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A microbiological study of Mozzarella cheese made by *Lactobacillus kefiranofaciens* ZW3 throughout cheese making and ripening

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Abstract

A novel exopolysaccharide producing strain *Lactobacillus kefiranofaciens* ZW3, isolated from kefir grain Tibet, was used for the first time for Mozzarella cheese preparation. The survival of lactic acid bacteria (LAB) during different cheese preparation phases, and ripening period of 4 weeks, was investigated and compared with the Mozzarella cheese prepared with industrially used strains. The results demonstrated that The LAB counts remained almost constant throughout the coagulation and cutting phases of Mozzarella cheese preparation. However, the counts were decreased during cooking temperature (45 ˚C) and stretching temperature (60**º**C). The M.C LAB count decreased to a greater extent as compared to ZW3 and L.C. However this sharp decrease in MC LAB count was increased significantly during cheese ripening period of first 2 weeks. The ZW3 and L.C LAB counts increased gradually during ripening. Furthermore the study also determined the *E. coli*, yeast and mold counts during ripening period of 4 weeks. The yeast and mold count increased gradually from the date of manufacture up to 4 weeks of storage in A.C, L.C, M.C and ZW3 made Mozzarella cheese. However this increase was not significant. *E. coli* count remained zero throughout ripening period. The pathogenic microbial counts were minimum in ZW3 made cheese compared to L.C and M.C.

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Introduction

Probiotic bacteria are defined as 'living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition (Guarner and Schaafsma, 1998). Foods containing probiotic bacteria are categorized as 'functional foods' and such products are gaining widespread popularity and acceptance throughout the developed world. A number of health benefits for product containing live probiotic bacteria have been claimed including alleviation of symptoms of lactose intolerance, treatment of diarrhea, ant carcinogenic properties, blood cholesterol reduction and improvement in immunity (Ballongue, 1993; Shah, 2000; Shah, 2000). Daily consumption of high levels of probiotic bacteria, however, is required to confer health benefits. For dietary organisms to be beneficial in food systems, they should maintain viability in the food until the time of consumption and be present in significant numbers, at levels of at least 10^7 viable cells per gram or milliliter of product (Sulieman *et al*., 2013). For this reason, changes in the numbers of viable bacteria during ripening period should be known. Probiotic foods are currently restricted to fermented milk drinks and yoghurt, which have limited shelf life in contrast to cheese. Incorporation of probiotic cultures in cheeses provides potential not only to improve health status and quality of products but also to increase the range of probiotic products. Cheeses have a number of advantages over fresh fermented products such as yoghurt as a delivery system for viable probiotic to gastrointestinal tract as they tend to have higher pH, more solid consistency and relatively higher fat content. This provides protection to probiotic bacteria during storage and passage through the gastrointestinal tract. Cheeses also have higher buffering capacity than yoghurt (Ong *et al*., 2006).

Microbial contamination, causing approximately one-fourth of the world's food supply loss, has become an enormous economic and ethical problem worldwide (Huis in't Veld, 1998). Dairy products are an excellent growth medium for a wide range of microorganisms and, thus, display a reduced shelf life (Ruegg, 2003). The microbiological quality of dairy products is influenced by the initial flora of raw milk, the processing conditions, and post heat treatments. Spoilage bacteria and various bacteria of public health concern can be found in these products and their concentrations should be kept as low as possible (Varga, 2007). In contrast, lactic acid bacteria (LAB), occurring in the indigenous microflora of raw milk and being the major components of starter cultures used in fermentation, contribute to the quality of fermented cheese products by improving the taste and texture and inhibiting food spoilage bacteria by producing growth-inhibiting substances and large amounts of lactic acid (Jana and Mandal, 2011). Thus, to be confident of fermented cheese quality, LAB concentration should be monitored during cheese production. Our lab has isolated a novel strain of *Lactobacillus kefiranofaciens* ZW3 from kefir, a traditional dairy product that is known to provide many health benefits to humans (Wang *et al*., 2008). It is a viscous, slightly carbonated dairy beverage obtained from kefir grain collected in Tibet, China. It acts as a probiotic as it provides many health benefits (Rodrigues *et al*., 2005) and uses kefir grain as the starter culture. The exopolysaccharide (EPS) produced by the kefir starter culture are believed to be involved in imparting health beneficial aspects (Pigeon *et al*., 2002). *Lactobacillus kefiranofaciens* is one of the major microbial constituents of kefir grain that forms highly viscous colonies. Wang *et al*., (2011) reported the complete genome sequences of *L. kefiranofaciens* ZW3, representing the first genome of *L. kefiranofaciens* ZW3.

