



EVALUATION OF *LACTOBACILLUS REUTERI* IN RIPENING OF BUFFALO MILK FETA CHEESE

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Abstract

Feta-type cheese was made from buffalo milk using isolated local adjunct culture of *Lactobacillus reuteri* along with Feta starter (*Lactococcus lactis sub. lactis*, *Lactococcus lactis sub. cremoris* and *Streptococcus thermophiles*). The manufactured Feta cheese was subjected to microbiological and sensory evaluation through the 60 days of ripening. pH values of the experimental cheeses were found to be significantly ($P < 0.05$) higher than of those in control. The microbiological analyses showed a noticeable decrease in population of *Staphylococcus aureus* and coliforms bacteria after the first day of production, thereafter were inactivated for the rest of the ripening period. Whereas the count of lactococci and streptococci decreased gradually by the increasing of adjunct level and progressed of the ripening period. In contrast, counts of lactobacilli increased until day 15 and then decreased up to 30 days and remained constant until the end of ripening approximately $8 \log$ cfu/g. Sensory evaluation showed that the cheese control and containing *Lb.reuteri* along with Feta culture were very good accepted by the sensory evaluation panelists in comparison of samples containing *Lb.reuteri* only.

Key words: *Lactobacillus reuteri*, Feta cheese, buffalo milk, ripening, sensory evaluation.

Introduction

Among the many probiotic strains used in the food industries at the present, many of them belong to Lactobacilli group. In general, they are regarded as safe and many strains with advantage health effect of *Lb. acidophilus* and *Lb. plantarum* have been isolated and belong to the group of lactic acid producers (Alrubeii and Alalaaq, 2018, Azhari Ali, 2010) *Lactobacillus* species are the most common reported adjunct cultures for enhancing the sensory quality of different varieties of cheeses (Hashemi *et al.*, 2009). There is no information about the effect on the quality of buffalo milk Feta cheese of lactobacilli as adjunct cultures. Among the species of *Lactobacillus* claimed to be of probiotic, *Lb. reuteri* is one of the dominant species in the gastrointestinal tract (GIT) of all vertebrates, including human. It is reported to be the most well documented probiotic species and had been widely utilized as a probiotic in humans and animals (Hou *et al.*, 2014, Oh *et al.*, 2010). Several studies have demonstrated that *Lb. reuteri* have the capacity to colonize, and can adhere to mucin and intestinal epithelial cells (Hou *et al.*, 2015). They are also able to

produce antimicrobial substances, such as bacteriocin (reuterin). Reuterin has been found to be inhibitory against a number of pathogens, for example for *Escherichia coli* O157:H7, *Listeria monocytogenes* (Ortiz-Rivera *et al.*, 2017), *Staphylococcus aureus*, *Salmonella choleraesuis ssp. choleraesuis*, *Yersinia enterocolitica*, *Aeromonas hydrophila ssp. hydrophila* and *Campylobacter jejuni*. (Arqués *et al.*, 2004). Other antimicrobial compounds that inhibit growth of pathogenic bacteria include lactic acid and hydrogen peroxide (Connolly, 2004). One aspect of the incorporation of *Lb.reuteri* in cheese could be the inhibitory effect on the mentioned microorganisms, therefore improve the hygienic quality of the cheese. Adjunct cultures are non-starter lactic acid bacteria, which are used along with a standard starter culture to further improve and enhance the flavor of cheese by increasing the concentration of desirable flavor compounds (Fox *et al.*, 2017). Ripening of cheese is a complex process necessary for the overall development of cheese flavor and texture. Flavor development in buffalo milk cheese is considerably slower than that in cheese made from cow's milk (Kumar *et al.*, 2012). Feta cheese is a semi-soft, matured in brine, with

