Survival of B. lactis During Ripening of Probiotic Lighvan Cheese

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ABSTRACT

The aim of this research was to determine the survival of B. lactis and the effect of acidity development and salt concentration in Lighvan cheese on the viability of B. lactis. This type of cheese is produced from ewe milk or the mix of ewe and goat. The effect of time on survival of B. lactis during the ripening of probiotic Lighvan cheese was significant (p<0.05). B. lactis cells survived in cheese samples at concentrations up to 6.84 log10 cfu/g for at least 60 days of storage time at 4 °C. High concentration of salt and acid production from fermentation decreased viability of B. lactis during ripening of Lighvan cheese.

INTRODUCTION

Survival in fermented milk products during long-term storage is a complex process because it is influenced by other stress factor including oxygen, light, nutrient exhaustion and metabolites other than organic acids produced during fermentation [1]. The success of the incorporation of bifid bacteria into cheeses is dependent on the bifid bacteria strain, the activity of lactic acid bacteria used in the manufacture of the cheese, the composition of the cheese, and the conditions of processing and ripening. Although numerous studies report great losses in viability of probiotic strains during the storage of yoghurt-like products, the data on cheese shows that bifid bacteria incorporated can be stable during storage thus, the pH value seems to be a critical factor in the stability of probiotic strains during storage. There are significant differences between species and strain with respect to the bacterial survival in an acid environment [2].

The cheeses having higher pH (between 4.8-5.6) than fermented milks such as yoghurt milk with a lower pH range (3.7-4.3) can provide a more stable medium to support the long term survival of probiotics. Moreover, the cheese matrix and the high contents of fat and proteins especially in cheese produced from sheep milk having higher amount of both components. Cheeses can protect them during processing, ripening and digestion [3, 4]. In addition, an anaerobic environment will be developed due to the metabolism of lactic flora within a few weeks of ripening, favoring the survival of bifidobacteria [5]. Bifidobacteria have been used as probiotic culture to produce Cottage cheese, Crescenza, Cheddar, Fresco, fresh or white-brined cheeses. Lighvan is a semi hard cheese with a large market demand. It is mostly produced from ewe's or goat's milk, or a mixture of the two. Traditional Lighvan cheese which ripened in Brine is a major component especially in the diet of consumers in north-west of the country. Some efforts have been done to produce Lighvan cheese plant using pasteurized milk. The ripening period of this type of product is about 90 days but the cheeses made from raw milks in small, rural production units may be ripened for six to eight months. Incorporating bifidobacteria in cheese through the cheese milk is not difficult, as cheese offers the necessary anaerobic conditions and a suitable pH. The growth and acid production of bifidobacteria in milk under conditions typical of cheese-making were studied, and evaluated the survival of the selected strains during storage in the presence of starters used for cheese-making [6].

OBJECTIVE:

In this work, we tried to produce traditional cheese supplemented with B. lactis and study both the survival and factors influencing survival of probiotic bacteria of probiotic traditional cheese during ripening period.

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MATERIAL AND METHODS

Bifidobacterial Cultures:
A lyophilised culture of B. lactis was supplied by CAMINOX, Spain. The manufacturer claims in its technical literature that this strain has probiotic effects. B. lactis was cultured in MRS broth (Merck, Germany), under anaerobiosis at 37 °C for 24 hours. Cells as seed cultures were harvested by centrifugation (SANYO, MISTRAL, Germany) at 10,000 × g for 10 minutes, washed twice with sterile skim milk, and resuspended in cheese-milk at a concentration of $9.0 \log_{10} \text{cfu/ml}$ for B. lactis. The different steps of cheese manufacture are summarized in the Fig. 1.

* The concentration of B. lactis in the cheese milk was always ca. $9.0 \log_{10} \text{cfu/ml}$

Fig. 1: The production steps of traditional, probiotic Lighvan cheese

Cheese Manufacture:
Ewe's milk was supplied from an animal husbandry in Varamin region from the Zandy breed. Experimental cheese samples were made in three replications at the Tehran Pegah dairy plant (Tehran, Iran). Lighvan cheese was produced using raw milk according to the above protocol. The raw milk was coagulated with fungal rennet (Rennet force was 1/100000) for less than 60 minutes; coagulated milk, the curd was cut into small cubes, approximately 1 cm³, and left to rest for 15 minutes after the separation of whey, the curds were collected and put under pressure. The applied pressure was one kg according the final produced curd. Then pieces of curds were cutting into $10 \times 10 \times 7$ cm dimensions. these pieces were immersed into brine with 22% concentration for 6 hours at room temperature then placed into tin-plate containers with brine at about 12% concentration. The containers were sealed and stored at 4°C for 60 days.

