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Application of Bacteriocin-Like Inhibitory Substances (BLIS)-Producing Probiotic Strain of *Lactobacillus plantarum* in Control of *Staphylococcus aureus* in White-Brined Cheese Production

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ABSTRACT

The aim of this study was to investigate the antimicrobial activity of an autochthonous probiotic strain of bacteriocin-like inhibitory substances (BLIS)-producing *Lactobacillus plantarum*, previously isolated from a Tulum cheese and satisfied technological criteria as adjunct culture in cheese production, in reducing *Staphylococcus aureus* during production and ripening of white-brined cheeses. Cheeses were manufactured in two trials from pasteurized milk artificially contaminated with *S. aureus* to the mean level of 6.243 log MPN mL^{-1}. *Lb. plantarum* BG33 was added at 1% as adjunct to the starter culture. The study was also carried out with control group cheeses produced without the adjunct culture. *S. aureus* counts were monitored for up to 90 days by BAM's 5-tube MPN method and each positive tube of MPN (most probable number) method was confirmed by PCR amplification of a 400 bp fragment of the *nuc* gene, which encodes the thermostable nuclease of *S. aureus*. The capacity of *Lb. plantarum* BG33 to reduce *S. aureus* count was found as 0.9 log unit on the 18th day of ripening. After 39 and 59 days of ripening, *Lb. plantarum* BG33 lowered *S. aureus* count by 1.9 and 2.0 log units, respectively, when compared to control group cheeses in which it was lowered by 0.5 and 1.0 log units, respectively. As a result, the BLIS activity of *Lb. plantarum* BG33 throughout ripening of white-brined cheese could make it useful as bioprotective adjunct culture in white-brined cheese production to prevent *S. aureus* growth which is an important foodborne pathogen in respect of safe cheese production.

Keywords: *Lactobacillus plantarum*; *Staphylococcus aureus*; White-brined cheese; Bacteriocin; Bio-control

1. Introduction

Traditionally manufactured cheeses especially white-brined cheeses made from thermized or raw cow's milk with a soft or semi-hard texture and a salty and acidic taste, play an important role in nutrition of people in Turkey besides being the most popular part of dairy products export of Turkey (Hayaloglu et al 2002; Temelli et al 2006). *Staphylococcus aureus* is a common commensal of the skin and mucosal membranes of humans. White-

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brined cheese production requires an extensive manual processing of the curd by the cheese-maker. This represents a possible route resulting in the risk of *S. aureus* contamination in white-brined cheeses and hence they could be a major cause of staphylococcal food poisoning (Rilla et al 2004; Mercanoglu Taban et al 2017).

Although there has been an improvement in cheese production facilities in dairy industries, *S. aureus* is still one of the leading pathogen that contaminates cheeses (Le Loir et al 2003; Charlier et al 2009). The frequency of *S. aureus* contamination in cheeses and the impact of staphylococcal food poisoning on public health have focused on the researches to control this pathogen. Therefore, the behaviour of *S. aureus* during production and ripening of some cheeses has been well studied (Nunez et al 1988) and many studies have been documented regarding the control of this pathogen by the direct application of bacteriocins (primarily nisin) to the cheeses (Abdalla et al 1993; Cintas et al 1998; Rilla et al 2004; Trmčić et al 2010). The use of bacteriocin-producing starter cultures in cheese production has gained the greatest interest from a food safety and human health point of view in addition with the consumers' preference of eating foods including minimum levels of chemically-synthesized additives (Rilla et al 2004). Besides, many papers have been published in combined use of various hurdles, including bacteriocins (Capellas et al 2000; Al-Holy et al 2012) or bacteriocin-producing cultures (Arques et al 2005) to inhibit foodborne pathogens in cheeses. Although the potential of bacteriocin-producing starters or adjunct cultures to control *Listeria* spp. in cheese production has been evaluated by a considerable amount of researches (Buyong et al 1998; O'Sullivan et al 2002; Rodriguez et al 2005), little has been known about the efficacy of using bacteriocin-producing starter or adjunct cultures on the growth and survival of *S. aureus* in cheese production (Rodriguez et al 2000; Rodriguez et al 2005; Favaro et al 2015), which may result in a better and economic way to control this pathogen in addition to the necessity of their contribution to the typical sensory characteristics and nutritional value of cheeses. For example; Rodriguez et al (2000) showed a reduction on the counts of *S. aureus* in a semi-hard cheese made with a nisin-producing starter and Rilla et al (2004) designed nisin-producing dairy starters to specifically inhibit *S. aureus* in acid-coagulated cheeses.

