to the conversion of lactose into lactic acid, fermented dairy products can be consumed also by those people, who are lactose intolerant. In addition, they have a high nutritional value resulting from the combination of the presence of biological active compounds and the activity of probiotic microorganisms (Ebringer et al., 2008).

The bioactive compounds (such as vitamins, conjugated linoleic acid, bioactive peptides and others) have anti-inflammatory (Penedo et al., 2013), antiatherogenic and antioxidant properties (Chinnadurai et al., 2013), as well as antihypertensive and immune-modulatory activities. Some of these compounds, for example galactooligosaccharide (GOS), have an important role in the regulation of the gastrointestinal tract thanks to their prebiotic effect on the intestinal microbiota, and hence on: (i) promotion of selective growth of bifidobacteria (Padilla et al. 2012, Schwab et al. 2011); (ii) defence against pathogenic bacteria; (iii) enhancement of different systems (immune, digestive, cardiovascular and nervous); and (iv) stimulation of the antioxidant activity (Ebringer et al., 2008).

Probiotic microorganisms are live microorganisms that have a beneficial effect on the host by influencing the composition and or the metabolic activity of the gastrointestinal microbiota. These microorganisms have a positive effect on the immune system, modulating the production of cytokines and antimicrobial peptides (Trebichavsky and Splichal, 2006). Moreover, they are able to metabolize cholesterol reducing its resorption in the gastrointestinal tract by incorporating it into membranes, deconjugating and precipitating the bile acids (Gilliland et al., 1995; Tahri et al., 1996; Pereira and Gibson, 2002). It has been demonstrated that a long-term daily consumption of fermented milk products increases the HDL cholesterol and improve the ratio LDL/HDL (Kiessling et al., 2002). However, this hypocholesterolemic effect does not belong to all species and strains of bacteria typically occurring in milk.

Another important beneficial effect of these microorganisms is due to their antioxidant activity. The presence of free radicals in the human body plays an important role in the development of pathologies such as cancer, cardiovascular diseases, allergies, atherosclerosis and others (Haliwell and Guteridge, 1989; Bergendi et al., 1999, Agerholm-Larsen et al., 2000). The antioxidant defence systems naturally present in the human body can compensate to a certain degree the oxidative stress, until an imbalance in favour of the reactive oxygen molecules leads to cell damage. The presence of antioxidant

compounds can help to counteract the damage caused by these molecules. Probiotic microorganisms have the ability to trap these reactive forms of oxygen, affecting the human health in a positive way. By comparing sour milks and unfermented milk products, the first are characterized by an improvement of the antioxidant status (Ebringer et al., 2008).

1.1.1.1 Toxic compounds released by LAB, yeasts and moulds during fermentation

As mentioned above, fermented milk products have important beneficial effects, but in some cases metabolic activities can result in the production of two types of toxic compounds: mycotoxins and biogenic amines.

Mycotoxins are chemical hazards synthesized by *fungi* and considered secondary metabolites because they are not essential for the normal growth and development. In milk and dairy products, the most important mycotoxin is the aflatoxin M1. This mycotoxin results from the metabolic conversion in the liver of the aflatoxin B1, a type of mycotoxin that can be present in contaminated feed.

The biogenic amines are low-molecular weight nitrogenous organic bases and the most important are histamine, tyramine and putrescine. Many bacteria have the ability to produce by decarboxylation these compounds, especially Gram-negative. In dairy products, the main biogenic amines producers belong to the genera *Enterococcus, Lactobacillus, Leuconostoc, Lactococcus* and *Streptococcus* (Fernandez et al., 2004; Cruz Martin et al., 2005; Bonetta et al., 2008; Calles-Enriquez et al., 2010; Ladero et al., 2011).

The possible occurrence of the aflatoxin M1 or of the biogenic amines in dairy products can lead to serious diseases in humans. In the case of aflatoxin M1, the occurrence of diseases in children is well documented by Prandini et al. (2009).

1.1.2 Technological aspects

In general, during the production of fermented milk, the best possible growth conditions must be created for the microorganisms responsible of the fermentation process. These are achieved by heat treatment of the milk to destroy any competing microorganisms. Then, the milk must be held at the optimum temperature for the relevant starter culture and finally, when the desired flavour and aroma have been achieved, the fermented milk must be cooled quickly, to stop the fermentation process.

Indeed, the fermentation time should not be too long or too short in order to avoid problems related to flavour and consistency of the final product. The latter is another important feature of fermented milks which is determined by the choice of pre-treatment parameters, for example, in the production of yoghurt the homogenization of milk is essential for the construction of the coagulum during the incubation period (Dairy Processing Handbook).

Each fermented milk product has its own process, but there are some stages, the principal ones, which are in common, as summarized by Tamime (2002).

Preparation of the milk

The fat and the non-fat solid (SNF) contents are standardized to ensure compliance with the legal standards. For example, according to the FAO/WHO code, yoghurt may have a fat content of 0 to 10 %, however a fat content of 0.5 – 3.5 % is the most typical, while the minimum non-fat solid fraction is about 8,2%

The aim is to improve the viscosity of the final product by the fortification of the non-fat solid fraction. To achieve this goal, different techniques are available such as the addition of powders or the concentration by evaporation or filtration. In the case of adding powders, it is important to increase the temperature to about 40° C and then filtrate and deaerate the milk in order to remove possible undissolved particles and the air, respectively. In fact, the presence of air in milk may hinder the growth of *Lactobacillus acidophilus* and bifidobacterial, increases heat-exchanger fouling and affects the characteristics of the gel.

Homogenization

This step leads to desired physical-chemical changes of the raw material which are:

- Reduction in the diameter of fat globules to avoid coalescence
- Whitening
- Increase in viscosity thanks to the interaction between fat globules and casein micelles
- Decrease in syneresis of the gel for an increase in its hydrophilicity and water-holding capacity as a result of the interactions of the protein

In the case of fermented milks, usually the homogenizer is placed upstream.

Heat treatment

Depending on the type of fermented milk, different combination of time and temperature can be used. The thermal treatment affects the physical and chemical composition of the product, but it is also responsible of the elimination of pathogens and other microorganisms and it is involved in the production of components which could be stimulatory to the starter cultures.

Inoculation

After the thermal treatment, there is a cooling step and then the inoculation phase. At this point, pre-selected microorganisms (starter cultures) or a small portion of a previous fermentation product (back-slopping) are inoculated into the batch of fermentation.

The **back-slopping** is a technique based on the use of a small quantity of the previous fermentate as the raw material for the next fermentation step, resulting in the dominance of the best adapted strains. It is a cheap and traditional method mainly used for small-scale productions (Di Cagno et al., 2013).

For large-scale productions, **starter cultures** are used. They are made up of the highest possible number of viable and harmless microorganisms, mainly lactic acid bacteria, which are intentionally grown in the product to impart predictable texture and flavour to fermented milk products. Depending on the type of product, starter cultures can be divided into mesophilic cultures, which grow best at 25-30° C, and thermophilic cultures, which grow at higher temperature (37-45° C) (Surono et al., 2011).

Cooling and miscellaneous handling

After heat treatment, the milk is cooled to the temperature favourable for the growth of desired microorganisms. Indeed, cooling is used to control the metabolic activity of the starter cultures in order to retain an abundant count of these organisms in fermented milks. The primary objective is to cool the product to 5° C, and the process of cooling commences at 4.6 pH, which is carried out using a one- or two-phase cooling (the latter is used in case of fruit-flavoured fermented milks).

1.1.3 Microbiota

For its characteristics, fermented milk has been an important component of nutrition and diet. The typical fermented milk is the yogurt, but there are many types produced all over the world that differ

each other so much that it is possible to classify them according to the method of fermentation and processing, which are related to the microorganisms involved.

The fermentation process involves primarily the conversion of lactose in lactic acid by lactic acid bacteria (LAB), resulting in the reduction of the pH that makes growth conditions of microorganisms other than LAB increasingly unfavourable (Fernandez et al., 2015).

The LAB in fermented dairy products belong to different genera and for this reason each one is characterized by different requirements in terms of nutrition, metabolism and culture but also by different technological properties. The most common species included in these products belong to the genera *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc* and *Enterococcus* (Quigley et al., 2011).

Lactococcus lactis ssp. *lactis* and ssp. *cremoris*, in particular are primarily known because of their role as starter cultures for the cheese industry.

The genus *Lactobacillus* plays two main roles in fermented dairy products: as starter (for example *Lactobacillus delbrueckii* ssp. *bulgaricus* and ssp. *lactis* in yoghurt production) or as secondary microbiota (nonstarter LAB or NSLAB).

Also streptococcal genera are used in dairy products formulation, although some of the species belonging to this genera are considered pathogenic. *Streptococcus thermophilus* carriers a GRAS status (Facklam, 2002) and for this reason it is widely used as starter culture in the manufacture of dairy products so much that it is considered the second most important industrial dairy starter after *Lactococcus lactis*.