mild rancidity, slightly acid taste and firm, creamy, smooth and sliceable texture. These special sensory properties are attributed to world-wide recognition of Feta cheese and its annual consumption is estimated at 12 kg per person in Greece (Panagou *et al.*, 2013). Efforts have been made, for the production of probiotic Feta cheese using commercially probiotic strains, since Feta can act an appealing delivery vehicle for probiotics (Angelopoulou *et al.*, 2017). probiotic Feta cheese using commercially probiotic strains, since Feta can act an appealing delivery vehicle for probiotics (Angelopoulou *et al.*, 2017). Feta cheese is the most known Greek Protected Designation of Origin (PDO), It is a high quality white brined-cheese produced from sheep milk or a mixture of sheep and goat milk, the latter not exceeding 30%. (Manolopoulou *et al.*, 2003). The production of sheep and goat milk is very limited and its production is around 2% of total annual world's milk production (Kumar *et al.*, 2014). Therefore, it becomes almost impossible to fulfill the growing demand for Feta cheese from ewe milk and goat milk mixtures. In order to fulfill the ever growing demand for Feta cheese worldwide, the technology for the manufacture of Feta-type cheese with acceptable quality from cow's milk has been developed in European countries. The aim of the study came out to solving these issues, Such as the limited production of ovine and ewe milk around the world, slow flavor development in buffalo milk cheese and intervention of adjunct with probiotic potential properties.

Materials and Methods

Starter Culture for Feta Cheese

a. Feta starter culture was purchased from MAYSA Turkish Company, as lyophilized packaged with direct inoculation, which consist of the following strains: *Lactococcus lactis sub. lactis*, *Lactococcus lactis sub. cremoris* and *Streptococcus thermophiles*.

b. Adjunct culture A local isolate *Lb.reuteri* as probiotic adjunct culture was acquired as lyophilized capsule from College of Agricultural Engineering Sciences-University of Baghdad. It was activated on MRS broth at 37°C for 48 h. Adjunct culture *Lactobacillus reuteri* was prepared by 12% reconstituted skim milk, sterilized at 121°C for 5 minutes, subsequently cultured in 10% and incubated at 37°C for 24-48 h., this process was repeated three times for activation of *Lb.reuteri* to maintain high activity.

Manufacture of Feta Cheese

Feta cheese was manufactured according to the method described in (Fox *et al.*, 2017) with some modifications as followed:

Buffalo milk was purchased from farms of Abu-Gharib/west of Baghdad and pasteurized at 62.8°C and holding for 30 minutes, cooled down to 35°C. Starter culture 2% (v/v) was added at this point and wait for 50 min to ensure rapid acidification. Calcium chloride solution 0.02% and microbial rennet (1gm/100kg) produced by fungus *Mucor miehei* from Co. ltd. (Japan) were added at 32°C after allowed to set for 45 min before the coagulum was cut. Curd was cut into approximately (2-3 cm) cubes and left undisturbed for 10-20 min. Curds were transferred into perforated molds lined with muslin cloth for further removal of whey and texturization of cheese curd without pressure. Cheese molds were inverted every 2-3 hour to shaped a firm curd, the molds were left settled overnight at 16-20°C. The curds mass were expelled from molds and were cut into uniform size which are dry salted before being transferred to other container. The containers was filled with brine (8% NaCl) and held at 14–16°C until the pH of cheese has decreased to pH 4.8 Cheeses were ripened at 4°C for 2 months.

Microbiological analyses

Representative 10g of cheese samples during (1, 15, 30, 45, 60) days of ripening were homogenized thoroughly with 90 ml of a sterile 2% (w/v) sodium citrate to make an initial dilution (10^{-1}). Suspension was then submitted to 10 decimal serial dilution with sterile 0.1% peptone water. A suitable dilutes were cultured on appropriate medium in triplicate by pouring plate method. Colonies were enumerated, recorded as colony forming units (cfu) per g of cheese. The following microbiological analyses were performed on the cheese samples. Total coliforms were enumerated on MacConkey agar at 37°C for 24 h., *Staphylococcus aureus* on Manitol salt agar at 37°C for 48 h., Lactococci were enumerated on Elliker agar (Himedia) and incubated aerobically at 37°C for 48-72 h. (Moon *et al.*, 1974), *Lb.reuteri* counts were enumerated on MRS agar (oxiod) for 72 h. at 37°C in anaerobic condition (anaerobic jar). Yeasts and molds were counted on Potato dextrose agar acidified with 10% HCl (BDH-England) at 25°C for 5 days. Finally, psychrophilic bacteria were enumerated on Nutrient agar (Himedia) and incubated at 10°C for 7 days. All cell count were expressed as Log of mean colony forming unit (cfu).

