

*Full Length Research Paper*

# Microbiological changes in Lighvan cheese throughout its manufacture and ripening

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The aim of this research was to monitor the microbiological changes throughout the manufacture and ripening of Lighvan cheese. In summer 2009, ten independent batches of cheese were manufactured by experienced workers without any intervention by the researcher. Duplicate samples of raw milk, coagulum and 0, 15, 30, 60 and 90 days old cheese were analyzed. The main groups, such as mesophilic *Lactococci*, thermophilic *Lactococci*, mesophilic *Lactobacilli*, thermophilic *Lactobacilli*, *Enterococci*, and the total aerobic count reached its highest levels during the first 15 days and then declined approximately 2 to 3 log units until the end of ripening. The number of coliforms, *Micrococci*, and *Staphylococcus* increased at early phases of manufacturing and at the end of the manufacturing and ripening phase they showed a falling trend. The number of yeasts throughout this phase decreased at the rate of 3 log units. Generally, we may claim that in ripening Lighvan cheese a large number of different microbes were involved and its hygienic matters call for more attention.

**Key words:** Lighvan cheese, microbiological changes, manufacturing, ripening.

## INTRODUCTION

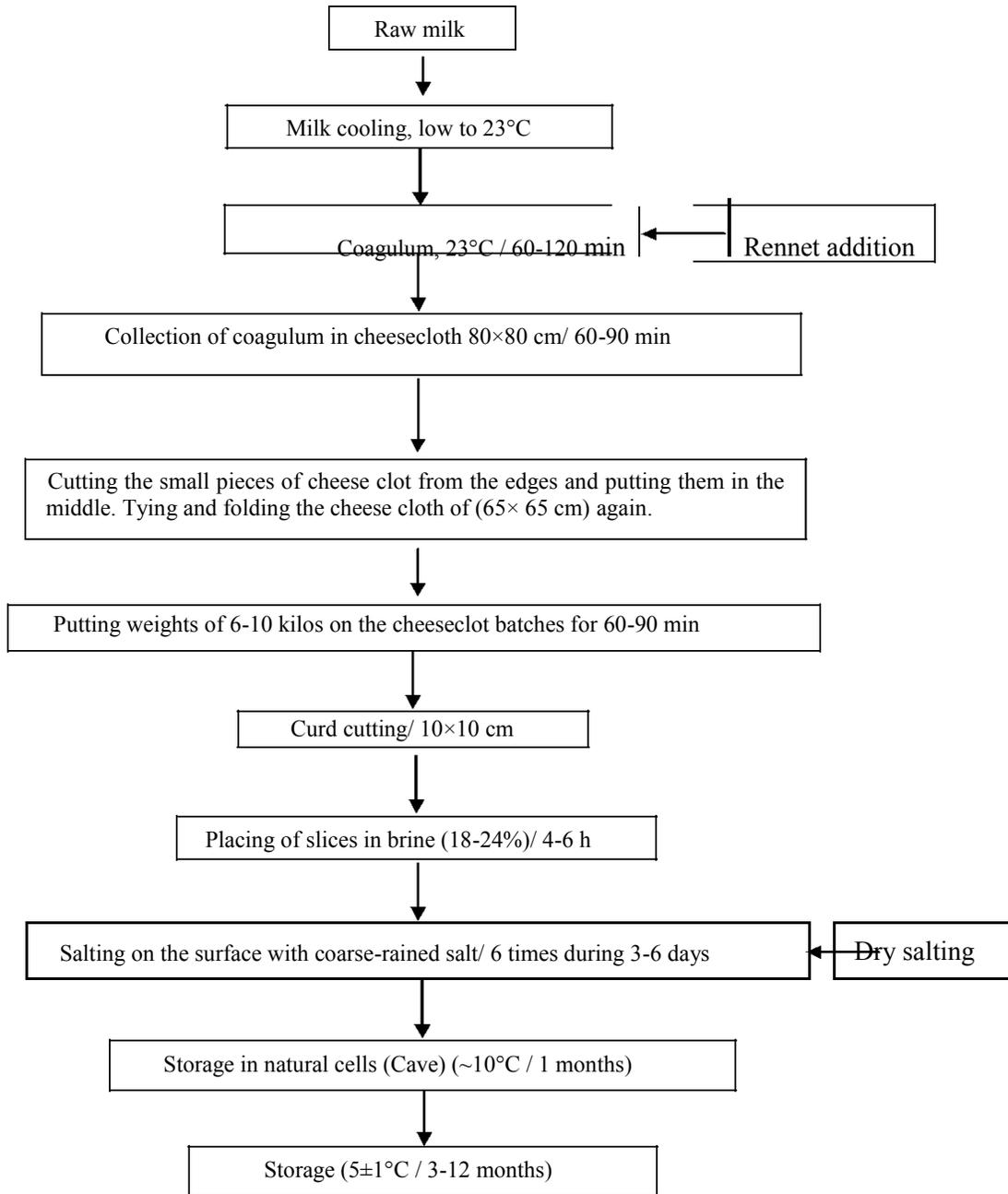
In spite of advances and developments in industrial equipments and techniques, and the rapid growth in the production and variety of industrial cheese in Iran, several traditional cheese types are produced and consumed in different regions of the country; Lighvan cheese is one of the most significant among them. Because of its pleasant organoleptic properties, this type of cheese is popular and widely consumed all over Iran and is enjoying high economical and nutritional value (Mirzaei et al., 2008).