Enterococci are the most controversial group of food associated LAB: they are index of fecal contamination for water, meat products, and vegetables, but they are not considered in such way in dairy products, being part of the milk microbiota (especially, *Enterococcus faecium* and *Enterococcus faecalis*). According to some authors, high amount ($> 10^8$ CFU / g) in food could cause intoxication due to the ability to produce biogenic amines. Moreover, they are potentially dangerous due to their resistance to numerous antibiotics.

Regarding the species belonging to the genera *Leuconostoc*, they are important for their activity as flavouring because they are able to ferment citrate with diacetyl production (for example: *Leuconostoc mesenteroides*).

In addition to lactic acid bacteria, there are another important group of microorganisms included in fermented milks: bifidobacteria. These microorganisms have health-promoting properties and,

although they have a growth rate slower than that of lactic acid bacteria, their multiplication contribute to increase the level of lactate in the final product (Fernandez et al., 2015).

Also yeasts and moulds are considered important microbial populations in the manufacture of dairy products, especially in some types of cheeses in which they play a key role in the enhancement of texture and flavour. Among the yeasts the most important species found in dairy products are *Candida* spp., *Geotrichum candidum* and *Debaryomyces hansenii*, while among moulds *Penicillium, Aspergillus, Mucor* and *Fusarium* are the most common genera (Lavoie et al., 2012).

Thus, the identity of each type of fermented milk is the result of the specific microorganisms involved which, through their metabolic activity, supply a wide range of metabolites giving individual characteristics to the final product, but also the process conditions providing a suitable environment for their growth.

According to the type of fermentation, fermented milk products can be classified into: products of mesophilic/thermophilic lactic fermentation, products of alcohol-lactic fermentation and products in which, in addition to the other two types of fermentation, there is the mould growth (Surono et al., 2011).

Mesophilic lactic acid fermentation

Generally, for this type of fermentation, the starters used belong to the genera *Lactococcus*, whose *Lactococcus lactis* sub. *Cremoris* is the main acid-producer, and *Leuconostoc* which, together with *Lactococcus lactis* sub. *Lactis*, is the main flavour-producer (Robinson et al., 2000).

The lactose is transformed in L-lactate by lactococci, while, in the case of leuconostocs, it is converted in D-lactate. In the first case, lactose is transported into cells by the phosphoenolpyruvate system as lactose phosphate and then it is hydrolysed to glucose and galactose 6-phosphate, while, leuconostocs directly hydrolyse lactose resulting in the production of glucose and galactose.

For the production of flavour, microorganisms metabolize the citrate and produce diacetyl, acetoin, 2-3-butylene glycol, which confer the typical milk flavour to the product, and also CO_2.

Example of fermented milk obtained by mesophilic starters are: buttermilk, ymer and Nordic ropy milks.

Thermophilic lactic acid fermentation

Typical of the yogurt and yogurt products, this kind of fermentation uses as starter cultures *Streptococcus thermophilus* and *Lactobacillus delbruekii* subsp. *Bulgaricus*. An interaction between these two microorganisms is established and it is called protocooperation.

Streptococcus thermophilus obtains the energy necessary for its growth from lactose but its development is further stimulated by some amino acids and peptides released from the milk proteins by *Lactobacillus bulgaricus*. On the other hand, *Lactobacillus* exploits formic acid and carbon dioxide for its development, two products synthesized by the *Streptococcus* already about 40 minutes after the start of fermentation.

Mould-lactic acid fermentation

Normally, mould is not a component of the starter cultures, but in the case of viili, a product obtained by mould-lactic fermentation, *Geothricum candidum* is used together *Leuconostoc mesenteroidees* and *Lactococcus lactis*. The latter is responsible of the formation of capsules, giving to the product the typical ropy character, while *Geothricum candidum* gives to the product a velvet-like appearance.

Yeast-lactic acid fermentation

This fermentation process is characteristic of kefir, koumiss and others such as Gioddu. Yeasts (*Saccharomyces, Pichia, Candida…)* and lactic acid bacteria (*Lactococcus, Lactobacillus, Leuconostoc*) are used and they are able to ferment lactose. The most important volatile components are acetaldehyde, propionaldehyde, acetone, ethanol, 2-butanone, n-propyl alcohol, diacetyl and amyl alcohol. The yeasts also exhibit proteolytic activity, contributing to the flavour of the final product (Robinson et al., 2000).

1.2 Overview of international and Italian fermented milk products

There is evidence that fermented milk products have been produced since around 10,000 BC. Initially, they were produced by spontaneous fermentation carried out by wild starter cultures (naturally fermented milk – NFM). The problem with these starters is that the quality of the end products is unpredictable; hence, since the end of the XIX century the industrial production of fermented milks has moved to the introduction of selected starter cultures and controlled processing conditions. On the one hand, the use of these latter starters has increased tremendously the quality of the end products but, on the one other hand, it has reduced the microbial diversity of fermented dairy products.

Milk fermentation has several advantages, including the prolongation of shelf life of the end product, but also the improvement of its taste and digestibility.

Puhan et al. (1994) report that as early as thirty years ago the consumption of dairy products, and especially of fermented milks, was very popular all around the word.

Approximately 400 generic names referred to traditional or industrialized fermented milks are used worldwide (Kurmann et al., 1992). In the year 1984, Kurmann tried to make a classification of these products with a "family tree", created on the basis of the optimum growth requirements of the starter cultures used (Figure 1).

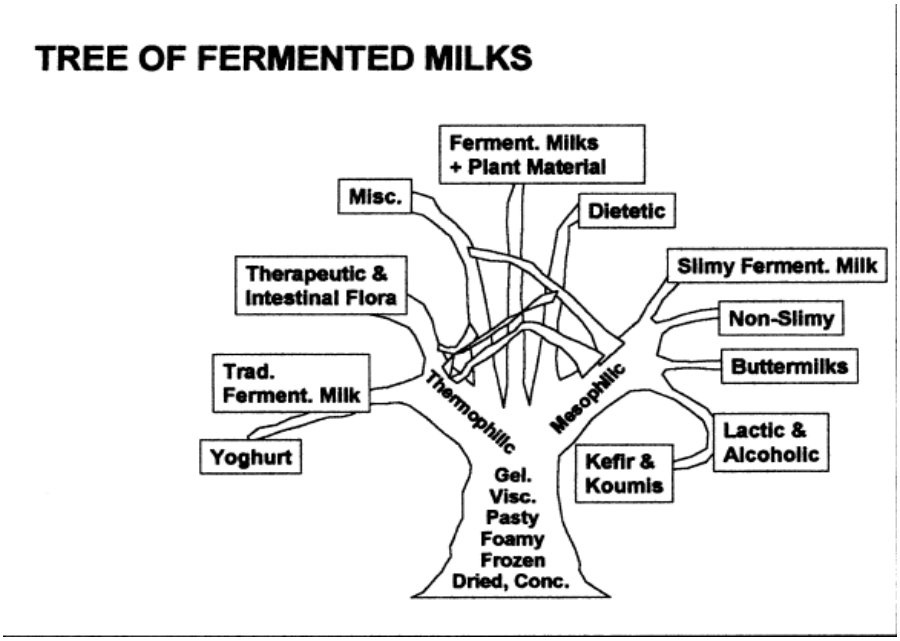

Figure 1 - Family tree (Kurmann, 1984)

However, as mentioned in the previous chapter, based on the microorganisms involved in the production process, fermented milks can also be grouped into 4 categories, being (i) products from mesophilic lactic acid fermentation; (ii) products from thermophilic lactic acid fermentation; (iii) products from mould-lactic acid fermentation; (iv) products from yeast-lactic acid fermentation

Mesophilic lactic acid fermentation

Cultured buttermilk

Cultured buttermilk is a low-acid fermented milk with a soft white appearance without gas holes or whey separation. The strains responsible for acidification are *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*, while the characteristic flavour and aroma are due to *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc mesenteroides* subsb. *cremoris*. The incubation step at 22° C is essential to enable the starter culture to produce the desirable features of cultured buttermilk. Higher temperature would favour the growth of *Lactococcus lactis* subsp. *lactis*, hence, an excess in acid production (Surono et al., 2011). Cultured buttermilk is typically produced and consumed in Germany and Scandinavian countries.

Nordic (Scandinavian) fermented milks

Långfil (Sweden), tettemelk (Norway), filmjőlk (Sweden) are examples of Nordic fermented milks, which are characterized by a high viscosity and ropiness. These traits are essentially due to the presence of capsule-forming lactococci, mainly *Lactococcus lactis* subsp. *cremoris.*

Thermophilic lactic acid fermentation

Yogurt

Yogurt is the typical fermented milk consumed in Italy, but originally produced in Eastern Mediterranean countries over thousands of years. It is a medium-acid fermented milk, which results from the fermentation (42° C for 3-5 hours) of cow milk by *Streptococcus thermophilus* and *Lactobacillus delbruekii* subsp. *bulgaricus.* Based on the method of production, there are three types of yogurt, being (i) Balkan-style or set-style yogurt, which is produced into containers without any stirring; (ii) Swiss-style or stirred yogurt, which is manufactured in large vats, where after fermentation and cooling, it is stirred for a creamy texture; (iii) Greek-style yogurt, a very thick yogurt

that is either produced from milk that has undergone partial removal of water or by straining whey from plain yogurt to make it thicker and creamier.