Considering the fact that there has still been technological drawbacks in using them as starters in industrial cheese production due to their poor acidifying, proteolytic and lypolytic characteristics and thus it is not easy to select appropriate strains having both strong technological and antimicrobial activities as starters in cheese production (Sarantinopoulos et al 2002; Favaro et al 2015), the present study aimed to evaluate antistaphylococcal ability of using autochthonous probiotic plantaricin-like bacteriocin producing *Lactobacillus plantarum* BG33 as adjunct culture in white-brined cheese production. The use of antistaphylococcal adjunct culture in white-brined cheese production like in this study is important in respect of dairy technology as well as food safety since there is an increasing amount of public demand for high-quality cheeses that are free of both pathogens and artificial additives.

2. Material and Methods

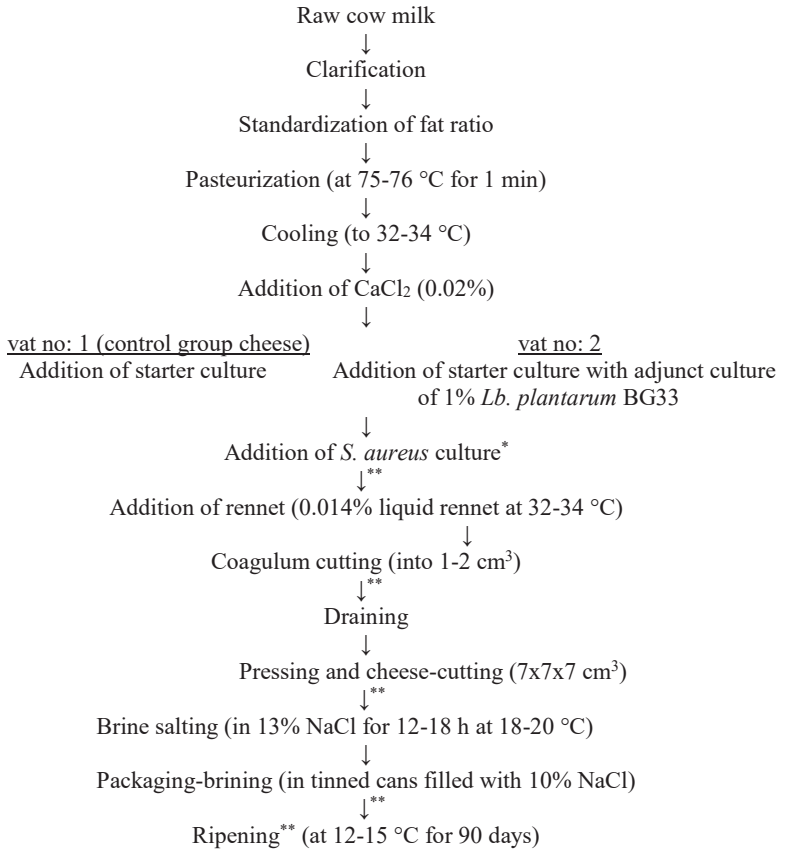
2.1. Cultures

Lb. plantarum BG33, previously isolated from traditional Turkish Tulum cheese and selected on the basis of its proven technological capability as adjunct culture in cheese production by Dr. M. Akcelik (Biology Department, Faculty of Science, Ankara University), were used as adjunct to the starter culture in white-brined cheese production in this study. It was cultivated routinely in MRS broth (De Man, Rogosa & Sharpe) (Merck, Germany) at 35-37 °C for 18-24 h and subcultivated twice in sterile reconstituted skim milk before use in white-brined cheese production. The synthesis of plantaricin-like bacteriocin (400 AU mL^{-1}) with low heat stability but high resistancy to lipase by *Lb. plantarum* BG33, was defined in the previous study,