Lighvan cheese is a semihard, white cheese ripened in brine, manufactured from sheep's milk, or a mixture of sheep's and goat's milk in which the proportion of the latter is not higher than 20 to 30%. Since no starter culture is added, the native microflora plays an important role during cheese ripening (Tavaria et al., 2006). Methods and tools of cheese making are rigorously artisanal and traditional; all the phases of manufacturing are manual.

Lighvan cheese is made at Lighvan village, located in the south east of Tabriz, the north west of Iran. It has a mild acidic salty taste, and a pleasant flavour and smell. Approximately 70,000 sheep is raised in the region; each sheep gives milk amounting to one litre, at a daily basis.

From 3.5 to 4 L of milk, 1 kg of cheese is obtained. At this village, from mid-winter to mid-summer (during almost 180 days of lactation), nearly 3150 tons of Lighvan cheese is produced. Today, Food and Drug Office, encourages the cheese manufactures of the village to use pasteurized milk to make cheese. However, most traditional cheese producers believe that using raw milk produces pleasant taste and flavour in their cheese; this is due to the activities of proteolytic and lipolytic enzymes of microflora in raw milk (Little et al., 2008).

Cheese is currently considered as one of the safest foods consumed; however, pathogenic bacteria that can be transmitted through the dairy products, including cheese, are important from public health point of view. Historically there have been outbreaks of infection associated with the consumption of cheese, and the predominant organisms responsible for the problem. These outbreaks included *Salmonella*, *Listeria monocytogenes*, verocytotoxin producing *Escherichia coli* (VTEC), and *Staphylococcus aureus* (Razavilar, 2002; Karim, 2006; Tamagnini et al., 2005). Detailed investigations have demonstrated that the sources of contamination were raw milk, inadequately pasteurized milk, or post-pasteurization contamination with organisms originally



**Figure 1.** Flow diagram of Lighvan cheese production.

derived from raw milk or from manufacturing environment (Tamagnini et al., 2006).

The native microflora existing in raw milk and their number changes throughout the manufacture and ripening plays an important role in controlling pathogenic microbes and also creation of organoleptic characteristics in traditional cheeses manufactured from raw milk. In spite of Lighvan cheese popularity; there have been very few studies on the characteristics of this famous cheese. The microbiological and chemical quality of traditional cheese presented in Tabriz market, have been examined

in a cross-sectional study (Mirzaei et al., 2008); however, no research has ever been performed on microbiological changes throughout its manufacture and ripening. Therefore, the purpose of the present study was to monitor microbiological changes throughout the manufacture and ripening of Lighvan cheese.