Sensory evaluation

Organoleptic characteristics of Feta cheese was conducted to evaluate the influence of *Lb.reuteri* on sensory properties. Organoleptic evaluation was done by five trained panelists randomly from Food Science Department as described in (IDF, 1987). Cheese samples were evaluated through (1, 15, 30, 45, 60) day of storage.

The evaluated attributes included (Appearance, Body-Texture and Flavor). Samples were presented at random and evaluated separately.

Statistical Analysis

Data from each experiment were analyzed by tow-way ANOVA to determine the main effects (Ripening period, Adjunct level) and their interactions by using GLM procedure of SAS Software (V 9.1). Comparison of the means was conducted with Duncan's test. Differences of $P < 0.05$ were considered to be significant.

Results and Discussion

Microbiological analysis of Feta cheese

Total count of *Lactobacillus reuteri* incorporated with Feta cheese was significantly ($P \leq 0.05$) affected as shown in fig. 1.

Counts of lactobacilli augmented during the first 15 day (8.1 log cfu/g) and then marginally declined after 30 day. In all probiotic cheese samples (B, C, D and E) after one day of manufacturing, *Lb.reuteri* was displayed the highest counts roughly 9.1 log cfu/g in treatment E. Afterward, a decline of their counts were observed after one month of ripening reaching approximately 8.8 Log cfu/g in cheese samples incorporated with *Lb.reuteri* only. Likewise, the population of *Lb.reuteri* in trail C were remained almost unchanged reaching roughly 7.6 log cfu/g at the end of ripening. The unfavorable conditions such as lack of carbohydrates, low pH and probably unfavorable ambient temperature during 60 days of the storage could be responsible for this reduction (Ong and Shah, 2009). The probiotic strain *Lb.reuteri* used in the present study however known to be acid resistant, this is accordance with (Mu *et al.*, 2018). In general, *Lb.reuteri* counts were present in high enough levels (≥ 6 Log cfu/g) throughout the storage at 4°C in all experimental cheeses, which is required for probiotic potential and having enough viability.

Lactococci are considered native milk microflora as they dominate raw milk in about 24 h. of production. It is noteworthy that lactococci count showed high gradually increasing rates in cheese control compared with samples treated with *Lb. reuteri* after 1 day of manufacturing. Subsequently, a decline

of cocci count was noted after 15 day of ripening.

Results showed in fig. 2, a high count of cocci reached 6.9 Log

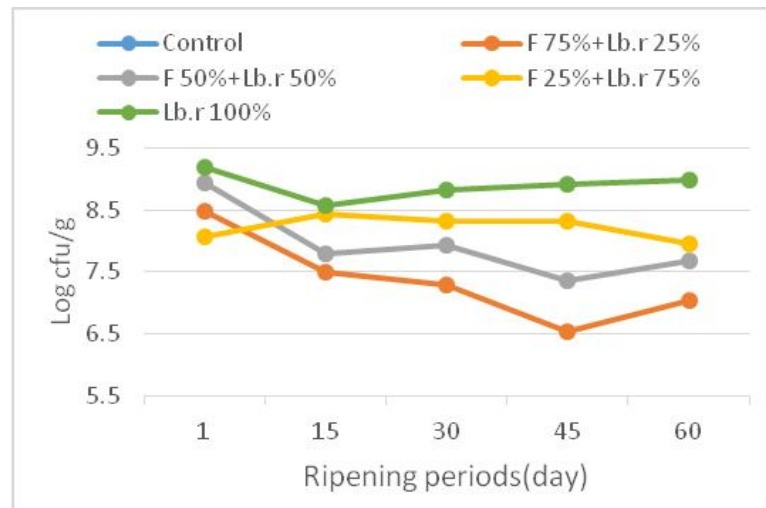


Fig.1: Total count of *Lb.reuteri* of Feta cheese that prepared with *Lb.reuteri* as adjunct culture.