including its antimicrobial and probiotic potential (Uymaz et al 2011). The reference strain of *S. aureus* ATCC 6538 was cultivated in tryptic soy broth (TSB; Merck, Germany) at 35-37 °C for 18-24 h and subcultivated twice in sterile reconstituted skim milk before use in white-brined cheese production. Frozen glycerol stock of each strain was made in sterile reconstituted skim milk supplemented with 30% glycerol and maintained at 80 °C. The initial number of strains used to inoculate pasteurized cow milk was determined by streaking on the appropriate media incubated at 35-37 °C for 18-24 h.

2.2. Cheese production

To study the antimicrobial ability of BLIS-producing *Lb. plantarum* BG33 to control *S. aureus* during production and ripening of white-brined cheeses, two independent vats of cheeses for each trial were conducted. Cheese production was carried out as given at Figure 1. White-brined cheeses were experimentally prepared from pasteurized cow's milk (at 75-76 °C for 1 min). After warming pasteurized milk to 32-34 °C, 0.02% $CaCl_2$ (Merck, Germany), commercial mesophilic homofermentative lactic acid bacteria (LAB) (mixture of *Lactococcus lactis* subsp.



*, Vat no:1 and vat no:2 were inoculated with *S. aureus* to the mean level of 6.243 log MPN mL^{-1}; **, steps of *S. aureus* counts (just after addition of *S. aureus* culture, coagulum cutting, pressing and moulding, and packaging-brining) and on the 1st, 6th, 18th, 25th, 39th, 59th, 80th, and 90th days of ripening.

Figure 1- The flow diagram of white-brined cheese production with adjunct culture, including the steps of *S. aureus* counts in this study

lactis and *Lc. lactis* subsp. *cremoris*, CHR Hansen R-708, Denmark) was added to the milk in each vat. Since vat no: 1 was served as control to determine the effect of production procedures for white-brined cheese on the growth of *S. aureus* added to milk at the start of the process, adjunct culture of *Lb. plantarum* BG33 was only added to the vat no: 2. In other words; control cheese from pasteurized milk was made without any LAB culture, just with *S. aureus* in vat no: 1. Then, vat no: 1 and vat no: 2 were inoculated with *S. aureus* to a final concentration of 6.243 log MPN mL^{-1}. Next, liquid rennet (CHR Hansen Naturen® Mandra 175, 175 IMCU mL^{-1}, Denmark) was used as a rate of 14 mL 100 L^{-1} milk to obtain coagulum within 90 min. After pressing, the cheese masses were divided into blocks of about 7x7x7 cm^3 and these blocks were salted in brine (13% NaCl for 12-18 h at 18-20 °C). The brined blocks were then placed in tinned cans filled with 10% NaCl and ripened at 12-15 °C for 90 days.