Besides to cow's milk, yogurt can also be manufactured with milks deriving from other animals, such as goats or sheeps; depending on the country of origin, it has different names such as "zabady" in Egypt, "dahi" in India, "dadih" in Indonesia and so on. In the past, but still today, the greatest fraction of published papers about the nutritive value of fermented milks refers to yogurt.

Bulgarian buttermilk

Bulgarian buttermilk is a high-acid fermented milk obtained by the use of *Lactobacillus delbruekii* subsp. *bulgaricus* as starter culture. This product is popular exclusively in Bulgaria.

Yakult

Very famous in Japan for its health-promoting properties, Yakult is made by using the probiotic strain *Lactobacillus casei* subsp. *casei* Shirota, which remains viable in the gastrointestinal human tract.

Mould-lactic acid fermentation

Viili

Viili is a Finnish product fermented by *Lactococcus lactis* and *Leuconostoc mesenteroides* subsb. *cremoris.* It is characterized by the presence of the mould *Geotrichum candidum* on its surface, giving the product a velvet-like appearance.

Yeast-lactic acid fermentation

Kefir

Kefir is a very old fermented milk originally produced in the Northern Caucasus region withkefir grains. These latter contain a complex microbial population, which includes streptococci, leuconostocs, thermophilic lactobacilli, mesophilic lactobacilli, yeasts and also acetic acid bacteria. The grains are incubated in milk, thus allowing the microorganisms to be shed from the grains and to begin to ferment. Kefir is a refreshing and nutritious drink which is also used as an aid in the therapy of gastrointestinal diseases (Kurmann et al., 1992).

Kumiss

Probably originating from central Asia, the traditional manufacture of kumiss involved the use of raw mare's milk and a starter culture made of *Lactobacillus delbruekii* subsp. *bulgaricus* and lactose-fermenting yeasts, derived from previously produced kumiss.

Gioddu

This is the only variety of fermented milk originating in Italy, typically in Sardinia (South Italy). Traditionally, it is prepared with sheep's or goat's milk and it is characterized by a firmer consistency than that of cow's yoghurt, a translucent white colour and an acid taste, mainly conferred by its raw material, but also by the presence of live lactic ferments (ONAF, 2018). The home-made product tends to acquire an acid and sparkling taste after about 2 days, thus it can be considered an acid-alcoholic milk. This fermented milk has a long tradition of being regarded as a healthy product as other fermented milks, whose functional properties are attributed to the metabolic products of the microbial content (Lopitz-Otsoa et al., 2006). The microbiota can include *Lactobacillus delbruekii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactococcus lactis*, *Enterococcus faecalis* and *Enterococcus faecium*, but even yeasts. In the industrial products, yogurt starter cultures are used for the inoculum of the milk, together with (occasionally) baker's yeast (Kurmann et al., 1992).

Gioddu technology is not well defined because different methods are used according to the local tradition and the raw material employed, but, basically, milk is kept boiling for the achievement of the typical Gioddu consistence, then it is cooled down to 30-40°C and inoculated with 1-2% of Gioddu from the previous day. In order to promote the growth of lactic acid bacteria, the milk is kept at constant temperature in metallic container (Arizza et al., 1983).

Gioddu samples analysed in this master thesis were prepared using sheep milk as raw material. After filtration with woollen sheets, they were boiled and consequently cooled down to 25-30° C. For the inoculation, natural starters from Fruhe cheese, a Sardinian soft cheese, were used. A temperature of 25-30°C was maintained for the fermentation step and the final products were stored at 5°C. The following flow chart shows schematically the Gioddu production process (Figure 2).

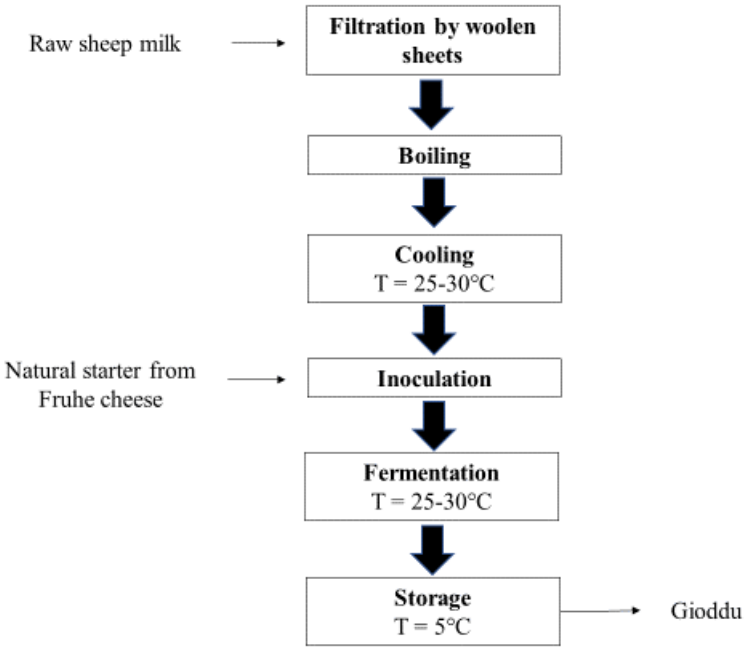

Figure 2 – Gioddu flow chart

1.3 Investigation into the microbial composition and dynamics of fermented milks and related products

Different types of fermented milks have been developed in all parts of the world, thus each one has its own characteristics that depend on several factors such as the type of milk used, the pre-treatment of the raw material, the fermentation conditions and the technological treatments they undergo. From a microbiological point of view, fermented milks contain lactic acid bacteria, which play a key role in the fermentation process, but other bacteria and yeasts could be involved, as well (Wouters et al., 2002).

Knowledge about the microbial composition and the potential interactions of microorganisms in food matrices is crucial to ensure production of safe and high-quality food, which are the primary features requested by consumers, industries and government (Juste et al., 2008). Moreover, the monitoring of the microbial population in a given food allows the management of the microbial processes involved in its processing and ripening (Barriga et al., 1991; Walls and Scott, 1997; Delfini and Formica, 2001) as well as the improvement of its microbiological safety (Liu, 2004).

In the past decades, the identification of food microorganisms has been carried out by using culture-dependent techniques. The problem with this approach is that it allows to exclusively detect cells capable of replication without taking into account microorganisms, which are alive but cannot replicate, such as microbes subjected to environmental stress in a viable but not culturable state (VBNC). This limitation has led to the development of culture-independent techniques, most of which rely on polymerase chain reaction (PCR) (Yang et al., 2001). Compared with traditional methods, culture-independent techniques have the potential to provide a rapid and direct enumeration/detection of viable cells by evaluating the metabolic activity, measuring the cellular integrity or revealing the presence of nucleic acids.

1.3.1 Culture-dependent approaches

The culture-dependent techniques allow the quantification/detection of bacteria able to replicate on synthetic media and under specific conditions. The quantification of bacteria with this approach is achieved by counting the number of colony forming unit (CFU) grown on dilution plates per gram or millilitre of the original samples. The enumeration can be performed by using two alternative

approaches, each with advantages and drawbacks, being (i) the pour plate method and (ii) the spread plate method.

The pour plate method is usually the method of choice for counting and isolation of bacteria occurring in liquid specimens. One millilitre of a bacterial suspension is placed in the centre of a sterile Petri dish, then the melted agar medium is poured into the Petri dish and slowly mixed. The advantage of this technique is that it does not require the preliminary preparation of agar plates, whereas the main disadvantages are the loss of viability of heat-sensitive microorganisms, the difficulties in counting embedded colonies and the low growth rate of obligate aerobes in the depth of the agar medium.

Similarly, the spread plate method can be used for both counting and isolation of microorganisms. With this technique, 0.1 millilitres of a microbial suspension are placed onto the centre of a Petri dish containing a solid medium. The inoculum is spread over the growth medium surface using a L-shaped spreader and hence the plate is incubated at the opportune temperature. The main advantage of this technique is that colonies have all the same size, but agar plates have to be previously prepared and the inoculum distribution is laborious and time-consuming.

The use of both techniques has different challenges. First, standardized methods are only available for a limited number of species to be enumerated/isolated from a few dairy products (e.g. enumeration standards for *Lactobacillus acidophilus* and *Bifidobacterium* published by the International Organisation of Standardization. Secondly, a consensus on the definition of live viable cells is yet to be established. By convention, the scientific community typically considers a cell viable if it reproduces on an agar plate with key nutrients, where it forms a colony. However, recent advances have revealed that this is a limited definition considering that microbes exist in different growth phases and metabolic states, which in turn depend on the environmental conditions and stress sources they have to cope with; thereafter only a subset of these states involve active replication, as elucidated in Table 1.