Microbiological Analysis:
Ten grams of cheese was first diluted in 90 ml of 2% sodium citrate solution and homogenised in a Stomacher Lab-Blender 400(Seward, England) for 1 minute. Subsequent serial dilutions were made in Ringer's solution and plated on specific media for viable counts. mMRS agar (Merck, Germany) was modified with L-cysteine.HCl (0.05%) for reducing the ox-repotential and 60 mg of lithium mupirocin(Sigma-Aldrich, USA) for its inhibitory effect [7]. Cultivation was carried out using the pour-plate technique, and the plates were incubated, under anaerobiosis, for 72 hours at 37 °C for B. lactis. The B. lactis in the cheese samples was enumerated after 5, 25, 45 and 60 days. Each experiment was done in triplicate.

Chemical Analysis:
Samples of cheese were analyzed for titratable acidity and also salt AOAC (2000).

Statistical Analysis:
The data were statistically analyzed using a completely randomized design (CRD) with three replications. Data were subjected to analysis of variance using the SAS statistical software package. Mean comparison was performed with LSDs test at the $P<0.05$ level of significance.
RESULTS AND DISCUSSION

Enumeration of Bifidobacterium lactis:

Table 1 shows the number of cells of *B. lactis* during ripening of probiotic *Lighvan* cheese. Different culture media have been reported for the selective enumeration of bifidobacteria in dairy products [8]. The traditional technology was modified slightly to favor the survival of probiotic microorganism. According to statistical analysis, significance differences (*p*<0.05) were observed in the enumeration of *B. lactis* during 60 days of ripening. After 25 days, the cheese contained 8.0 $\log_{10}$ cfu/g of bifidobacterium, and after 60 days of ripening, the survival of *B. lactis* was 6.84 $\log_{10}$ cfu/g.

After comparison of several selective media for isolation and enumeration of *B. lactis*, under the conditions in this study, mMRS agar modified with L-cysteine HCl (0.05%) and lithium mupirocin was the best for cell recovery. Because Mupirocin susceptibility showed that bifidobacteria were consistently resistant to mupirocin, whereas all Lactobacilli were susceptible. *B. lactis* bacteria decreased slightly throughout cheese ripening: a fall of only 2. $\log_{10}$ cfu/g during the 60 days. The minimum concentration of probiotic microorganisms that must be contained in a food product to exert a beneficial effect is unclear. The Fermented Milks and Lactic Acid Bacteria Beverages Association in Japan introduced a standard that stipulates that the minimum concentration of viable bifidobacteria per gram or milliliter of product defined as a probiotic food should be at least 7.0 $\log_{10}$ cells. This concentration should ensure the therapeutic minimum dose of 5.0 $\log_{10}$ viable cells/g or ml of product. Other international food associations and results from several studies have proposed that the concentration should range between 6.0 to 7.0 $\log_{10}$ cfu/g or ml [9]. Intrinsic characteristics of the *Lighvan* cheese matrix (low aw and pH, high concentration of NaCl) could have caused severe cellular stress that reduced cell recovery.

When added individually, *B. lactis* showed a significant (*p*<0.05) decrease during 60 days of ripening. Several factors which can influence the capacity of probiotics to survive in cheese and to remain active must be considered. These factors include (1) physiological state of probiotic cultures (if the cells are in logarithmic or stationary phase of growth), (2) physical conditions of storage of the product (for example, temperature), (3) chemical composition of the product to which probiotics are added (for example, acidity, content of sugars available, nitrogen sources, mineral content, water activity and oxygen content), and (4) possible interactions between probiotic cultures and starters (for example, bacteriocin production, antagonism and synergism) [2]. Similar to our result, Ketney *et al.* [10] reported that Bifidobacteria had a satisfactory viability in the Feta cheese during 60 days of refrigerated storage also Corbo *et al.*, [11] reported that after 56 days of ripening of CanestratoPugliese Hard cheese supplemented with Bifidobacteria, the survival of Bifidobacteria was 6.0 $\log_{10}$ cfu/g. The use of bifidobacteria as a starter adjunct to produce probiotic cheeses was recently applied. Not all the strains exhibited the same stability during ripening and storage of the dairy products [6, 12-16], suggesting that strain survival should be evaluated individually prior to commercial use. Indeed, in cottage cheese, *B. infantis* reached levels of approximately 7.0 $\log_{10}$ cfu/g of cheese after 1 day of storage, but large viability losses were observed after 15 days at 4 °C [12]. Other reports showed that bifidobacteria added in Cheddar [13] or Cheddar-like cheese [17] survived up to 24 weeks at approximately 7.3 $\log_{10}$ cfu/g, or remained above 6.5 $\log_{10}$ cfu/g.