2.3. Staphylococcus aureus counts

In each trial, all cheeses were sampled at the production steps of just after addition of *S. aureus* culture, coagulum cutting, pressing-moulding, and packaging-brining and at the 1^{st}, 6^{th}, 18^{th}, 25^{th}, 39^{th}, 59^{th}, 80^{th}, and 90^{th} days of ripening. The counts of *S. aureus* were monitored by BAM's recommended 5-tube MPN method. Twenty five grams of each sample were mixed in sterile plastic bag for 1 min with 225 mL of 0.1% Butterfield's phosphate buffer in stomacher (Stomacher 400, the UK). One mL portions of decimal dilutions of each sample homogenate was inoculated into 5 tubes of tryptic soy broth (TSB) (Merck, Germany) containing 10% NaCl and 1% sodium pyruvate (Merck, Germany) and these tubes were incubated at 35-37 °C for 48 h. One loopful from each tube showing growth (turbidity) was spreaded onto the surface of prepared Petri plates on duplicate with Baird-Parker agar (Merck, Germany) and all plates were incubated at 37 °C for 48 h. At least 1 colony suspected to be *S. aureus* from each plate was transferred to TSB and was confirmed for *S. aureus* by polymerase chain reaction (PCR) amplification of a 400 bp region of the *nuc* gene.

2.4. PCR confirmation of S. aureus

The DNA isolation was performed as in the study of Mercanoglu Taban & Aytac (2009) with a high-pure PCR template preparation-HPPTP kit (Roche, Germany). The resulting template DNAs were subjected to PCR. Each PCR contained 4 mM $MgCl_2$ (with 1×PCR buffer, containing 10 mM Tris, 50 mM KCl, pH 8.3) (Roche, Germany), 200 µM of dNTP mix, each PCR primer at a concentration of 0.4 µM [based on the sequence of thermostable nuclease gene (*nuc*), F166: (5′- AGT TCA GCA AAT GCA TCA CA-3′) and R565: (5′-TAG CCA AGC CTT GAC GAA CT-3′) Cremonesi et al (2005)] (Roche, Germany), 0.04 µM 5 U μL^{-1} FastStart Taq DNA polymerase (Roche, Germany), and 3 µL target DNA. The final volume was adjusted to 50 µL by adding sterile ultrapure water. DNA amplification was performed in Primus 96 thermal cycler (THE-MWG, Germany) using the following conditions: initial denaturation for 5 min at 95 °C followed by 35 cycles of denaturation (95 °C for 30 s), annealing (56 °C for 30 s), and extension (72 °C for 30 s). A final extension step (72 °C for 7 min) was performed after the completion of the cycles. As positive control, PCRs containing template DNA extracted from the reference strain *S. aureus* ATCC 6538 was carried out. Some PCRs received ultrapure water instead of template DNA to provide negative control. Aliquots of the PCR products, along with a 100-bp GeneRuler DNA ladder plus (ready-to-use, Fermentas, Lithuania), were loaded into 1% agarose gel (Sigma-Aldrich, the USA) containing ethidium bromide (1 mg mL^{-1}-Invitrogen, the USA) and submitted to electrophoresis in Trisborate EDTA buffer for 40 min at 125 V. The amplified DNA fragments were visualized with InGenius gel visualization and analysis system (Syngene, the UK). The expected size of the *nuc* PCR product is 400 bp.

2.5. Statistical analysis

Comparison of means by "T test, Mann-Whitney, one way ANOVA, and Kruskal-Wallis" tests were performed using SPSS 11.5 program (SPSS Inc., the USA). Statistically significant comparative results are achieved when the significance level was $P<0.01$.

3. Results and Discussion

Pasteurized milk in vat no: 1 and vat no: 2 were inoculated with *S. aureus* to the mean level of 6.243 log MPN mL^{-1}. *S. aureus* count in pasteurized milk inoculated with 1% adjunct culture of *Lb. plantarum* BG33 in vat no: 2 was firstly increased only 0.4 log unit from pasteurized milk to 1-day-old cheese and then reduced by 0.9 log unit (to the mean level of 5.309 log MPN mL^{-1}) on the 18th day (432 hours) of ripening whereas it was increased 0.7 and 0.3 log units from pasteurized milk to 1-day-old and to 18 days (432 hours) of ripened control group cheeses, respectively. After 39 days (936 hours) of ripening, *Lb. plantarum* BG33 lowered *S. aureus* count by 1.9 log units with respect to control group cheeses in which it was lowered by only 0.5 log units (Figure 2). According to the analysis of variance, *S. aureus* counts in white-brined cheese were influenced ($P<0.01$) by addition of adjunct culture of *Lb. plantarum* BG33 during the ripening period. This is an important reduction unit when it is considered that there is always a risk of enterotoxin accumulation at high levels of *S. aureus* contamination in foods (Lindqvist et al 2002; Akineden et al 2008; Mercanoglu Taban et al 2017).