Physiological state	Phenotype
Viable (live)	Intact cytoplasmic membrane, functional synthesis of proteins and other cell components (nucleic acids, polysaccharides, etc.) and energy production necessary to maintain cellular metabolism, and, eventually, growth and multiplication (Breeuwer and Abee, 2004)
Culturable (replicating)	Capable of division, resulting in the formation of a colony on an agar plate or proliferation in a broth medium.
Non-replicating (in stationary phase; inhospitable conditions for replication; or injured)	Will not form a colony on an agar plate nor proliferate observably in liquid medium; but may have active physiologic activity and intact cytoplasmic membrane. Cells may be inhibited by the medium or injured but capable of repair (Le et al., 2008).
Starving	Cells undergo dramatic decreases in metabolism, but remain fully culturable (Mahdi et al., 2012).
Dormant (viable but not culturable)	In a state of low metabolic activity and unable to divide or to form a colony on an agar plate without a preceding resuscitation phase. A protective response. Also seen in "post-acidification" (Lahtinen et al., 2008, Shah, 2000)
Irreparably damaged cells	Will not grow with vigour under any conditions due to progressive metabolic decline. These cells may be irreparably injured (Le et al., 2008)
Non-viable (dead)	No metabolic activity (Lahtinen et al., 2008, Le et al., 2008)

Table 1 - Physiological states of cells (Davis, 2014).

If only culture-dependent techniques are applied, underestimation of the effective number of strains capable of biological activity would occur. Some stressed or injured cells may not be immediately capable of replication, but by using repair mechanisms their capacity may be re-established. By contrast, those bacteria seriously damaged, which undergo an irreversible metabolic decline, should be excluded from the enumeration of live cells.

In the last years the application of culture-independent molecular methods based on the DNA extraction directly from a sample followed by the amplification of the 16S rRNA gene by Polymerase Chain Reaction (PCR) have been successfully applied. Of note, besides to these methods, even culture-dependent molecular approaches relying on the extraction of the bacterial DNA from bacterial colonies grown on solid media, have been applied, as well (Pogačic et al., 2009).

1.3.2 Culture-independent approaches

Due to their limitations, results from culture-dependent approaches might not reflect the effective composition of the microbiota of a given food (Ampe et al., 1999, Ercolini et al., 2001). For example, the detection of bifidobacteria by using culture-dependent techniques is difficult for the lack of selective media able to differentiate among *Bifidobacterium* species. For this reason, in the last decades, numerous culture-independent approaches have been developed to overcome the problems resulting from conventional cultivation of microorganisms (Vaughan et al., 2002).

Culture-independent approaches typically rely on (i) the use of dyes to differentiate live and dead cells by direct observation (e.g. *Fluorescent in Situ Hybridization* – FISH); (ii) the measurement of the membrane integrity (e.g. Flow cytometry); or (ii) the detection of specific target molecules (e.g. nucleic acids) associated to the metabolic activity of microorganisms (e.g. PCR).

To date, several culture-independent molecular methods have been applied in microbial ecology studies, including denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP) (Spiegelman et al., 2005) or Sanger sequencing of 16S rRNA gene clone libraries. Sanger sequencing, being considered a "first-generation" technology, has been the dominant sequencing method for almost two decades, leading to key results, such as the final sequencing mapping of the human genome. Nevertheless, it has some limitations, which led to the development of new technologies, referred to as next generation sequencing (NGS) (Metzker, 2009).

1.3.2.1 Next generation sequencing

Also known as "High-Throughput Sequencing", NGS is the term commonly used to describe various technologies aimed at sequencing DNA and RNA molecules much more quickly and cheaply than the previously used Sanger sequencing. They focus on one or a few phylogenetic marker genes and provide a cost-effective culture-independent method to study the composition and diversity of complex microbial communities. The most widely used phylogenetic marker gene is the 16S rRNA gene; this latter consists of nine different variable regions, which are used as target regions, flanked by conserved regions, which are used for primers design (Herzyk, 2014).

The basic characteristics of NGS sequencing technologies are: (i) the generation of many millions of short reads in parallel; (ii) the speed up of the sequencing process in respect with the first generation

sequencing technologies; (iii) the low cost of sequencing and (iv) the obtaining of sequencing output without the need for electrophoresis.

NGS platforms include: Roche 454 sequencing, Ion torrent sequencing, ABI/SOLiD sequencing, and Illumina (Solexa) sequencing.

Roche 454 sequencing

Pyrosequencing using the Roche 454 platform was initially the most successful because it provided long reads (longer than 400 bp) that could generate high-resolution results when identifying taxa at the species level (Herzyk, 2014). It appeared on the market in 2005; it relies on the use of the pyrosequencing technique, which is based on the detection of pyrophosphate released after each nucleotide incorporation in the new synthetic DNA strand (Kchouk et al., 2017).

The main drawback of this technology is the occurrence of insertion and deletion mutation events, which are due to the presence of homopolymeric regions (Margulies et al., 2005; Huse et al., 2007).

Ion torrent sequencing

Ion torrent sequencing is similar to 454 pyrosequencing technology but it does not use fluorescent labelled nucleotides. It relies on the detection of the hydrogen ion released during the sequencing process (Rotheberg et al., 2011).

The major advantages of this sequencing technology are the length of reads (200 bp, 400 bp and 600 bp), and the fast sequencing time (2-8 hours). However, it is difficult to interpret homopolymer repeats (more than 6 bp) (Reuter et al., 2015; Loman et al., 2012) which causes insertion and deletion errors with rates around 1% (Kchouk et al., 2017).

ABI/SOLID sequencing

SOLiD (Sequencing by Oligonucleotide Ligation and Detection) is a next-generation DNA sequencing technology developed by Life Technologies and commercially available since 2006. This technology generates hundreds of millions to billions of small sequence reads at one time (Goodwin et al., 2016; Alic et al., 2016). The main advantage of this platform is the high accuracy (each base is read twice), while its main drawbacks are the relatively short length of reads and long run times.

Illumina sequencing

Illumina technology is the most used technology in the NGS market. It is based on reversible dye-terminators enabling the identification of single bases as they are introduced into new synthesized DNA strands. Currently, it is used for whole-genome and region sequencing, transcriptome analysis, metagenomics, small RNA discovery, methylation profiling, and genome-wide protein-nucleic acid interaction analysis (Dohm et al., 2008).

2. Materials and methods

2.1 Origin and maintenance of Gioddu samples

Gioddu samples were obtained from three different Sardinian producers labelled as A, M and P. From each producer three different batches were sampled and analysed (Table 2). Samples were delivered under refrigerated conditions and processed for microbiological and chemical analysis immediately after arrival at the laboratory.

Producer code	**Batch**		
A	1	2	3
M	1	2	3
P	1	2	3

Table 2 - Gioddu samples.

2.2 Colour measurements

The colorimetric profile of Gioddu samples was determined with a colorimeter (Chroma Meter CR-200, Minolta, Japan) that had previously been calibrated. Colour of the Gioddu samples was analysed according to CIELab system using a Minolta CR 200 equipped with a D65 illuminant. Lightness, red(+)/green(−) colour attribute and yellow(+)/blue(−) colour attribute were determined through L, a* and b* parameters; chroma index (C), which expresses the quantitative attribute of colourfulness, was calculated as $\sqrt{(a*2 + b*2)}$ whereas whiteness indexes (WI) were calculated as 100 - $\sqrt{(100 - \mathrm{L})2 + \mathrm{a}*2 + \mathrm{b}*2}$. All the measures were performed in triplicate.

2.3 Determination of pH and total titratable acidity

pH values of the Gioddu samples were measured with a model 300 pH meter equipped with an HI2031 solid electrode (Hanna Instrument, Padova, Italy). For the determination of total titratable acidity (TTA), 1 mL of each Gioddu sample was homogenized with 9 mL of distilled water and titrated with 0.1 N NaOH. The results were expressed as the total volume (mL) of 0.1 N NaOH used to reach a pH of 8.3. For each sample, the measurements were performed in duplicate and the results were expressed as mean value ± standard deviation.

2.4 Lactic acid bacteria enumeration

One mL of each Gioddu sample was added with 9 mL of a sterile peptone water (0.1% peptone, w/v) solution and homogenized by vortexing. The obtained homogenates (dilution 10^{-1}) were further ten-fold diluted and subjected to viable counts of lactic acid bacteria (LAB). In particular, LAB were inoculated by pour plate method in de Man Rogosa and Sharpe (MRS) Agar (VWR Prolabo, Leuven, Belgium) and in M17 agar (Merck KGaA, Darmstadt, Germany), for the enumeration of lactobacilli and lactococci, respectively. The agar plates were incubated at 37°C under aerobic conditions. Both growth media were added with 200 mg L^{-1} of cycloheximide to inhibit the growth of yeasts.

The results were expressed as colony forming units (CFU) per mL g of sample ± standard deviations.

2.5 DNA extraction

The microbial DNA was extracted directly from each Gioddu sample using the "PowerFood microbial DNA isolation kit" (MO BIO laboratories, Carlsbad, California) according to the manufacturer's instructions with slight modifications:

The DNA extracts were stored at -20° C.