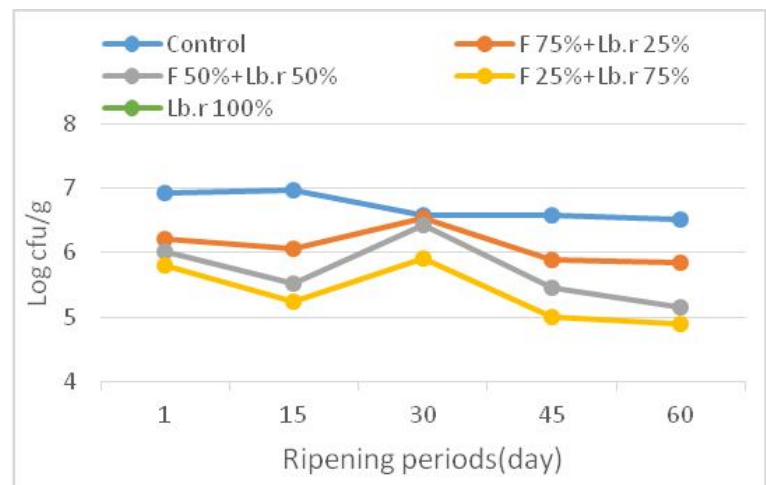


Fig. 2: Total count of Lactococci/Streptococci of Feta cheese that prepared with *Lb.reuteri* as adjunct culture.

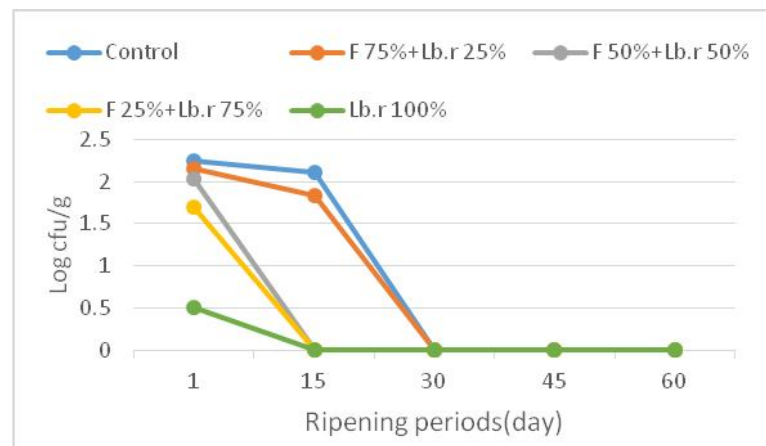


Fig. 3: Total count of Coliforms of Feta cheese that prepared with *Lb.reuteri* as adjunct culture.

cfu/g in control cheese after one day of cheese production. Whereas, the results showed low counts of standard culture by gradually increasing ratio of *Lb. reuteri* as adjunct starter until the end of ripening period. After 30 days of storage period, cocci counts were 6.57 Log cfu/g in control sample and remained constant until the end of storage. Lactococci counts were significantly affected ($P \leq 0.05$) by time of ripening as well as by the combination with *Lb. reuteri* culture. Specifically, during the ripening and refrigerated storage period at 4°C.

The results in the present study was in agreement with (Mantzourani *et al.*, 2018) when they pointed to use potential probiotic *Lb. paracasei* in Feta cheese production. This result is also in accordance with former studies indicating that lactic acid bacteria (*Lactobacillus*) dominate against other microorganisms when they are active in the curd during cheese manufacture (Gobbetti *et al.*, 2018, Terpou *et al.*, 2016).