In this study Mozzarella cheese was prepared for the first time by using our lab isolated strain *Lactobacillus kefiranofaciens* ZW3 as an adjunct strain. Being lactic acid bacteria (LAB) and to analyze the proteolytic and lipolytic pattern during ripening, it was necessary first to investigate the survival of this bacterium during different stages of cheese manufacture and ripening at 4°C. Therefore the present study was aimed to determine the impact of different processing phases of Mozzarella cheese

preparation and ripening temperature of 4°C, for a period of 4 weeks, on the survival of ZW3 and other LAB. The other part of this study was focused on the numeration of total viable count of food born pathogens such as *E. coli*, yeast and mold during cheese ripening period of 4 weeks.

Materials and methods

Reagents

Milk coagulant enzyme rennet was obtained by Zhengzhou Wanjiu Chemical products Co. Ltd, Henan Province, China. Fresh cow milk was obtained from local dairy farm (Tianjin Tanggu dairy farm). Mozzarella cheese was prepared in stainless steel cheese maker purchased from Guangzhou WELLMAX Industry, China. All chemicals were purchased from local market.

Mozzarella cheese preparation

Mozzarella cheese was prepared as described before (Scott, 1981) with little modifications. Fresh cow milk was procured from local dairy farm. The milk was standardized to 3% fat, pasteurized to 72°C for 15 sec, followed by cooling to 30°C. 2% adjunct strain of *Lactobacillus kefiranofaciens* ZW3 along with *Lactobacillus delbrueckii* ssp. *bulgaricus* CGMCC 1.2470 and *Streptococcus thermophilus* CICC6058, were inoculated to 1 L milk. The L.C batch used 1 L milk inoculated by 2% of *Lactobacillus delbrueckii* ssp. *bulgaricus* CGMCC 1.2470 and *Streptococcus thermophilus* CICC6058 while M.C batch used 0.016g (as per recommendations of the company) of industrial grade starter culture MA14, constituting *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *Lactis*, in 1L milk to prepare Mozzarella cheese. After inoculation, the milk was stirred and incubated at 30°C for an optimum fermentation time to reduce the pH level to 5.4. The direct acidification method implied the usage of 10% acetic acid solution to lower down the milk pH to 5.4. The rennet was added at concentration of 0.005-0.007g/L of milk and incubated at 30°C. The cutting of coagulated milk was done with the help of cutter into small cubes. The curd was kept at 30 ˚C for 20 to 25min to permit the whey separation, followed by cooking at 45°C for 45min. The whey was drained, milled at pH 5.2 and the curd was salted (1.5% w/w) and held for 30min. The cheese was stretched in hot water (60°C) at pH 5.1, shaped into round balls and the cheese was stored at 4°C.

Lactic acid bacteria counts

The LAB counts of Mozzarella cheese prepared by L.C, M.C and ZW3 during preparation and ripening was assessed by plate count method as determined by (Nicholson and Setlow, 1990) with slight modification. The medium was prepared as follows. 10% skim milk was prepared. 200mL of 2% yeast extract was prepared followed by adding 0.8mL bromocresol purple; the pH was maintained between 7.2-7.4 and the agar (4%) was added. At the same time, normal saline solution was prepared, followed by adding 100mL normal saline solution in 4 flasks; the glass beads were put in 3 of 4 flasks. The medium and normal saline solution was sterilized at 121°C for 20 minutes while the skim milk was sterilized at 115°C for 20min. The skim milk was added into the medium and the medium was put into petri dishes. 1g cheese sample was added into 100mL normal saline solution into a flask containing glass beads and was stirred. 100µL of this solution was added into 900µL of normal saline solution to get dilution of 10^1 and the dilution process was extended to 105. 100µL from each dilution factor sample was added into petri dishes containing solidified yeast agar medium. The plates were kept at 30°C for 72h. The colonies were counted and the total viable counts were evaluated.