2.6 DNA spectrophotometric quantification

The quantification of the extracted DNA was performed by using the Spectrophotometer UV-1800 *(*Schimadzu Corporation, Japan). In more detail:

- 5 µL of the extracted DNA was added to 495 µL of deionized distilled water (100 X dilution);
- The absorbances were read at 260 nm against deionized distilled water and recorded.

DNA concentration of was calculated according to the following formula:

$$1 : 50 \text{ ng } \mu L^{-1} = X \; A_{260\,nm} : Y \text{ ng } \mu L^{-1}$$

$$Y \text{ ng } \mu L^{-1} \times 100 \text{ (dilution factor)} = [DNA] \text{ ng } \mu L^{-1}$$

2.7 PCR amplification

The effective extraction of the bacterial DNA was checked by PCR amplification of the V3 region of the 16S rRNA gene, using the following primers:

- 27f (5′-GAG AGT TTG ATC CTG GCT CAG-3′)
- 1495r (5′-CTA CGG CTA CCT TGT TAC GA-3′) (Weisburg et al., 1991)

Aliquots (3 µL) of each DNA extract was amplified in a final reaction volume of 50 µL. The PCR mixture and the cycling conditions used for the amplification are illustrated in Table 3 and Figure 3, respectively.

	Ci	**Vi** (µL for each sample)	**Cf**
Buffer	10 X	5 µL	1 X
DNTPs	10 mM	1 µL	0.2 mM
27f	10 µM	1 µL	0.2 µM
1495r	10 µM	1 µL	0.2 µM
Taq Polimerase	5 U/µL	0.2 µL	1 U/50 µL
H_2O	Up to 50 µl volume		

Table 3 - PCR mixture for the amplification of the V3 region of the 16S rRNA gene.

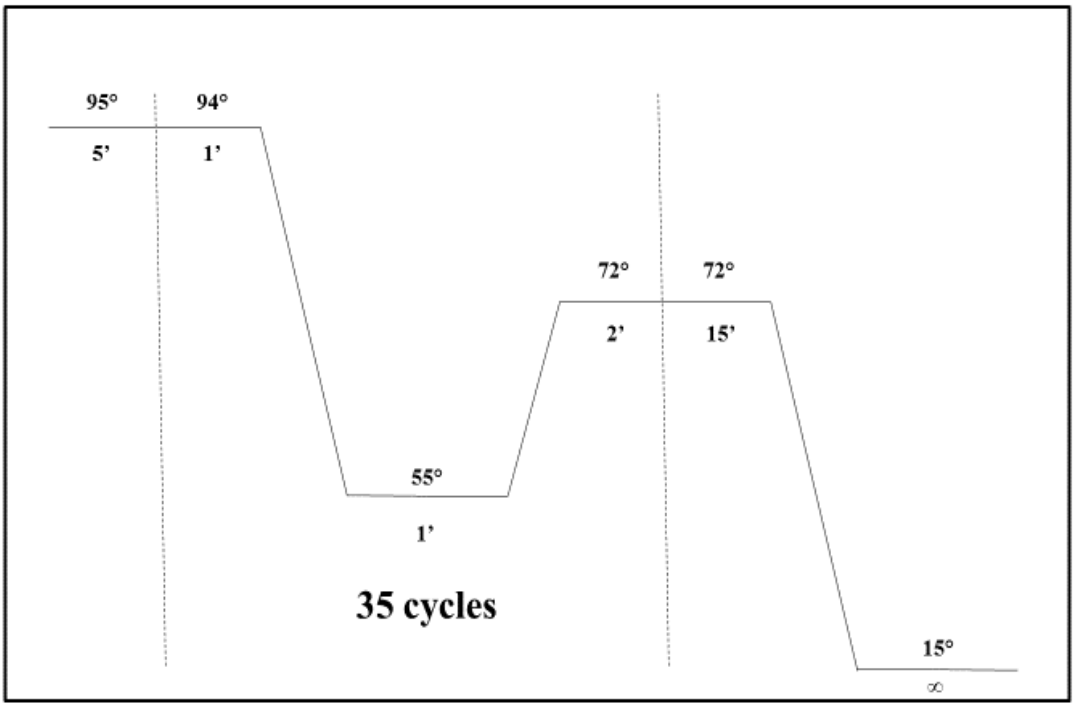

Figure 3 - PCR cycling program for the amplification of the V3 region of the 16S rRNA gene using primers 27f-1495r.

The D1/D2 regions of the 26S rRNA gene were also amplified using the MyFi^{TM} mix (BioLine, London, UK) with primers:

- NL1 (5′-GCG GGC CGC GCG ACC GCC GGG ACG CGC GAG CCG GCG GCG GGC CAT ATC AAT AAG GGG AGG AAA AG-3′)
- LS2 (5′-GGTC CGT GTT TCA AGA CGG-3′) (Cocolin et al., 2000)

Aliquots (2 µL) of each DNA extracts were amplified in a final reaction volume of 50 µL. The PCR mixture and the cycling conditions used for the amplification are shown in Table 4 and Figure 4, respectively.

Vf = number of samples x 50 µL	**Ci**	**Vi** (µL for each sample)	**Cf**
MyFi^{TM} mix	10 X	25 µL	2 X
NL1	10 µM	1 µL	0.2 µM
LS2	10 µM	1 µL	0.2 µM
H_2O	Up to volume		

Table 4 - PCR mixture for the amplification of the D1/D2 regions of the 26S rRNA gene.

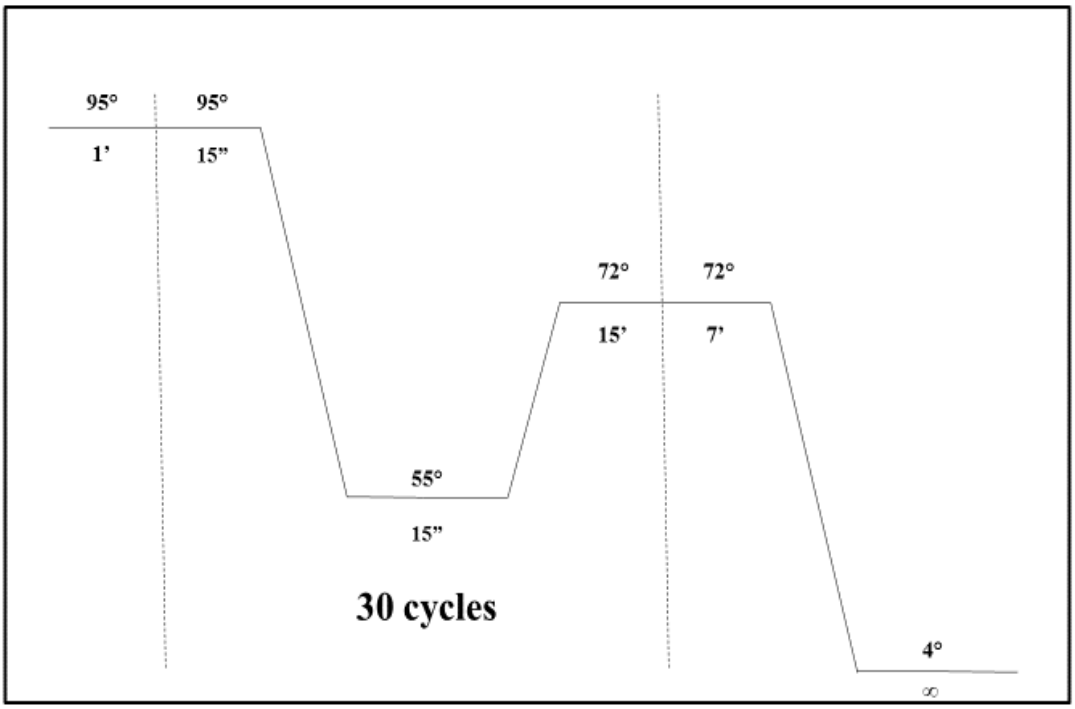

Figure 4 - PCR cycling program for the amplification of the D1/D2 regions of the 26S rRNA gene using primers NL1-LS2.

All the PCR amplifications were carried out with the thermocycler My cycler Thermal cycler (BIO-RAD laboratories, Hercules, USA).

2.8 Agarose gel electrophoresis

An aliquot (5 µL) of each PCR product was mixed with 2 µL of Atlas Load 6x Loading Dye Bromophenol Blue (Bioatlas, Estonia and checked by electrophoresis using 1.5% (w/v) agarose gel in 0.5 X Tris Borate-EDTA (TBE) running buffer containing 0.5 µg mL^{-1} of ethidium bromide.

The electrophoretic run was carried out at a voltage of 4 V cm^{-1} for 45 minutes using a molecular weight standard (HyperLadder^{TM} 100bp Mix, BioLine, London, UK). The amplicons were visualized under UV light using a transilluminator and photographed with the Complete Photo XT101 system (Explera, Jesi, Italy).

2.9 Library preparation and sequencing

Aliquots of the PCR products (40 µL) were sent to the Department of Agriculture, Forest and Food Sciences (DISAFA), University of Turin for Illumina sequencing. Library preparation was carried out according to the Illumina metagenomic procedure. Sequencing was performed by MiSeq Illumina sequencing platform using V3 chemistry with the generation of 250-bp paired-end reads, following the producer's instructions.

