Genotypic identification of some lactic acid bacteria by amplified fragment length polymorphism analysis and investigation of their potential usage as starter culture combinations in Beyaz cheese manufacture

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ABSTRACT

In this study, 2 different starter culture combinations were prepared for cheesemaking. Starter culture combinations were formed from 8 strains of lactic acid bacteria. They were identified as *Lactococcus lactis* ssp. *lactis* (2 strains), *Lactobacillus plantarum* (5 strains), and *Lactobacillus paraplantarum* (1 strain) by amplified fragment length polymorphism analysis. The effects of these combinations on the physicochemical and microbiological properties of Beyaz cheeses were investigated. These cheeses were compared with Beyaz cheeses that were produced with a commercial starter culture containing *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp. *cremoris* as control. All cheeses were ripened in brine at 4°C for 90 d. Dry matter, fat in dry matter, titratable acidity, pH, salt in dry matter, total N, water-soluble N, and ripening index were determined. Sodium dodecyl sulfate-PAGE patterns of cheeses showed that α _S-casein and β -casein degraded slightly during the ripening period. Lactic acid bacteria, total mesophilic aerobic bacteria, yeast, molds, and coliforms were also counted. All analyses were repeated twice during d 7, 30, 60, and 90. The starter culture combinations were found to be significantly different from the control group in pH, salt content, and lactobacilli, lactococci, and total mesophilic aerobic bacteria counts, whereas the cheeses were similar in fat, dry matter content, and coliform, yeast, and mold counts. The sensory analysis of cheeses indicated that textural properties of control cheeses presented somewhat lower scores than those of the test groups. The panelists preferred the tastes of treatment cheeses, whereas cheeses with starter culture combinations and control cheeses had similar scores for appearance and flavor. These results indicated that

both starter culture combinations are suitable for Beyaz cheese production.

Key words: starter culture, lactic acid bacteria, amplified fragment length polymorphism analysis, Beyaz cheese

INTRODUCTION

White-brined cheeses (also known as white-pickled cheeses) are the most popular varieties of cheeses manufactured in the Northeast Mediterranean area and the Balkans. Such cheeses include Beyaz Peynir (Turkey), Beli Sir U Kriskama (Yugoslavia), Bjalo Salamureno Sirene (Bulgaria), Feta (Greece), Teleme (Romania), Domiati (Egypt), and Halloumi (Cyprus). Turkish Beyaz cheese is a soft or semi-hard cheese that is manufactured from raw sheep's milk, raw cow's milk, or a mixture of the two and is then ripened in brine. Salt content is the main difference in the cheese varieties produced in Northern European countries. Traditionally, Beyaz cheese is manufactured from raw milk in small dairy plants or on farms and at private houses (Bintsis and Papademas, 2002; Hayaloğlu et al., 2002; Öner et al., 2006a). Starter culture is not added to the cheese; therefore, natural lactic acid microflora predominate over the other microbial groups during ripening and may regulate the ripening characteristics (Öner et al., 2006a). The produced cheeses are consumed after a 3-mo ripening period. This ripening period is also necessary for pathogen inhibition.

Because the demand for Beyaz cheese has rapidly increased during the past 3 decades, Beyaz cheese is now produced in large dairies. In industrial-scale production, pasteurization of raw milk is essential for cheese-making. However, the pasteurization process destroys pathogens as well as the indigenous flora of raw milk. In cheeses that are made from pasteurized milk, the formation of flavor components delays or develops at a low level (Yılsay, 2000). Although several imported

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commercial starter cultures have been commonly used for the manufacture of Beyaz cheese and they contain subspecies of *Lactococcus lactis*, a combination of lactococci, and even yogurt cultures (Dağdemir et al., 2003; Dağdemir and Özdemir, 2008; Başığit Kılıç et al., 2009), presently there is still no commercial starter culture production in Turkey. In plants using starter cultures, there are problems with culture usage. The main problem is that only a few studies have investigated the suitability of imported cultures to Turkish Beyaz cheese. In such cases it is unavoidable to have cheeses of different quality on the market. Cheeses with these starter cultures do not satisfy the expectations of the consumer because traditional Beyaz cheese contains a wide variety of lactic acid bacteria (Kayagil, 2006). For this reason, the composition of the lactic bacterial flora of Turkish Beyaz cheese was investigated in several research studies. In a study conducted on Beyaz cheese, *Lc. lactis* ssp. *lactis* and enterococci (*Enterococcus faecalis* and *Enterococcus faecium*) were the predominant species at the beginning of ripening, whereas *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Leuconostoc lactis*, and *Leuconostoc mesenteroides* ssp. *dextranicum* were seldom present. The presence of lactococci declined during ripening whereas *Lactobacillus* species such as *L. casei* and *L. plantarum* became predominant (Karakuş et al., 1992; Öner et al., 2006a; Dağdemir and Özdemir, 2008). Tzanetakis and Litopoulou-Tzanetaki (1992) also reported that lactobacilli (especially *L. plantarum*) predominated over enterococci and pediococci in Feta cheese during ripening.