On the other hand, a population of coliforms, Staphylococci and psychrophilic bacteria are presented in (fig. 3, 4, 5) and significantly affected by ripening period and addition of adjunct levels of *Lb. reuteri*. Coliforms count in probiotic samples was decreased significantly ($P \leq 0.05$) to undetectable levels after 15 days of ripening and remained constant until the end of the experiment. Whereas the complete absence of *S. aureus* and psychrophilic bacteria was recorded after 30 day and 60 day of ripening respectively. These findings were in accordance with previous studies reporting depression of spoilage and pathogenic bacteria by probiotic lactic acid bacteria (Madureira *et al.*, 2008, Schoina *et al.*, 2014). A possible explanation for this observation could be related to the high population of the adjunct culture which through the production of bacteriocins or antagonism in nutrients can lead to the reduction of spoilage or other possible pathogenic microorganisms in milk products (Pappa *et al.*, 2019, Woraprayote *et al.*, 2016). The similar results was obtained by (E. Connolly, 2004, Ortiz-Rivera *et al.*, 2017) that some strains of *Lb. reuteri* are able to produce anti-microbial substances like reuterin, organic acid and hydrogen peroxide, these substances have been found to be inhibitory against a number of pathogens, for example (*Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus*

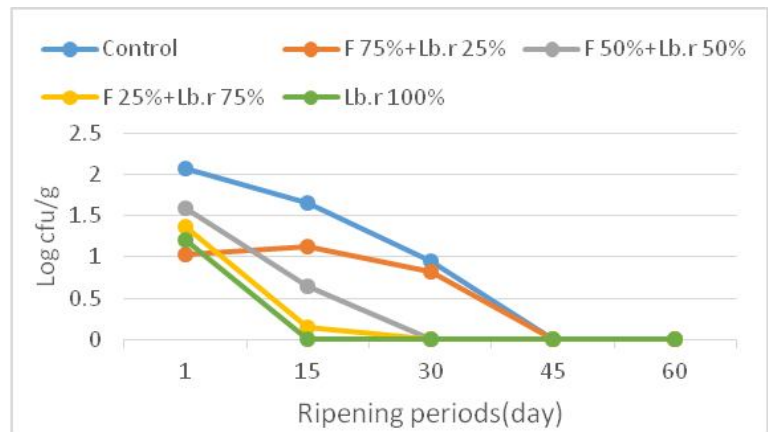


Fig. 4: Total count of *Staphylococcus aureus* of Feta cheese that prepared with *Lb.reuteri* as adjunct culture

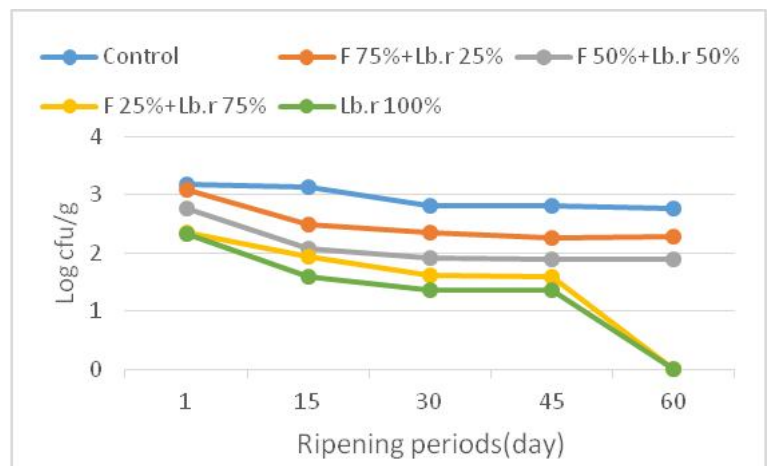


Fig. 5: Total count of Psychrotrophic bacteria of Feta cheese that prepared with *Lb.reuteri* as adjunct culture.