2.10 Alignment of sequences and identification of microbial species

After sequencing, reads were assembled and processed by using the QIIME 1.9.0 software (Knight et al., 2010). The pipeline was described by Ferrocino et al. (2017) for amplicons of the 16S rRNA gene and from Mota-Gutierrez et al. (2018) for amplicons of the 26S rRNA gene.

2.11 Statistical analysis

The analysis of variance (ANOVA-oneway) of colorimetric parameters was performed on three different levels: producer A (A), producer M (M), and producer P (P). The Tukey-Kramer test ($P \leq 0.05$) was carried out to detect differences through multiple mean comparisons. The statistical analysis was conducted using JMP software (version 11.0).

In addition, pH, TTA and bacterial count data collected were subjected to one-way analysis of variance (ANOVA) using JMP software (version 11.0), and differences were considered non-significant at $P < 0.05$.

3. Results and discussion

3.1 Colour measurements

The nine samples of Gioddu analyzed in this research study were subjected to colour determination using the Chroma Meter CR-200 (Minolta, Japan) system. UHT goat milk was used as an external control. The results of statistical analyses carried out on colorimetric data are shown in Table 5.

Producer	L	a*	b*	C	WI
A	85.35±1.14 ^{B}	-8.13±0.43 ^{A}	9.54±0.78 ^{A}	12.53±0.84 ^{A}	80.69±0.92 ^{A}
M	87.64±1.22 ^{A}	-8.67±0.78 ^{A}	10.45±1.97 ^{A}	13.60±1.99 ^{A}	81.52±1.17 ^{A}
P	87.12±1.09 ^{A}	-8.43±0.37 ^{A}	10.55±0.86 ^{A}	13.51±0.79 ^{A}	81.31±0.82 ^{A}
External control	90.52±0.01	-7.68±0.02	9.63±0.01	12.83±0.90	84.46±0.01
F test	***	n.s.	n.s.	n.s.	n.s.

Table 5 - Colorimetric parameters mean comparisons of Gioddu products (LSD test) and ANOVA F test. A= Producer A; M= Producer M; P= Producer P.
Means within the same column with different letters are different at $P < 0.05$.
L= lightness; a*= redness-greenness (+ red; - green); b*= yellowness-blueness (+ yellow; - blue); C= Chroma Index; WI= whiteness Index.

Overall, ANOVA results showed significant differences between producers only for Lightness (L) parameter.

The external control was characterized by a higher Lightness (L), redness (a*), and Whiteness Index compared to the three samples analyzed. Gioddu made by producers M and P (samples M and P) highlighted significantly higher mean values for L than Gioddu manufactured by producer A.

To date, no data are available on the scientific literature about colour determination of Gioddu, thus explaining the further comparison of our data with those referred to similar fermented milk-based products.

As a general trend, colorimetric parameters of Gioddu were quite different from those reported by Cais-Sokolinska and colleagues (2014) in kefir made with goat milk added with a mixture of lactic acid bacteria and yeasts and those observed by Vargas and colleagues (2008) in yogurt made with 100% goat milk.

The Whiteness index is commonly used for the evaluation of dairy products, since it can be directly correlated to the consumer's preference towards white colors (Ghasemlou et al., 2011; Gul et al., 2018; Pathare et al., 2013). According to Vargas et al. (2008), this parameter can be affected by several physical-chemical variables, including: (i) dimension of fat globules; (ii) casein ratio; and (ii) casein micelles aggregation.

3.2 pH and total titratable acidity

The results of pH and TTA determination are shown in Table 6. The first parameter is a measure of the product acidity whereas TTA refers to the content in organic acids as well as proteins, salts and inorganic compounds.

As it emerges from Table 6, Table 7, and Table 8, pH values range from 3.55 to 4.32, whereas TTA values were comprised between 1.10 and 2.23 mL NaOH per mL of sample.

		A	
	1	**2**	**3**
pH	4.27 ± 0.08 ^{A}	4.08 ± 0.14 ^{A}	4.32 ± 0.28 ^{A}
TTA (mL NaOH)	1.55 ± 0.07 ^{A}	1.24 ± 0.01 ^{B}	1.10 ± 0.00 ^{B}

Table 6 - pH and TTA values comparisons in the three production batches (1,2, and 3) from producer A.
pH values were obtained by using a pH meter; TTA values (expressed as mL NaOH) were obtained by titration with 0.1 N NaOH.
Means ± standard deviations of triplicate independent experiments are shown.
Means within the same raw with different letters are different at $P < 0.05$.

		M	
	1	**2**	**3**
pH	3.89 ± 0.02 ^{A}	4.05 ± 0.13 ^{A}	3.89 ± 0.11 ^{A}
TTA (mL NaOH)	2.23 ± 0.00 ^{A}	2.21 ± 0.01 ^{A}	1.75 ± 0.07 ^{B}

Table 7 - pH and TTA values comparisons in the three production batches (1,2, and 3) from producer M.
pH values were obtained by using a pH meter; TTA values (expressed as mL NaOH) were obtained by titration with 0.1 N NaOH.
Means ± standard deviations of triplicate independent experiments are shown.
Means within the same raw with different letters are different at $P < 0.05$.

	P		
	1	**2**	**3**
pH	4.01 ± 0.10 ^{A}	3.55 ± 0.27 ^{B}	3.85 ± 0.11 ^{A}
TTA (mL NaOH)	1.56 ± 0.01 ^{C}	2.05 ± 0.07 ^{B}	2.30 ± 0.01 ^{A}

Table 8 - pH and TTA values comparisons in the three production batches (1,2, and 3) from producer P.
pH values were obtained by using a pH meter; TTA values (expressed as mL NaOH) were obtained by titration with 0.1 N NaOH. Means ± standard deviations of triplicate independent experiments are shown.
Means within the same raw with different letters are different at $P < 0.05$.

As shown in Table 6, pH values in the three different batches are not significantly different, while TTA analysis revealed differences between batch 1 and the other two batches (2 and 3).

Similar results were observed for the batches from producer M. pH data are not statistically different, but batch 3 showed a lower pH value with respect to batch 1 and batch 2 (Table 7).

A totally different picture emerged for the production batches of Gioddu from producer P, which significantly differed for both variables (pH and TTA). In particular, batch 1 showed a TTA value lower than that of batch 2, which, in turn, was lower than that of batch 3 (Table 8).

	A	**M**	**P**
pH	4.22 ± 0.16 ^{A}	3.94 ± 0.08 ^{B}	3.80 ± 1.97 ^{B}
TTA (mL NaOH)	1.29 ± 0.02 ^{B}	2.06 ± 0.02 ^{A}	1.97 ± 0.03 ^{A}

Table 9 - pH and TTA values means comparisons of the three producers (A, M, and P). pH values were obtained by using a pH meter; TTA values (expressed as mL NaOH) were obtained by titration with 0.1 N NaOH.
Means within the same raw with different letters are different at $P < 0.05$.

Table 9 shows data comparisons between the three manufacturers (A, M, and P). pH and TTA values of Gioddu produced at plant A were different with respect to those produced at plants M and P. More in detail, Gioddu from producer A showed a pH value higher than Gioddu from producers M and P, but a TTA value lower than that of the other two producers.

When compared to the available literature, pH data collected for producer A were similar to those previously found in yogurt (Behrad et al., 2009; Amirdivani et al., 2011) and kefir (Megalhales et al., 2011). Concerning the samples from producer M and P, pH values comparable to those found in low fat and low cholesterol milk products (Kahn et al., 1990) were seen.

Regarding TTA, a higher acidity than that found in yogurt by Amirdivani et al. (2011) was seen in the Gioddu samples analysed, in particular if the samples from producer M are considered. According to Billard et al. (2007), TTA positively correlates with the accumulation of organic acids.

3.3 Microbial counts

In Table 10, Table 11 and Table 12, the results of viable counts of presumptive lactococci and mesophilic lactobacilli are shown.

	A		
	1	**2**	**3**
		log CFU mL^{-1}	
Lactococci	4.92 ± 0.12 ^{A}	5.72 ± 1.29 ^{A}	5.54 ± 0.76 ^{A}
Mesophilic lactobacilli	4.66 ± 0.16 ^{A}	4.66 ± 0.88 ^{A}	4.95 ± 0.22 ^{A}

Table 10 - Viable counts assessed in the three production batches (1, 2, and 3) from producer A.
Presumptive lactococci were enumerated on M17 incubated at 37 °C, whereas presumptive mesophilic lactobacilli were counted on MRS incubated at 37° C.
Means ± standard deviations of triplicate independent experiments are shown.
Means within the same raw with different letters are different at $P < 0.05$.