#### **MATERIALS AND METHODS**

Figure 1 shows the steps in manufacturing Lighvan cheese. In summer 2009, ten independent batches of Lighvan cheese in five

small cheesemaking factories in Lighvan village were made. The work in each factory was done in its routine way by experienced Workers, without any involvement on the part of the researcher. In each trial, nearly 50 L of raw milk was filtered and its temperature was adjusted to 23°C by putting the milk container in cold water. The milk was later poured into a stainless steel milk vat. 5 to 10 ml of commercial calf rennet (1:10.000 strength) for every 100 L of milk was added to the milk and homogenized. The coagulum after a lapse of 60 to 120 min was usually put into a cheesecloth of 80 × 80 cm, the cloth corners were turned inwards and the cheese inside was squeezed in a way that in 60 to 90 min it would lose most of its whey.

Later a smoothly-cut flat piece of plank weighing 6 to 12 kilos was put over the cheesecloth for 60 to 90 min so that the maximum amount of whey would ooze out. Then, the resulting cheese-clot was cut into pieces of 10 × 10 cm, and after a time lapse of 10 min was immersed in brine (18 to 24%) for 4 to 6 h. Later, the pieces were transferred into special containers for brining, and in a period of 3 to 6 days, they were dry-salted twice a day to reach the needed firmness. Finally the resulting cheese pieces were put in tin containers holding 17 kilos of cheese, and the tins were filled with brine (11%) and their lids were caulked. The cheese-filled tins were kept in caves with temperature of nearly 15°C for a month; the tins were later preserved in refrigerator with a temperature of  $5 \pm 1^\circ\text{C}$  for a period of 2 months. Usually, before being distributed to the consumption market, the cheese is transferred to small tins.

### Sampling

Duplicate samples (1000 ml or g each) of raw milk, coagulum and 1, 15, 30, 60 and 90 day old cheese were taken from each bath. Samples were then transferred to the laboratory under refrigerated conditions (4°C) and analyzed immediately.

### Microbiological analysis

For this purpose, 10 ml/g of each milk, coagulum or cheese samples were homogenized for 2 min with 90 ml of sterilized sodium citrate (2%, w/v; Merck, Darmstadt, Germany), in a stomacher, Lab-Blender (PBI International, Milan, Italy). Decimal dilutions were prepared in sterilized 0.1% (w/v) peptone water (Sigma Chemical, St. Louis, MO, USA), and each dilution was plated in duplicate on the specific media required for the different microbial groups to be examined. The following analyses were completed on different samples.

Aerobic mesophilic flora (TBC) on plate count agar (PCA) medium (Difco, Detroit, Michigan, USA) at 30°C for 72 h (Salmeron et al., 2002). Coliforms on violet red bile agar (VRBA), after incubation at  $31 \pm 1^\circ\text{C}$  for  $24 \pm 2$  h; for the determination of *E. coli*, 5 typical coliform colonies were inoculated on lactose broth in sterile tubes with Durham's tubes following incubating at 44.5°C for 24 to 48h, the cultures monitored for gas (CO<sub>2</sub>) formation within Durham's tubes and also analyzed for IMViC tests. The gas formation and indole positive, methyl-red positive, Voges-Proskauers negative, and citrate negative isolates after 24 to 48h incubation at 37°C were assessed as positive (Cetinkaya and Soyutemiz, 2006). Yeast on Potato Dextrose Agar (Oxoid, CM 139) acidified with 10ml/l of 10% tartaric acid (Merck, Darmstadt, Germany) incubated at 25°C for 3 to 7 days (ISIRI 997, 1994); mesophilic and thermophilic *Lactobacilli* respectively at 22°C for 96 h and at 45°C for 48 h, on deMan Rogosa Sharpe (MRS) agar (Oxoid) acidified to pH 5.4 with acetic acid and incubated anaerobically (gas pack plus anaerobic system, BBL, Becton Dickinson, Sparks, MD) (Caridi et al., 2003). Mesophilic and thermophilic *Lactococci* on M17 agar (with 10% w/v lactose) at 30