The effects of starter culture addition on Turkish Beyaz brined cheese were investigated in several studies (Karakuş and Alperden, 1995; Dağdemir et al., 2003; Hayaloğlu et al., 2004, 2005; Goncu and Alpken, 2005; Kesenkas and Akbulut, 2006). In these studies, mixed mesophilic or thermophilic starter cultures (i.e., in different ratios *Lactococcus* plus *Lactococcus* or *Lactococcus* plus *Lactobacillus*) from several culture collections and even yeasts or lyophilized commercial cultures such as direct vat set were used for Beyaz cheesemaking. In another study on Beyaz cheese, the use of single strains of *Lactococcus* as a starter in cheese manufacture was investigated. The performance and relative contribution of 4 different single strains of lactococci on ripening characteristics and grading of Beyaz cheese during ripening were compared by Hayaloğlu et al. (2007). Although some efforts have been made in the determination of the natural flora, only a limited number of investigations have been conducted regarding the development of starter cultures from this flora for the manufacture of Beyaz cheese (Kayagil, 2006).

In this study, 8 lactic acid bacteria strains isolated from traditional cheeses were identified genotypically by 16S rRNA gene sequence analysis and combined to form 2 different starter cultures. The effects of these starter culture combinations on the properties of Turkish Beyaz cheese were then evaluated from manufacture to the end of the ripening period.

MATERIALS AND METHODS

Source of Lactic Acid Bacteria

A total of 8 lactic acid bacteria were used in this study. These strains were previously isolated from different cheese samples and chosen from 135 lactobacilli, lactococci, and enterococci isolates (Öner et al., 2006b) based on the following criteria. Identification was carried out by biochemical tests (Kandler and Weiss, 1986). Lactic acid production ability of single-strain cultures during growth in skim milk for 6 and 24 h, antimicrobial activity against *Micrococcus luteus*, proteolytic activity in skim milk for 24 h, biogenic amine, and production of flavor compounds were determined. The best 8 strains were chosen according to acid production, hydrolysis of milk proteins in sterile skim milk, production of low contents of biogenic amine, and best flavor compounds for Beyaz cheese production.

Identification of Starter Cultures

Molecular characterization of 8 bacterial strains was further performed by amplified fragment length polymorphism analysis (AFLP) at Ghent University (Belgium). The cultures were cultivated and checked for purity after incubation at 28°C for 24 h under anaerobic conditions on M17 agar base (Oxoid CM785, Cambridge, UK) supplemented with 0.5% (wt/vol) lactose for lactococci or de Man, Rogosa, and Sharpe agar (MRS; Oxoid CM361) for lactobacilli. All cultures showed homogeneous growth. A well-isolated single colony was used as starting material for analysis. Genomic DNA was isolated by the method of Gevers et al. (2001) and digested simultaneously by 4-base cutter and 6-base cutter restriction enzymes. Using this method, only a limited number of fragments of suitable size with 2 different cohesive ends were obtained. Small double-stranded DNA molecules (15–20 bp) containing 1 compatible end were ligated to the appropriate sticky end of the restriction fragments. Both adaptors were restriction half site-specific. These adaptors serve as binding sites for PCR primers. In the analysis, *EcoR* I (hexacutter) and *TaqI* (tetracutter) were used as restriction enzymes and the adaptors 5'-CTCGTA-

GACTGCGTACC-3'; 3'-CTGACGCATGGTTAA-5' and 5'-GACGATGAGTCCTGAC-3'; 3'-TACTCAG-GACTGGC-5' were used for *EcoR* I- and *TaqI*-digested ends of fragments, respectively.

The PCR primers were designed according to adaptor sequences and selective amplification of restriction fragments was accomplished with the use of single selective nucleotides. The PCR primers were hybridized to the adaptors and the selective base, extending beyond the restriction site into the fragment on the 3' end, led to the amplification of restriction fragments that had the appropriate complementary base adjacent to the restriction site. The primer combinations E01: 5'-GACTGCGTACCAATTCA-3' and T01: 5'-CGATGAGTCCTGACCGAA-3' were used for the hybridization of *EcoR* I- and *TaqI*-specific adaptors, respectively.

The PCR products were separated on a high-resolution polyacrylamide gel using a DNA sequencer (ABI 377, GMI, Dusseldorf, Germany). Fragments that contained an adaptor specific to the restriction half site created by the 6-bp cutter were visualized because of the 5'-end labeling of the corresponding primer with the fluorescent dye FAM. The resulting electrophoretic patterns were tracked and normalized using GeneScan 3.1 software (Applied Biosystems, Norwalk, CT). Normalized tables of peaks, containing fragments of 50 to 536 bp, were entered into BioNumerics 4.5 software (Applied Maths, Sint-Martens-Latem, Belgium). For numerical analysis, a data interval was delineated between the 75- and 500-bp bands of the internal size standard. Phylogenetic data analysis was based on band matching using the Dice coefficient and the UP-GMA (unweighted pair group method with arithmetic mean) algorithm. The profiles were compared with the reference profiles of the lactic acid bacteria taxa (including bifidobacteria) that were currently available in the database (Ghent, Belgium).