E. coli, yeast and mold counts

Mozzarella cheese was also analyzed for the *E. coli*, yeast and mold count during ripening period of 4 weeks as described previously (ISIRI, 1998) with little modifications. *E. coli* count was made in LB media: containing yeast extract powder (0.5%), NaCl (1%) and peptone (1%). These ingredients were dissolved in distilled water and the pH was set at 7. While yeast count was calculated by preparing YPD medium containing yeast extract powder (1%), peptone (2%) and glucose (2%). Mold count was measured in potato's agar medium containing Magnesium

sulphate (1g), potassium dihydrogen phosphate (2g), peptone (5g), glucose (20g) and potatoes (200g). The potatoes were chopped and were added in 1000mL of distilled water followed by boiling for 20min. Potatoes extract was sieved through a muslin cloth. The extract was then added into a beaker containing above mentioned chemicals and then made the volume up to 1000mL. 2% agar powder was added in all three medium before sterilization at 121°C for 20 min. The mediums were poured in sterilized petri dishes. 1g of each sample was used to make dilutions in sterilized normal saline water. The dilutions 10^2, 10^3 and 10^4 made to count all three parameters. 200µl samples were used to inoculate in plates containing respective media from each dilution. Samples were taken from freshly prepared cheese (0 d), 2 week and at the end of 4 weeks. For *E. coli* count, the plates were incubated at 37°C for overnight while for yeast and mold counts, the plates were incubated at 30°C for 48h.

Statistical data

3 different batches of Mozzarella cheese were prepared by using 3 different parameters. The parameters included the usage of experimental adjunct strain. In this study total 3 parameters were used with 3 repetitions. The data for LAB count and *E. coli*, yeast and mold counts of the Mozzarella cheese were statistically analyzed using mean and standard deviation.

Results and discussions

Survival of lactic acid bacteria during cheese manufacture and ripening

The samples were taken at different phases of Mozzarella cheese preparation and its ripening at 4°C. The Fig. 1 indicates that after inoculation of these inoculums, the LAB counts increased up to coagulation temperature (30°C). The LAB counts remained almost constant throughout the coagulation and cutting phases of Mozzarella cheese preparation. However, after treated at cooking temperature (45°C) and stretching temperature (60°C), the LAB counts decreased. The LAB counts of M.C decreased to a greater extent as compared to ZW3 and L.C. During ripening of 4 weeks, total LAB counts increased; The M.C counts increased sharply during first 2 weeks and then it increased gradually while a very little decrease was recorded during first 2 weeks of ripening and then it increased gradually up to 4 weeks of ripening while in case of L.C, the LAB counts increased gradually during ripening of four weeks at 4°C.

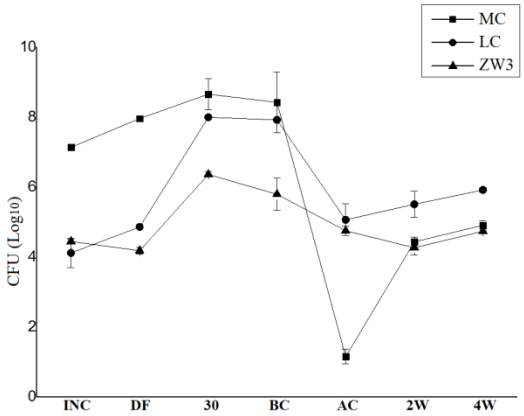

Fig. 1. The lactic acid bacteria (LAB) counts of 3 experimental groups including *L. kefiranofaciens* ZW3, Lab. Control (L.C) and Market available starter culture (M.C). at different stages of cheese manufacture and during storage at 4°C.

Optimum temperature is the basic requirement for the LAB growth. The LAB are highly sensitive to high temperatures and their growth is retarded under adverse temperature conditions. In this study the LAB count of L.C, M.C and ZW3 made Mozzarella cheeses were measured at different stages during manufacture and ripening. The LAB counts continued to increase from the time of inoculation, during fermentation and up to coagulation temperature (30°C), that is evident by the fermentation graph (data not shown). However, when the temperature was raised to 45°C (cooking temperature) and curd stretching temperature (60°C), the bacterial count was decreased indicating that ZW3 along with L.C and M.C are sensitive to high temperature. The M.C batch bacterial counts were decreased maximum as compared to L.C and ZW3, indicating that M.C bacterial strains are highly sensitive to high temperature. Several workers investigated the survival of bacteria in Mozzarella cheese during manufacture, as well as of market samples (Oberg *et al.*, 1991; Tunick *et al.*, 1993; Fife *et al.*, 1996) and