Table 1: Survival of *B. lactis* during ripening periods (days) of *Lighvan* cheese

<table>
<thead>
<tr>
<th>Frequency of sampling days for counting (day)</th>
<th>The number of <em>B. lactis</em> ($10^7$/cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>8.75 ± 0.02</td>
</tr>
<tr>
<td>25</td>
<td>8.09 ± 0.03</td>
</tr>
<tr>
<td>45</td>
<td>7.02 ± 0.01</td>
</tr>
<tr>
<td>60</td>
<td>6.84 ± 0.03</td>
</tr>
</tbody>
</table>

*Means in each column with a superscript differ significantly (*p*<0.05)

The main factors to be considered that may influence the ability of the probiotics to survive in cheese are the chemical composition of the cheese (acidity, salt).

Effect of acidity on survival of *B. lactis*:

Titratable acidity of the samples increased until the 60th day of ripening, while pH values decreased. Effect of time on acidity of probiotic *Lighvan* cheese was significant (*p*<0.05). The acidities (%) of probiotic *Lighvan* cheese was 2.21% at the day 5 of storage, and 2.27% at the day 60 of storage. The cheese acidity at a certain moment of the technological process is determined by the starting level of milk acidity and the lactic acid generated by the presence of the starter culture. The cheese acidity level has great importance, influencing the growth of microorganisms and enzymatic activity throughout the maturation process, as well as affecting rheological properties and flavor [18, 19]. The increase in titratable acidity during the 60 days of ripening in brine was due mainly to the near completion of lactose fermentation and the liberation of amino and free fatty acids following proteolysis and lipolysis. Similar to our results, Azarnia *et al.*, [20] reported that lactose is
converted into lactic acid during cheese-making by the starter culture. Therefore lactic acid is the most abundant organic acid in all types of cheese [21]. Acidity, pH and hydrogen peroxide have been identified to have an effect during manufacture and storage. Other factors, such as temperature of storage, oxygen content, concentrations of lactic acid and acetic acid also have been presumed to affect the viability of bifidobacteria [22, 23]. The main factors for loss of viability of bifidobacteria have been attributed the decrease in the pH of the medium and accumulation of organic acids such as a result of growth and fermentation [23]. Hence, the manufacture of these products containing bifidobacteria requires the selection of strain with low susceptibility to acid [24]. The problem of sensitivity to acidity of bifidobacteria in cheese is increased by the fact that acidity may increase during storage. In cheese, acidification may continue during cold storage, a phenomenon called post-acidification. Bifidobacterium lactis is acid tolerant species and hence, is the species of bifidobacterium most commonly used in acidic foods (Figure 2) [25].

![Fig. 2: Effect of acidity (%) of Lighvan cheese on survival of B. lactis](image)

**Fig. 2: Effect of acidity (%) of Lighvan cheese on survival of B. lactis**

*Effect of salt concentration on survival of *B. lactis*:

The salt (%) in probiotic Lighvan cheese was 3.2 % at day 5 and 3.5% at the day 60 of storage time. This characteristic showed significant (p<0.05) differences during ripening of probiotic Lighvan cheese. The salt (NaCl) penetration into the cheese was much faster during the early stage of storage than it had been during ripening. Salt is driven into cheese by the concentration gradient between the cheese blocks and brine; this gradient is much larger at the beginning of ripening [26]. Increase in salt content during ripening could be attributed to higher water content, as salt penetrates the cheese matrix in water. Salt was the one of the most important factor which influenced survival of *B. lactis*. Salt has been caused reduction of water activity of cheese and autolysation of cells of *B. lactis* which led to loss of survival of *B. lactis* during ripening of probiotic Lighvan cheese. However, survival of *B. lactis* was satisfactory during 60 day of ripening of probiotic Lighvan cheese (Figure 3).

![Fig. 3: Effect of Salt (%) of Lighvan cheese on survival of B. lactis](image)

**Fig. 3: Effect of Salt (%) of Lighvan cheese on survival of B. lactis**

*Conclusion:*
The production of functional cheese products was recently proposed as a suitable and promising alternative to fermented milks [4], because cheese could offer certain advantages as a carrier of probiotic microorganisms. Semi hard Lighvan cheese has intrinsic features (pH, moisture and aw) that may characterize it as unreceptive for microorganisms. However, the results of this study demonstrated that traditional Lighvan cheese proved to be an appropriate probiotic delivery vehicle for B. lactis. In particular, B. lactis cells survived in cheese at concentrations up to $6.84 \log_{10} \text{cfu/g}$ for at least 60 days of ripening. Besides meeting precise consumer demand, the production of functional or probiotic cheeses may be useful for differentiating and increasing the market popularity of various Iranian cheeses such as traditional Lighvan, which still have a strict regional tradition. If eaten daily, probiotic Lighvan cheese can be considered as a probiotic vector or as an additional variety supporting other probiotic foods that are eaten daily but we can conclude that in cheeses ripened in brine, a significant parts of ripening products are transferred into brine and their effects on the sensory properties of final product is limited.

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REFERENCES