According to the results obtained in this study, the amount of plantaricin-like bacteriocin produced by *Lb. plantarum* BG33 was sufficient enough for the inhibition of high levels of *S. aureus* cells present in white-brined cheeses by 1.9 log units although Abdalla et al (1993) concluded that *S. aureus* shows reduced sensitivity to bacteriocins in food matrices. Sarantinopoulos et al (2002) also concluded that the complex environment of Feta cheese, which is very similar to white-brined cheese, thoroughly interferes with bacteriocin production levels of bacteriocinogenic starter or co-cultures and there is no guarantee for their in situ antimicrobial efficiency. This is also confirmed by the study of Uymaz et al (2011) who demonstrated a greater and broad inhibitory activity of *Lb. plantarum* BG33 against all the tested indicator strains, including *S. aureus*, by the agar overlay assays. In addition, it might be considered that the antimicrobial effect of this adjunct culture might show more inhibitory effect than the effect obtained in this study if the milk was contaminated with lower levels of *S. aureus*. Therefore; the high counts of *S. aureus* (mean level of 4.380 log MPN mL^{-1}) in white-brined cheeses produced by autochthonous probiotic strain

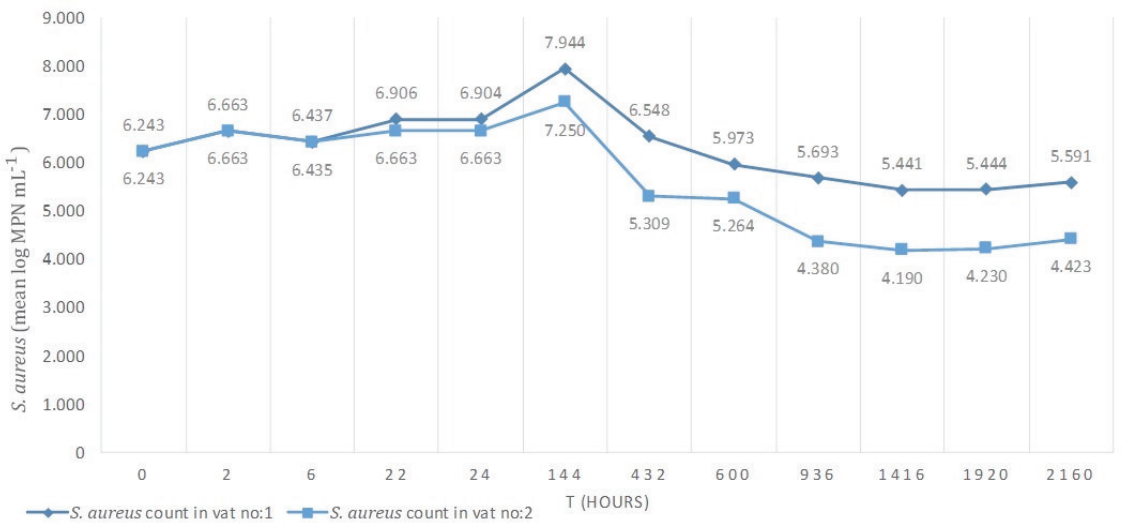


Figure 2- Survival of *S. aureus* (mean log MPN mL^{-1}) in control group cheeses (vat no: 1) and in white-brined cheeses produced with 1% adjunct culture of *Lb. plantarum* BG33 (vat no: 2)

of *Lb. plantarum* even at the 39th days (936 hours) of ripening can be based on the high inoculum level which was several log units above the levels that could be expected in naturally contaminated milk.