and 45°C for 48 h, (Marino et al., 2003). *S. aureus* were enumerated on Baird-Parker agar supplemented with egg yolk and potassium tellurite at 37°C for 48 h (Tamagnini et al., 2005). Colonies were examined by Gram stain, catalase test, anaerobic utilization of glucose and manitol, coagulase test (Miriam and Rosalia, 2004). *Mirococci* on manitol salt agar (oxoid, CM85) supplemented with cyclohexamide (100 g/ml; Sigma-Aldrich, C7698) at 30°C for 72 h (Manolopoulou et al., 2003). *Enterococci* were determined, according to published procedures (NMKL, 1992), at 45°C for 48 h on Slanetz-Bartley agar (Biokar Diagnostics, Ref. BK037HA). For the presence of *Salmonella*, a sample of 25 g was homogenized with 225 ml of lactose broth and was incubated at 37°C for 24 h; 1 ml of pre-enriched broth was subcultured in 10 ml of tetrathionate broth (TT) and another 1 ml in 10 ml selenite cystine broth and incubated at 37 and 43 °C respectively for 18 to 24 h. Subcultures were streaked on to Brilliant green phenol red agar and Hecktoen agar (Difco) and incubated at 37°C for 40 to 48 h (ISIRI 1810, 2002).

## RESULTS AND DISCUSSION

The results obtained from the microbiological analysis of the samples are shown in Table 1.

The number of coliforms in milk equals  $5.27 \pm 0.42$  log cfu ml<sup>-1</sup> and after 12 h, their amount in the resulting cheese clots, before being put in brine, increased to  $6.30 \pm 0.33$  log cfu g<sup>-1</sup> (Table 1). Then their number throughout manufacture and ripening declined, so after 90 days of ripening, their number reduced to log cfu g<sup>-1</sup>  $1.66 \pm 0.41$ . The high number of hygiene indicator microorganisms like coliforms in milk, the clot, and even the cheese throughout the ripening period can be attributed to the milk contamination in milking time and cheese manufacture, as well as to the improper cleaning of milk carrying containers. Research findings show that the number of coliforms in raw sheep milk in two artisanal Manchego cheese factories were  $2.63 \pm 0.00$  and log cfu g<sup>-1</sup>  $5.12 \pm 0.12$ , respectively; In the ripening period their amount declined, in a way that after 60 days of ripening period, the amount came to  $1.8 \pm 0.24$  and  $2.03 \pm 0.15$  log cfu g<sup>-1</sup> respectively, and after 120 and 180 days the amount was reduced to zero (Manolopoulou et al., 2003). In another research, it was reported that the number of coliforms and *E. coli* in 120 days of ripening of Feta cheese from raw sheep milk decreased from 2 log to zero (Sarantinopoulos et al., 2002). This can be explained by the development of inhibitory conditions, such as low pH, lack of oxygen, depletion of sugars by lactic acid bacteria and secondary microflora activity (Manolopoulou et al., 2003).

The average number of yeasts in raw milk equals to  $4.07 \pm 0.18$  log cfu ml<sup>-1</sup>, and their number in manufacturing and ripening period has a decreasing trend. Finally, after 90 days of ripening, the number came to  $0.88 \pm 0.34$  log cfu ml<sup>-1</sup>.

Relatively high counts of yeasts are frequently observed in many different types of cheese, especially in raw milk cheeses (Manolopoulou et al., 2003). The occurrence in cheese of some species of yeasts with high

**Table 1.** Log counts (mean  $\pm$  SE) of microbial groups in milk and during manufacture and ripening of Lighvan cheese.