	M		
	1	**2**	**3**
		log CFU mL^{-1}	
Lactococci	5.50 ± 0.22 ^{A}	4.51 ± 0.49 ^{A}	4.89 ± 0.66 ^{A}
Mesophilic lactobacilli	5.89 ± 0.58 ^{A}	5.36 ± 0.14 ^{A}	2.95 ± 0.07 ^{B}

Table 11 - Viable counts assessed in the three production batches (1, 2, and 3) from producer M.
Presumptive lactococci were enumerated on M17 incubated at 37 °C, whereas presumptive mesophilic lactobacilli were counted on MRS incubated at 37° C.
Means ± standard deviations of triplicate independent experiments are shown.
Means within the same raw with different letters are different at $P < 0.05$.

	P		
	1	**2**	**3**
		log CFU mL^{-1}	
Lactococci	4.47 ± 0.73 ^{A}	4.58 ± 0.98 ^{A}	4.74 ± 0.55 ^{A}
Mesophilic lactobacilli	5.14 ± 0.80 ^{A}	6.47 ± 0.23 ^{A}	5.78 ± 0.72 ^{A}

Table 12 - Viable counts assessed in the three production batches (1, 2, and 3) from producer P.
Presumptive lactococci were enumerated on M17 incubated at 37 °C, whereas presumptive mesophilic lactobacilli were counted on MRS incubated at 37° C.
Means ± standard deviations of triplicate independent experiments are shown.
Means within the same raw with different letters are different at $P < 0.05$.

In none of the 3 producers was observed a statistically valid difference between the individual batches, with the exception for M manufacture, in which batch 3 showed a presumptive mesophilic lactobacilli load significantly lower than that of the other batches (Table 11).

	A	M	P
		log CFU mL^{-1}	
Lactococci	5.39 ± 0.72 ^{A}	4.96 ± 0.42 ^{A}	4.60 ± 0.75 ^{A}
Mesophilic lactobacilli	4.75 ± 0.42 ^{A}	4.73 ± 0.26 ^{A}	5.80 ± 0.58 ^{A}

Table 13 - Viable counts means comparisons of the three producers (A, M, and P).
Presumptive lactococci were enumerated on M17 incubated at 37 °C, whereas presumptive mesophilic lactobacilli were counted on MRS incubated at 37° C.
Means within the same raw with different letters are different at $P < 0.05$.

Also comparing the three manufacturers (A, M, and P), significant differences were not seen for presumptive lactococci and mesophilic lactobacilli (Table 12).

Comparing these results with those reported in other published papers analysing fermented milks, a clear difference is immediately evident. In particular, Akabanda et al. (2010) reported a presumptive lactococci load of 8.82 ± 0.32, that is 4 logarithmic units higher than those recorded in this Master thesis.

Irigoyen et al. (2004) reported a lowering of microbial load in kefir during 30 days of storage, anyway the results they observed are higher than the microbial load assessed in Gioddu samples.

Similar values were instead recorded in the study on kefir grains from different Italian regions (Garofalo et al., 2015). In this scientific article a bacterial growth of the order of 10^5 on the M17 medium was observed, while in the MRS medium the results obtained were about 3 logarithmic units higher than the Gioddu samples.

3.4 Illumina sequencing

The total DNA extracted from the Gioddu samples was spectrophotometrically quantified. DNA concentrations of the extracts are shown in Table 14

	Gioddu samples								
	A			M			P		
	1	2	3	1	2	3	1	2	3
	ng µL^{-1}								
DNA concentration	15	35	17,5	25	30	10	25	15	5

Table 14 - DNA concentration (expressed as ng µL-1); 1, 2, and 3 refer to the production batches whereas A, M, and P refer to the three producers.

The extracted DNA underwent the amplification of the regions V3 (for bacteria) and D1/D2 (for yeasts) of the 16S and 26S rRNA genes, respectively; hence, PCR products were sent to DISAFA (University of Turin) for Illumina sequencing.

The total number of paired sequences obtained from the Gioddu samples reached 336855 raw reads. After quality filtering, a total of 237722 reads were used, with an average value of 26413 ± 7239 reads/sample, and a mean sequence length of 465 bp. Alpha diversity index showed a satisfactory coverage for all samples (> 98%) however did not showed different level of complexity based on the producers.

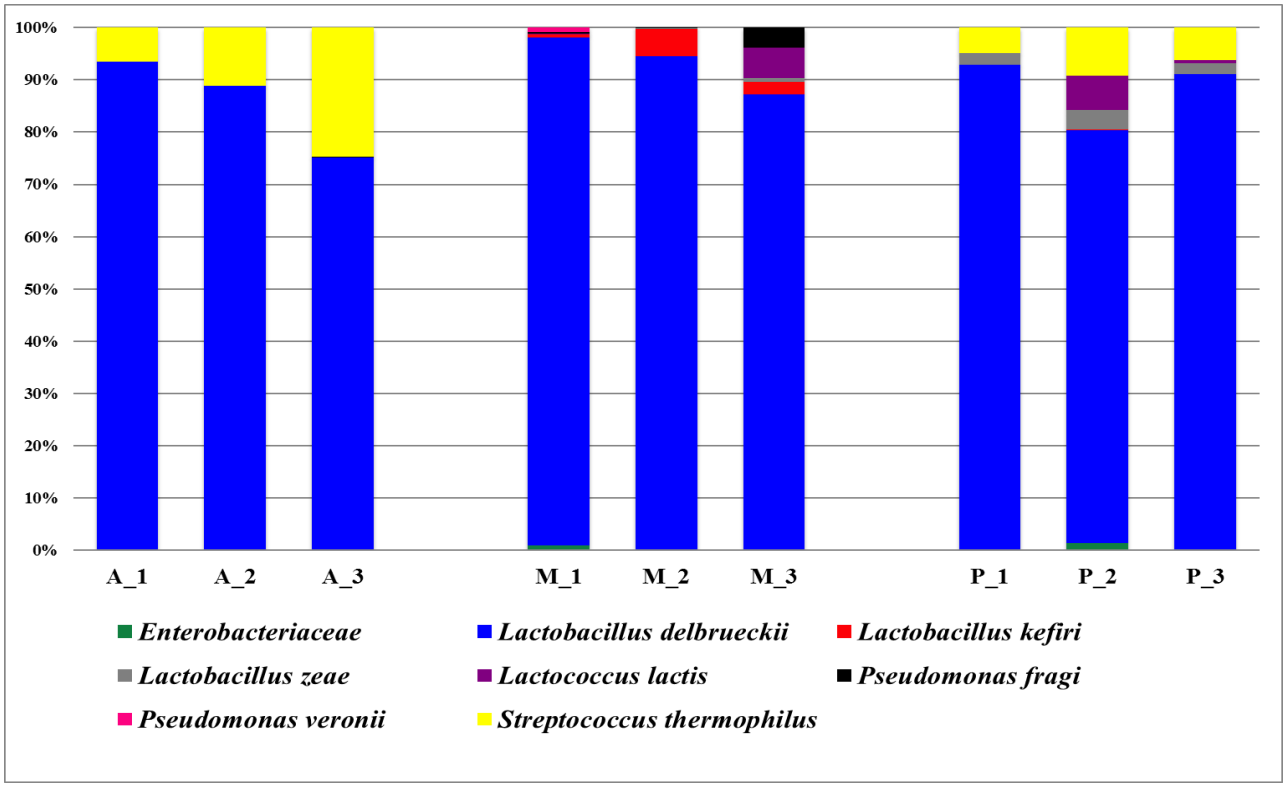

Figure 5 - Relative abundance of bacteria in the three production batches of Gioddu (1, 2, and 3) from the three producers (A, M, and P) revealed by sequencing with Illumina platform technology.
Only OTUs which showed an incidence above 0.2% in at least 2 samples are shown.

A simple microbiota composition was observed (Figure 5) with the neat dominance of *Lactobacillus delbrueckii* (occurrence higher than 85% in all the samples with the exception of batch 3 from manufacturer A), followed by – as expected - *Streptococcus thermophilus*, which was exclusively detected in Gioddu samples from producers A and P, (14% of the relative abundance in Gioddu from producer A, and 7% Gioddu from producer P). Regarding the minority fraction of the microbial populations investigated, two further lactic acid bacteria species were detected in Gioddu from producers M and P, namely *Lactobacillus kefiri* and *Lactococcus lactis* A contaminant species,

Pseudomonas fragi, was also detected in two batches (1 and 3) from producer M, though with very low, relative abundance.

The dominance of *Lactobacillus delbrueckii* in Gioddu has previously been reported by Arizza et al. (1983); however, in such a study, this species was found to dominate, whereas in the present investigation it was found as a minority component of Gioddu lactic acid bacteria population.

To the author's knowledge, this is the first report of *Lactobacillus kefiri*, *Lactococcus lactis*, and *Pseudomonas fragi* in Gioddu.

According to Arizza et al. (1983), the microbial diversity of Gioddu mainly depends on the peculiar production technology used and the environmental conditions.

Regarding Illumina sequencing of yeast target regions, the total number of paired sequences obtained from Gioddu samples reached 577662 raw reads. After quality filtering, a total of 509845 reads were used, with an average value of 56649 ± 31359 reads/sample, and a mean sequence length of 366 bp. Alpha diversity index showed a satisfactory coverage for all samples (> 99%) however did not showed different level of complexity based on the producers.