Beyond the direct use of bacteriocins as functional ingredients for the biopreservation of cheeses, nisin-producing lactococci have been of interest in the development of protective starter or adjunct cultures for cheese production due to their broad inhibitory activity. Therefore, most of the studies on the use of bacteriocin-producing cultures during cheese production related to them for the retardation of late gas blowing in Swiss style cheeses (O'Sullivan et al 2002). As an example; although Abdalla et al (1993) showed that *S. aureus* was not inhibited by nisin during production of white-brined cheeses from pasteurized milks and Cintas et al (1998) reported a very scarce inhibition of *S. aureus* by nisin A and pediocin PA-1, nisin-producing *Lc. lactis* ESI 515 which was also used as adjunct culture in cheese production was found to lower *S. aureus* count by 0.64 log units on the 30th day of cheese ripening (Rodriguez et al 2005), but a complete elimination of this foodborne pathogen was only achieved when nisin was added to process cheese spreads (Zottola et al 1994). In other words, Zottola et al (1994) showed significant reductions in numbers of *Clostridium sporogenes*, *Listeria monocytogenes*, and *S. aureus* when they used nisin-producing transconjugants of *Lc. lactis* ssp. *cremoris* JS102 and *Lc. lactis* ssp. *lactis* NCDO 1404 as starters in Cheddar cheese production. On the other hand in our study, 1.9 log unit reduction on *S. aureus* count was achieved by using adjunct culture of *Lb. plantarum* BG33 in white-brined cheese on the 39th days of ripening which was far beyond better than the reduction units obtained by transformant strain *Lc. lactis* CL1 of Rodriguez et al (2005) and by nisin-producing *Lc. lactis* TAB 50 of Rodriguez et al (2000) since these cultures showed 0.98 and 0.82 log units reduction on *S. aureus* counts in cheeses only after 30 days of ripening, respectively.

Likewise in our study, Rodriguez et al (2000) found that *S. aureus* count was firstly increased from pasteurized milk to 1-day-old semi-hard cheese, and then was decreased. On the contrary, El-Kholy et al (2014) evaluate the inhibition capacity of probiotic strains of *Lb. acidophilus* La-5 and *Bifidobacterium longum* ATCC 15707 on the growth of *S. aureus* and *Escherichia coli* O157:H7 during Domiati cheese production and storage and found that *Lb. acidophilus* La-5 reduced *S. aureus* and *E. coli* O157: H7 populations in Domiati cheese by about 3 and 1.88 logs after 14 days of storage, respectively, whereas *B. longum* ATCC15707 reduced *S. aureus* and *E. coli* O157: H7 populations in cheese by about 1.7 and 0.88 logs after 14 days of storage, respectively, compared with the control cheeses. Hence, we also showed almost the same reduction levels of *S. aureus* with the probiotic culture of *Lb. plantarum* BG33 in our study.

4. Conclusions

Considering the consumers' current demand for zero tolerance concerning the risk for foodborne pathogen contamination in dairy products, the use of antistaphylococcal starter cultures or adjunct cultures as an alternative to chemical additives in cheese production is a point of crucial importance both in respect of dairy technology as well as food safety. Therefore in this study, the use of BLIS-producing *Lb. plantarum* BG33 that was previously proven to have technological capability as adjunct culture in cheese production, to control growth and survival of *S. aureus* during white-brined cheese production and ripening was investigated. This study demonstrates the potential application of plantaricin-like bacteriocin-producing autochthonous probiotic strain of *Lb. plantarum* BG33 as adjunct culture in safe cheese production which is of crucial importance. Although this strain can slightly inhibit *S. aureus* growth in white-brined cheeses, due to the risk of enterotoxin production in cheese and of subsequent human intoxication, its inhibition potential of the expression of enterotoxin genes of *S. aureus* and antimicrobial potential in the control of other foodborne pathogens can also further be investigated.

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