have found that the LAB are sensitive to high temperature. Similar findings were also achieved previously (Kuchroo and Fox, 1982) who found a decrease of about 2 log of *Listeria monocytogenes* observed after stretching of the curd in hot water (95**o**C). Listeria could not survive in cheese after 24h when stored in the conditioning liquid (skim water from stretch, pH ~4.0). Similarly in another report it was found that stretching of Mozzarella cheese curd at 66°C for 5 min or 77**o**C for 1min could effectively control *L. monocytogenes* during the production of Mozzarella cheese (Laemmli, 1970). On the other hand, refrigeration (4**o**C) do not effect the survival the LAB growth. In our study cheese ripening at 4**o**C for 4 weeks had a positive impact on LAB growth in Mozzarella cheese as indicated by the Fig 1. The LAB continued to grow steadily from day 1 of ripening up to 4 weeks. Marco *et al.*, (2003) reported that the LAB continued to grow steadily during ripening period in Serrano cheese. Several reports have determined the survival of LAB during ripening (Centeno *et al.*, 1994; *Cuesta et al.*, 1996), indicating that LAB grow gradually during ripening.

E. coli, yeast and mould count of Mozzarella cheese during ripening

Evaluation of microbial quality of the different produced cheese samples in terms of determination of total *E. coli,* yeast and mold counts is depicted in Table 1. *E. coli*, yeast and mould accounts of Mozzarella cheese were calculated as colony forming unit (CFU Log_{10}) during a period of 4 weeks of storage at 4**o**C. The data represents that *E. coli* counts remained almost zeero for all the cheese samples during storage period of 4 weeks. The yeast and mold accounts however increased gradually from the date of manufacture upto 4 weeks of storage in A.C, L.C, M.C and ZW3 made Mozzarella cheese. Yeast, mold and *E. coli* play a critical role in cheese spoilage. An increased count of these may cause serious health problems in consumers. Presence of high counts of molds and yeasts in cheese can be also related to the unsanitary practices during cheese making, insufficient conditions of utensils, unacceptable controlling of pasteurization process and the use of low-quality milk and ingredients (Coveney *et al.*, 1994). Some yeasts and molds can also grow at refrigeration temperatures in the environment with atmosphere having lower oxygen concentrations, which are ideally suited for the role of contaminants of processed cheese. These microorganisms can be resulted to the surface discoloration, off-flavor and early blowing due to the action of their lipolytic and proteolytic enzymes (Buňková and Buňka, 2015). A good quality cheese must satisfy the consumer's acceptability. Therefore, it is necessary to evaluate their counts during storage of cheese. In our results, the yeast and mold counts increased during storage of cheese samples however these counts were not enough to make cheese contaminated (Table 1). *E. coli* counts were remained zero through the process of aging. The results are in line with the findings of Suleiman *et al.*, (2013) who reported a gradual increase in yeast and mold account during ripening of cheese. Similar results were also obtained by Sheida and Sanjabi,(2016) who reported an increase in yeast and mold account of the cheese during a period of 60 days. Furthermore, yeast, mold and *E. coli* count was lowest in ZW3 made cheese indicating that this bacterium might produce antimicrobial agents that helped to retard the growth of ZW3 made cheese.

Table 1. The average CFU (Log_{10}) values of *E. coli*, yeast and mold count during ripening of Mozzarella cheese at 4°C.

Account	Storage	L.C	M.C	ZW3
E. coli	0 Day	0	0	0
	2 Week	0	0	0
	4 Week	0	0	0
Yeast	0 Day	3.5	2.7	2.7
	2 Week	3.7	3	2.7
	4 Week	3.9	3.7	3.2
Mold	0 Day	3.5	3	3.4
	2 Week	3.9	3.5	3.6
	4 Week	4.1	4.3	3.7

Conclusion

This study demonstrated the survival of LAB in Mozzarella cheese made, for the first time, by *Lactobacillus kefiranofaciens* ZW3 during different manufacture stages and ripening. Inoculation and coagulation temperature did not affect the LAB growth while cooking and stretching temperature exerted an adverse effect. However this effect was normalized during cheese ripening of 4 weeks. The study also examined the growth of pathogenic microbes during cheese ripening. E. coli, yeast and mold counts remained low during cheese ripening and hence did not contaminate the cheese.

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Novelty statement

This is the first ever report on Mozzarella cheese made by a lactic acid bacterium strain *Lactobacillus kefiranofaciens* ZW3. This would lead to understand the microbiological study in Mozzarella cheese made with an EPS producing strain during cheese manufacture and ripening. Also this study determined the evaluation of pathogenic microbes in Mozzarella cheese made by *L. kefiranofaciens* ZW3 for the first time.

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