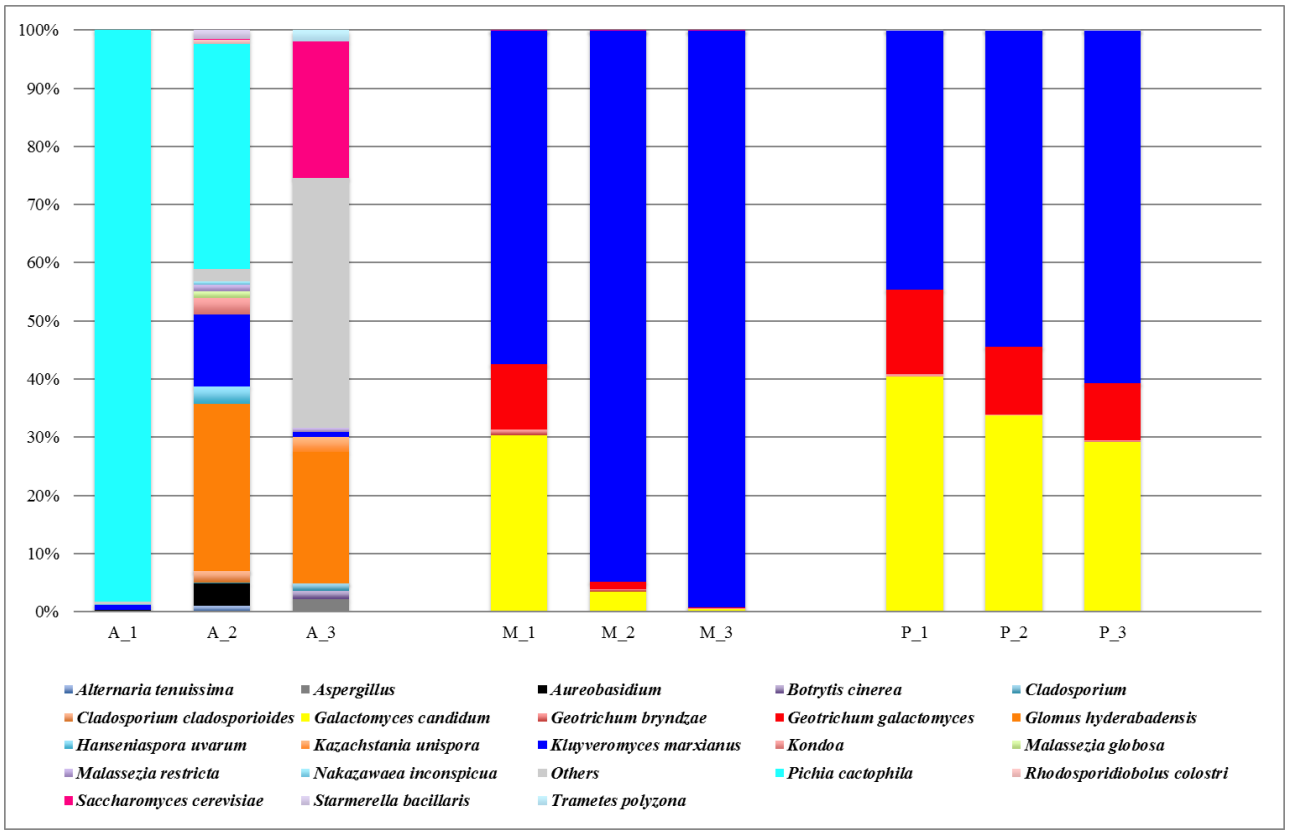

Figure 6 - Relative abundance of yeasts in the three production batches of Gioddu (1, 2, and 3) from the three producers (A, M, and P) revealed by sequencing with Illumina platform technology. Only OTUs which showed an incidence above 0.2% in at least 2 samples are shown.

As shown in Figure 6, samples from producers M and P showed the highest degree of similarity in term of yeast composition, with the predominance of *Kluyveromyces marxianus* (83% of relative abundance in samples from producer M, and 52% in samples from producer P), followed by *Galactomyces candidum* and *Geotrichum galactomyces*. Samples from producer A showed a highest presence of *Pichia cactophila* (45%), *Glomus hyderabadensis* (16%) and *Saccharomyces cerevisiae* (7 %). Minor taxa belonging to *Alternaria*, *Cladosporium* and *Aerobasidium* were also found.

Though numerous studies, focussed on yeast diversity of kefir grains, revealed the presence of *Kluyveromyces marxianus* (Simova et al., 2002; Angulo et al., 1993; Wyder et al., 1997; Wen et al., 1999), a quite different composition of the yeast community emerged in Gioddu, when compared to kefir. Indeed, Simova et al. (2002) reported the predominance of *Saccharomyces cerevisiae, Candida inconspicua*, and *Candida maris*, whereas Gioddu analysed in the present investigation revealed a marginal occurrence of *S. cerevisiae*.

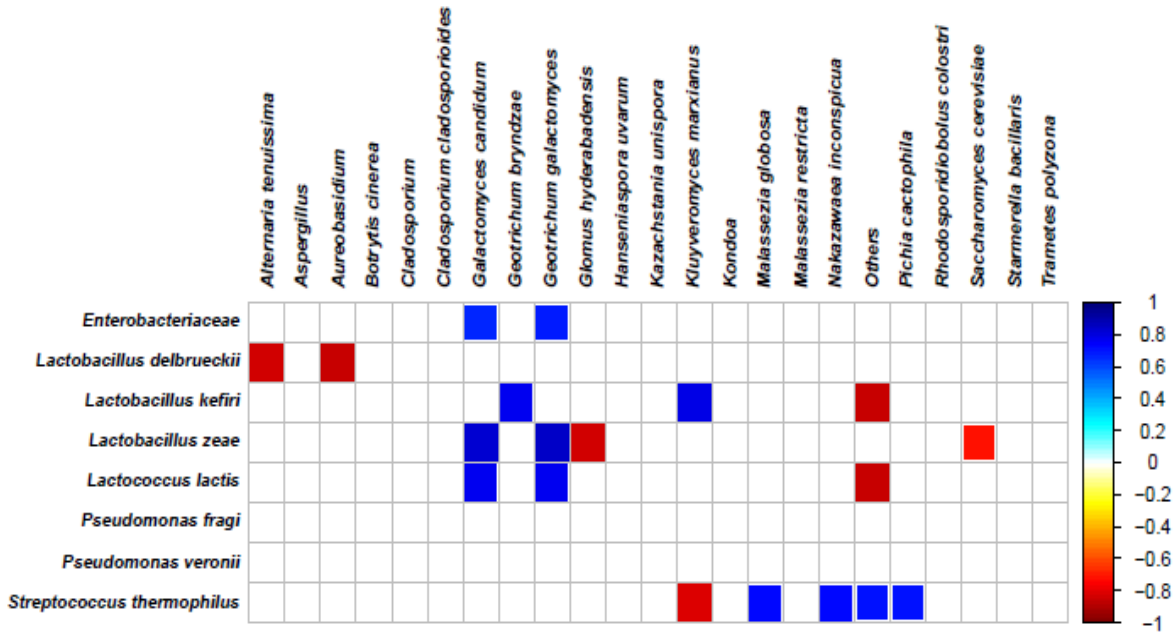

Figure 7 - Correlation plot showing Spearman's correlation between bacteria and yeasts composition. Only significance associations are shown (FDR< 0.05). The intensity of the colours represents the degree of correlation, where blue colour represents a positive degree of correlation and red colour a negative degree of correlation.

By plotting the correlation between bacteria and yeasts (Figure 7, FDR<0.05), it was observed that the presence of *L. delbrueckii* seems to exclude the occurence of *Alternaria tenuissima* and *Aerobasidium*, while the presence of minority species (*L. lactis* and *L. zeae*) might be associated with the occurence of *Galactomyces candidum* and *Geotrichum galactomyces* (Figure 7).

4. Conclusions

In this study, nine Gioddu samples from three different producers located in Sardinia were analysed through a polyphasic approach, based on culture-dependent and independent analyses. From the results obtained, interesting considerations are emerged.

The culture-dependent approach, based on the cultivation of lactic acid bacteria, revealed no numerical differences among the samples, but compared with other fermented milk-based products, namely yogurt and kefir, it was observed that the load of this microbial group was much lower in Gioddu. From sequencing of the amplified DNA regions, a notable microbial diversity was found between different manufactures.

In particular, Illumina sequencing showed the predominance – among bacteria - of *Lactobacillus delbruekii*, which undoubtedly represents a typical homofermentative species associated to the manufacturing of yogurt and fermented milks; by contrast, a low occurrence of *Streptococcus thermophilus* was seen. This latter finding was expected, since it is known that during fermentation of yogurt and yogurt-like products, a bacterial succession occurs, with streptococci being progressively replaced by lactobacilli.

Regarding yeasts, a quite high species diversity was seen in the three producers considered, with *Kluyveromyces marxianus* and *Pichia cactophila* dominating in two and one out of the three producers, respectively.

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