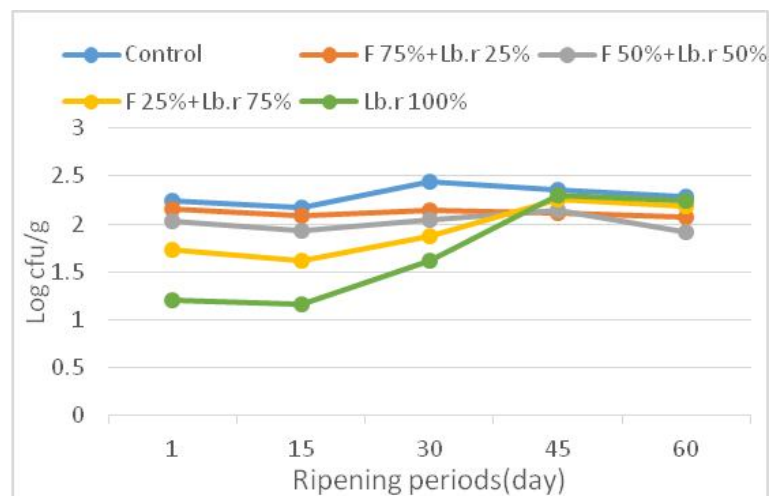


Fig. 6: Total count of molds and Yeasts of Feta cheese that prepared with *Lb.reuteri* as adjunct culture.

aureus and *Salmonella enterica*).

The changes in molds and yeasts count for Feta cheese during maturation are presented in fig. 6.

The molds and yeasts counts of cheese were

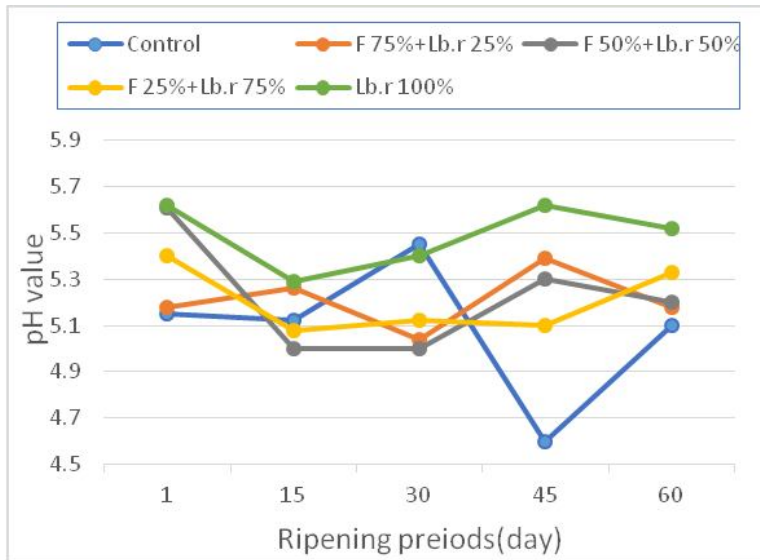


Fig. 7: pH values of Feta cheese manufactured from buffalo milk with *Lb. reuteri* as adjunct culture

decreased through 15 day of ripening period in cheese that treated with *Lb. reuteri* as co-starter. A similar observation was done by (Manolopoulou *et al.*, 2003) when noted a decline of yeast population during Feta cheese ripening. An increase in the population of yeasts/molds was noted after 30 days of storage at 4°C reaching a final population of 1.6 and 2.4 log cfu/g for probiotic and control samples, respectively. This could be attributed to the dominance of osmophilic species, capable of proliferating under extremist condition (low pH, high salt concentration and low temperature) and stayed in high numbers during the late storage periods.

The presence of molds and yeasts in cheese is closely related to organic acid utilization, while their contribution into ripening process is due to their lipolytic and proteolytic activities (Macedo *et al.*, 1995, Moubasher *et al.*, 2018), this explains the rise in pH values for a treatment containing *Lb. reuteri* only.

Yeasts and molds count were detected significantly ($P \leq 0.05$) higher in cheese produced without *Lb. reuteri* culture. As a result, it can be assumed that the development of conditions favors their growth while the action of *Lb. reuteri* culture could inhibit their growth during cheese maturation and storage. These findings are in accordance with other studies reporting that lactobacilli can provide an antifungal activity in cheese (Voulgari *et al.*, 2010).

pH The effect of starter type on pH Feta cheese during ripening were included in fig. 7. pH values of control cheese was significantly ($P \leq 0.05$) decreased faster than other treatments during all ripening and storage period.