Microbial groups	Raw milk	Manufacturing		Ripening (days)			
		Curd	Cheese before packaging	15	30	60	90
Coliforms	5.27 $\pm$ 0.42	6.30 $\pm$ 0.33	5.84 $\pm$ 0.35	4.69 $\pm$ 0.25	4.09 $\pm$ 0.38	2.43 $\pm$ 0.40	1.66 $\pm$ 0.49
TBC	7.01 $\pm$ 0.32	7.34 $\pm$ 0.56	7.53 $\pm$ 0.35	7.84 $\pm$ 0.62	6.65 $\pm$ 0.56	5.94 $\pm$ 0.75	5.84 $\pm$ 0.27
<i>Enterococci</i>	4.36 $\pm$ 0.49	4.72 $\pm$ 0.39	5.25 $\pm$ 0.54	6.12 $\pm$ 0.67	5.88 $\pm$ 0.76	4.55 $\pm$ 0.59	3.34 $\pm$ 0.45
<i>Micrococci</i>	3.66 $\pm$ 0.49	5.24 $\pm$ 0.31	5.48 $\pm$ 0.38	2.12 $\pm$ 0.29	0.84 $\pm$ 0.38	0.69 $\pm$ 0.12	0.34 $\pm$ 0.08
<i>Staphylococcus aureus</i>	5.03 $\pm$ 0.37	5.68 $\pm$ 0.15	5.45 $\pm$ 0.24	4.84 $\pm$ 0.21	3.75 $\pm$ 0.32	3.73 $\pm$ 0.22	2.66 $\pm$ 0.49
Mesophilic <i>Lactobacilli</i>	5.43 $\pm$ 0.40	6.75 $\pm$ 0.39	6.28 $\pm$ 0.34	7.88 $\pm$ 0.20	7.52 $\pm$ 0.32	6.45 $\pm$ 0.16	4.70 $\pm$ 0.50
Thermophilic <i>Lactobacilli</i>	4.63 $\pm$ 0.40	4.77 $\pm$ 0.27	5.16 $\pm$ 0.39	6.08 $\pm$ 0.28	2.75 $\pm$ 0.82	1.90 $\pm$ 0.77	0.65 $\pm$ 0.34
Mesophilic <i>Lactococci</i>	6.61 $\pm$ 0.24	7.26 $\pm$ 0.26	7.51 $\pm$ 0.31	7.71 $\pm$ 0.20	6.65 $\pm$ 0.12	6.21 $\pm$ 0.59	5.74 $\pm$ 0.15
Thermophilic <i>Lactococci</i>	5.96 $\pm$ 0.46	6.37 $\pm$ 0.33	6.16 $\pm$ 0.45	6.15 $\pm$ 0.27	6.14 $\pm$ 0.12	5.61 $\pm$ 0.19	4.84 $\pm$ 0.53
Feast	4.07 $\pm$ 0.18	3.79 $\pm$ 0.18	3.95 $\pm$ 0.15	3.83 $\pm$ 0.21	2.51 $\pm$ 0.41	2.12 $\pm$ 0.40	0.88 $\pm$ 0.34

counts is attributable to their tolerance towards low pH, reduced water activity and high salt concentrations, as well as to their ability to grow at low storage temperature environmental condition is recommended (Ferreira and Viljoen, 2003). Other important features able to explain their occurrence in cheese are the assimilation/fermentation of lactose and galactose, the assimilation of succinic, lactic and citric acids. In addition, they are widely distributed in the dairy environments and appear as natural contaminants in raw milk, air, dairy utensils, brine and smear water. Also the ability of dairy yeast strains to survive heat treatments and sanitizing agents has been reported (Gardinia et al., 2006). The recovery of yeasts in high numbers and their relatively strong proteolytic and lipolytic activities suggest that cheese organoleptical characteristics might be influenced by yeasts (Pereira-Dias et al., 2000). On the other hand, yeasts may also act as spoilage organisms causing typical defects like production of fruity, bitter or yeasty off-flavours and the appearance of a gassy, open texture, increased acidity due to stimulant effects on LAB

and brown surface discoloration (Pintadoa et al., 2008; Gardinia et al., 2006; Pereira-Dias et al., 2000). In addition, information is available regarding the contribution of yeasts to synthesis of biogenic amines in foods (Pintadoa et al., 2008; Gardinia et al., 2006).

The results of the present study indicate that the number of *enterococci* throughout the manufacturing phase has a rising trend, and throughout the ripening period has a falling trend. In the cheese of 90 days of ripening, it has a high level (Table 1). These high numbers are in accordance with counts of *Enterococci* found in other cheese varieties, manufactured from raw or pasteurized goats', ewes', water buffalo or cows' milk (Manolopoulou et al., 2003; Kagkli et al., 2007; Morandia et al., 2006). *Enterococci* are normal constituents of the intestinal flora of humans (Morandia et al., 2006) and nearly all animals, and are widely distributed in the environment. For this reason, *Enterococci* can be used as indicators of faecal contamination (Franza et al., 1999). *Enterococci* may enter the milk either directly from the faecal matter of humans or animals or

indirectly from contaminated water sources, from the surface of the animals, from milking equipment, and from bulk milk holding tanks (Ortigosa et al., 2008). The presence of a high number of *Enterococci* is because of poor hygiene during the manufacturing process and the resistance of *Enterococci* to high temperatures, freezing, drying and concentrations of salts or acids (Caridi et al., 2003; Buja et al., 2001). *Enterococci* have important implications in the dairy industry. They occur as nonstarter lactic acid bacteria (NSLAB) in a variety of cheeses. They play an acknowledged role in the development of sensory characteristics during ripening of many cheeses and have been also used as components of cheese starter cultures. The positive influence of *Enterococci* on cheese seems due to specific biochemical traits such as lipolytic activity, proteolytic and esterolytic activities (Morandia et al., 2006), ability to stimulate acid production by some *Lactococci* and gas production by *Leuconostoc* spp. (Manolopoulou et al., 2003), citrate utilisation, and production of aromatic volatile compounds (Giraffa, 2003).

The amount of Mesophilic *Lactobacilli* and

Thermophilic *Lactobacilli* throughout the manufacturing phases as well as the first 15 days of ripening had a rising trend, in average their number increased from the original amounts of  $5.43 \pm 0.4$   $4.61 \pm 0.39$  log cfu ml<sup>-1</sup> in milk respectively to  $7.88 \pm 0.19$  and  $6.08 \pm 0.28$  log cfug<sup>-1</sup> in 15 days cheese. In 90 days of ripening, the amount declined to  $6.08 \pm 0.28$  and  $4.7 \pm 0.51$  log cfug<sup>-1</sup> respectively. Mesophilic *Lactobacilli* constitute the majority of the non-starter lactic acid bacteria (NSLAB) present in most types of cheese (Mannu et al., 2000). NSLAB, predominantly mesophilic *Lactobacilli*, usually dominate the microflora of Cheddar-type cheese during much of its ripening, thus contributing to final cheese aroma. They may have entered adventitiously from the milk and the immediate surroundings during cheese manufacture. It is well known that mesophilic *Lactobacilli* play an important role during the ripening of cheeses (Mannu et al., 2000; Bouton et al., 2009). In fact, NSLAB possess a wide range of proteolytic and lipolytic enzymes (Kongo et al., 2009; Caridia et al., 2003) and increases the concentration of short peptides, free amino acids and free fatty acids (Kongo et al., 2009). The slower metabolism of *Lactobacilli* and their capacity to adapt to adverse conditions (acidity, low values of aw and higher concentrations of NaCl than for the rest of lactic acid bacteria), could contribute to their predominance in the last stage of ripening (Arenas et al., 2004). The lowest viable counts (but still above  $10^8$  cfu g<sup>-1</sup>) of both the *Lactococcus* and *Lactobacillus* genera in cheeses, may be related to the particularly high salt content and the concomitantly lower aw; both these conditions are unfavourable towards their growth. In fact, negative correlations between the *Lactobacillus* and the *Lactococcus* counts, and the NaCl content were observed (Pintadoa et al., 2008).

The number of mesophilic *Lactococcus* throughout the manufacturing period and till the 30th day of ripening had a rising trend; their number in 30th day of ripening rose from  $6.66 \pm 0.24$  log cfu ml<sup>-1</sup> in milk to  $7.65 \pm 0.09$  Log cfu mg<sup>-1</sup> in cheese, later it decreased to  $5.74 \pm 0.15$  log cfu mg<sup>-1</sup> in 90th day of ripening. The number of Thermophilic *Lactococcus* originally in the first phase of manufacturing (the change of milk into clot) increased, and their number went up from the primary value of  $5.96 \pm 0.46$  to  $6.37 \pm 0.33$  log cfu mg<sup>-1</sup>. Then throughout the manufacturing and ripening period, it had a falling trend, in a way that their amount until the 90th day of ripening decreased to  $4.86 \pm 0.53$ . *Lactococci* are an important group of lactic acid bacteria (LAB) and are used worldwide for the manufacture of fermented dairy products, e.g. cheese (Psoni et al., 2007). Our results agree with results of Caridi et al. (2003), in the Caprino d'Aspromonte cheese produced from raw goat's milk. In natural dairy microbial populations, only a few strains with good acidifying activity are probably present (Mannu et al., 2000). The wild strains present in the artisanal cheese during ripening may contribute to developing typical flavour in the traditional cheese through enzyme complexes (Psoni