Samples inoculated with different ratios of *Lb. reuteri*

exhibited slight decrease in pH values after one day of manufacturing which reached 5.18, 5.61, 5.4 and 6.55 for trails B, C, D and E, respectively. Likewise, the pH values in buffalo's cheese displayed in (fig. 7) showed a slight decrease at the end of ripening period at 4°C reaching 5.2, 5.3, 5.5 for treatments C, D and E, respectively. While in control treatment, pH value decreased to 5.1 until the end of ripening period. This may be due to that of *Lb. reuteri* has a low acidification ability, which is in accordance with (Cruz *et al.*, 2009) when pointed that many strains of *Lb. reuteri* incorporated with other probiotic bacteria are unable to ferment milk sufficiently due to their slow growth and low acidification level. In general, post acidification was noted during storage (4°C) of Feta cheese produced with *Lb. casei* and *Lb. planarum* starters which is in accordance with former

studies of white brined cheeses produced with the incorporation of lactic acid bacteria (Papadopoulou *et al.*, 2018, Terpou *et al.*, 2018).

Organoleptic properties

The results of sensory evaluation of Feta cheese samples were presented in table 1. Examiners evaluated the produced Feta cheese regarding Appearance, body-texture and flavor (odor and taste). In general, all cheese treatments were characterized by high rates of acceptance. The ripening process that takes place over time consists of proteolysis and lipolysis. Proteolysis is the breakdown of proteins into amino acids and lower molecular weight compounds which give the cheese its structure and texture. (Gunasekaran and A.K., 2003). While lipolysis the process of breaking down the fat into free fatty acids which are precursors for flavor compounds in cheese (Fox *et al.*, 2017). The use of Lactobacilli strains with probiotic potential has also been reported by other studies to provide desirable and robust technological properties (Papadopoulou *et al.*, 2018). In cheese production, the starter adjunct contributes to the texture and sensory profile of the end product by producing acetic acid, ethanol, aroma compounds and several enzymes (Azhari Ali, 2010).

Appearance and color: The effect of adjunct *Lb. reuteri* on color and appearance of Feta cheese during ripening is presented in table 1. It revealed that there was significant differences between color and appearance score of cheese manufactured from buffalo milk. The color and appearance scores of experimental cheeses were decreased with the progressive ripening period up to 60 days. Feta cheese color from buffalo milk was pure

Table 1: Effect of adjunct *Lb.reuteri* on sensory characteristics of Feta cheese during ripening

| Ripening (days) | Trials | Organoleptic attributes | | | |
|-----------------|--------|-------------------------|--------------|---------|------------|
| | | Appearance | Body-Texture | Flavor | Total |
| | | (10) | (40) | (50) | (100) |
| 1 | A | 9 ab | 32 cdb | 40 cb | 81 cadb |
| | B | 9 ab | 32 cdb | 41 cab | 82 cadb |
| | C | 9 ab | 32 cdb | 41 cab | 82.67 cadb |
| | D | 9 ab | 32 cdb | 42 cab | 83 cadb |
| | E | 9 ab | 33 cab | 43 ab | 84.67 cab |
| 15 | A | 10 a | 32 cdb | 41 cab | 82.67 cadb |
| | B | 9 ab | 33 cab | 42 cab | 84 cadb |
| | C | 9 ab | 33 cab | 42 cab | 84.67 cab |
| | D | 9 ab | 34 ab | 43 ab | 86 ab |
| | E | 8 cb | 34 ab | 43 ab | 84.67 cab |
| 30 | A | 9 ab | 31 cde | 41 cab | 81 cadb |
| | B | 8 cb | 32 cdb | 43 ab | 83 cadb |
| | C | 9 ab | 33 cab | 43 ab | 85 cab |
| | D | 9 ab | 34 ab | 43 ab | 86 ab |
| | E | 8 cb | 35 a | 44 a | 87 a |
| 45 | A | 8 cb | 31 cde | 40 cb | 79 cdb |
| | B | 8 cb | 31 cde | 41 cab | 80 cadb |
| | C | 9 ab | 32 cdb | 42 cab | 83 cadb |
| | D | 8 cb | 31 cde | 43 ab | 82 cadb |
| | E | 7 c | 31 cde | 43 ab | 81 cadb |
| 60 | A | 8 cb | 30 de | 39 c | 77 d |
| | B | 8 cb | 31 cde | 40 cb | 79 cdb |
| | C | 8 cb | 31 cde | 40 cb | 79 cdb |
| | D | 8 cb | 30 de | 42 cab | 80 cadb |
| | E | 7c | 29 e | 42 cab | 78 cd |
| Mean | | 8.48 | 31.96 | 41.76 | 82.21 |
| Root SME | | 0.959166 | 1.469694 | 1.56205 | 3.631345 |
| P v | | 0.923 | 0.3827 | 0.9995 | 0.9992 |