et al., 2007). In milk culture, and under cheese-like conditions, wild strains of *Lactococcus lactis* are able to produce Favours different from those produced by industrial strains. The major volatile compounds were methylalcohols and methylaldehydes, which are most likely derived from branched-chain amino acids (Morales et al., 2003; Ayad et al., 1999). Ayad et al. (1999) resulted that wild strains isolated from artisanal cheese are prototrophic for most amino acids whereas industrial strains are auxotrophic for 9 to 10 amino acids. Since wild strains are more dependent on their own synthesis of amino acids, these strains most likely harbour more amino acid convertases. Therefore, these strains have more active amino acid convertases which matches with their ability to produce unusual favours (Ayad et al., 1999).

Raw milk used in cheesemaking had total counts of  $7.01 \pm 0.32$  log cfuml<sup>-1</sup> (Table 1). Similar counts have been observed in ewe's milk by Salmeron et al. (2002). The average level of total mesophilic bacteria in the first day of ripening was  $7.53 \pm 0.35$  log cfu mg<sup>-1</sup> and later until the 90th day of ripening, the number decreased to  $5.84 \pm 0.27$  log cfu mg<sup>-1</sup>. Our results for total viable microorganisms are consistent with those reported by previous research groups, who worked with other artisanal cheeses manufactured from ewes' raw milk (Pintadoa et al., 2008; Albenzio et al., 2001).

According to the findings of this research, the average level of *Staphylococcus aureus* in raw milk and throughout the manufacturing process was over 5 log cfuml<sup>-1</sup>, and throughout the ripening period, it had a falling trend. At the end of 90 days of ripening, the amount declined to  $2.66$  log cfug<sup>-1</sup>. With regard to the process of change throughout the manufacturing and ripening period, the findings of this research confirm the findings of Psoni et al. (2007) in the production of a traditional cheese named Batzos, a traditional Greek cheese from raw goat's milk. Nevertheless, according to Estepar et al. (1999) in the traditional cheese, named as Penamellera, the number of *Staphylococci* increased from 4.7 logcfu/ml in raw milk to more than 7 in the cheese after 30 days of ripening period.

The number of *Micrococci* throughout manufacture rose from  $3.6$  log cfuml<sup>-1</sup> in raw milk to nearly  $5.5$  log cfug<sup>-1</sup>, and throughout the ripening period, it declined gradually with time, reaching zero. Sarantinopoulos et al. (2002) reported that the number of *Micrococci* in Greek Feta cheese from pasteurized sheep milk, in the first 4 days of ripening reached its maximum amount of 2 to 3 log cfu/g, and until the 60th day of ripening it fell down to 2.0 log CFU/ml. Manolopouloulou et al. (2003) reported that the number of *Micrococci* in traditional Feta cheese, throughout 120 days of ripening period, decreased from the original 4 log CFU/ml to less than 3 log CFU/ml. The present research nearly confirms their findings.

*Micrococci* are considered major components of the microflora of raw milk cheeses, occurring also in

significant numbers in cheese from pasteurized milk (Sarantinopoulos et al., 2002; Manolopoulou et al., 2003) and survive throughout ripening, probably due to their resistance to salt and dehydration. It has been suggested that the proteolytic and lipolytic activities of *Micrococci* play an important role during the ripening process (Manolopoulou et al., 2003).

Generally, we may claim that in ripening Lighvan cheese a large number of different microbes were involved and its hygienic matters call for more attention.

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