* N.S.: Non-Significant.** SEM: Standard Error Mean, a,b,c: means in the same columns with different superscripts differ significantly at probability value ($P \leq 0.05$). A: Control, B: 75% standard culture+25% *Lb.reuteri*, C: 50% standard culture+50% *Lb.reuteri*, D: 25% standard culture+75% *Lb.reuteri*, E: 100% *Lb.reuteri* only.

white, which is one of the typical characteristics of good quality Feta cheese. This observation could be attributed to absence of carotenes as mentioned by Fox *et al.*, 2017, in buffalo milk. The results in this study were in accordance with (Kumar *et al.*, 2012). It seems that the addition of probiotic *Lb.reuteri* culture in cheese, no desirable defects of appearance were detected such as cracks, sponge-like appearance and foreign materials. Whereas, wet and irregular shape was noted in treatment E which contain *Lb.reuteri* only. This finding was in the same line with (Karlsson, 2013).

Body-Texture: It is also apparent in (Table 1) that the texture and body values increased as the ripening period progressed up to 30 days. Afterwards, followed by a decreasing trend toward the end of the ripening. The maximum body and texture score was found at 30 days of ripening, as it was characterized for mellow and soft texture, which is desirable in good quality feta cheese. The maximum body and texture score as 35 was observed with cheeses E after 30 days of ripening, while the minimum score as 29 was observed with the same cheese sample (E) at the end of ripening period. This observation could be attributed to high moisture retention that resulted in cheese containing *Lb.reuteri* only as starter culture.

These findings were almost in accordance with (Kumar *et al.*, 2014) when they used some strains of Lactobacillus as adjunct culture in Feta cheese production. The buffalo milk Feta cheese was found to be crumple and hard, it could be attributed to low moisture retention or probably due to high calcium content and different kinds of casein micelles (Kumar *et al.*, 2012).

Flavor: The results of flavor scores are shown in table 1. The flavor value of the experimental cheeses was increased by progressed in ripening period up to 30 days. Afterward, a slight decreased of values towards the end of ripening period was observed. The interaction effect between ripening period and the proportion of adjunct culture was found significant ($p < 0.05$) and indicated the high flavor score of (44) was attained within 30 days of ripening in E trial, while the lower score of (40) was found on 1 day in trial A (control). The flavor development in Buffalo milk Feta cheese was faster when cheese cultured was incorporated with *Lb. plantarum* (Papadopoulou *et al.*, 2018) as was found in the present study. Similar

findings have been reported by other researchers (Hashemi *et al.*, 2009, Karlsson, 2013) for semi-hard and brined cheese. The long-term ripening period and slow flavor development in buffalo milk cheddar cheese is due to qualitative and quantitative differences amongst the major and minor components of buffalo and cow milk (Rafiq *et al.*, 2016)

Conclusion

The findings of the present study demonstrated that *Lb. reuteri* could be used in cheese manufacturing of

white brined cheese and can improve the microbiological analyses and sensory quality of Feta cheese. *Lb. reuteri* showed a good survival viability and high potential properties against undesirable microorganisms. Moreover, the sensory evaluators exhibited that cheese samples supplemented with adjunct *Lb. reuteri* had a high quality and a good satisfactory for all sensory characteristics.

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