Starter Culture Composition

To enable culture rotation during industrial-scale cheesemaking, 2 different starter culture combinations were prepared by using 8 strains of lactic acid bacteria. Combination 1 consisted of 3 strains of *L. plantarum* (16H, 23, and 87A) and *Lc. lactis* ssp. *lactis* YML62; combination 2 consisted of 2 strains of *L. plantarum* (1A and AK57B), *L. paraplantarum* 58A, and *Lc. lactis* A11i. A commercial starter culture (Wiesby Starter Cultures and Media, Niebuß, Germany) was used for the manufacture of the control cheeses. Before use, each lactococci strain was cultivated in M17 broth (Merck, Darmstadt, Germany) at 30°C for 24 h with 2 consecutive transfers [1%, (vol/vol), inoculum]; each lactobacilli

strain was grown in MRS broth at the same conditions. For starter propagation, the cultures were transferred to 10% reconstituted skim milk that had been sterilized at 110°C for 15 min. After incubation, an equal volume from each strain was taken and mixed to obtain the combinations under aseptic conditions.

Cheesemaking and Sampling

Raw cow's milk was clarified and pasteurized at 68°C for 15 min and cooled to 35°C. It was then divided into 2 equal parts and inoculated with starter culture combination 1 and commercial starter culture separately at 1% level (experiment 1). The same procedure was repeated for the production of combination 2 cheeses (experiment 2). Calcium chloride was added to the milk at a level of 0.2 g/L. During all stages of cheesemaking, precautions were taken to avoid cross-contamination. The inoculated milk (32°C) was held for approximately 30 min (until pH 6.3). The milk was coagulated by adding calf rennet (Mayasan, Istanbul, Turkey) for 90 min. The coagulum was cut into cubes (2 cm³) and the curds were allowed to rest in the whey for 5 to 10 min. The curds were pressed until whey drainage stopped. The pressure was provided by 20-kg weights for each 100 kg of cheese milk (Yetişmeyen, 1995). The weights were then removed and the cheese was cut into 7-cm³ blocks. The blocks were placed in brine (14 g/100 mL of NaCl) for 2 h. Tin cans were filled with cheese (2 × 500 g) and brine and then closed. The cheese was ripened in the cans at 4°C for 90 d. Cheese manufacture was performed in duplicate. Cheese samples were taken after 7, 30, 60, and 90 d of ripening (Öner et al., 2006a).

Chemical and Microbiological Analysis

The percentages of DM, fat, fat in DM, salt, and salt in DM were determined according to the Turkish Standards (TS, 1978, 1986, 1995). Total N and water-soluble N (WSN) contents were determined by the Kjeldahl method (Bayrakli, 1987). The ripening index (RI) was calculated from the ratio of soluble N to total N (Alais, 1984). The pH values of the cheeses were measured with a pH meter (Hanna Instruments, Woonsocket, RI). All physicochemical analyses were performed in duplicate. Microbial analysis was performed according to Gobetti et al. (1999) and Karahan et al. (2002). Polyacrylamide gel electrophoresis was performed using the method of Laemmli (1970). Sample preparations were made as described by Ong et al. (2006).

Sensory Analysis

The cheeses were graded at 7, 30, 60, and 90 d of ripening by 6 graders (from the permanent staff at the

Table 1. Identification results of lactic acid bacteria by classical and amplified fragment length polymorphism analysis (AFLP) methods

Strain	Name after classical phenotypic analysis	Name after AFLP analysis
16H	<i>Lactobacillus</i> sp.	<i>L. plantarum</i>
87A	<i>Lactobacillus casei</i>	<i>L. plantarum</i>
23	<i>Lactobacillus plantarum</i>	<i>L. plantarum</i>
58A	<i>Lactobacillus</i> sp.	<i>L. paraplantarum</i>
AK57B	<i>Lactobacillus</i> sp.	<i>L. plantarum</i>
1A	<i>Lactobacillus casei</i>	<i>L. plantarum</i>
A11i	<i>Lactococcus lactis</i> ssp. <i>lactis</i>	<i>Lc. lactis</i> ssp. <i>lactis</i>
YML62	<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>Lc. lactis</i> ssp. <i>lactis</i>

Food Engineering Department, SDU, Isparta, Turkey) familiar with Beyaz cheese. They graded the cheeses for appearance (0–20 scale), body and texture (0–35 scale), odor (0–10 scale), and flavor (0–35 scale) according to the Turkish Standards for Beyaz cheese as described in TS (TS, 1995).

Statistical Analysis

Multifactor ANOVA was used to compare the values of each microbiological and physicochemical change of cheese samples made using 2 types of cultures (combination 1 and combination 2) for 2 separate experiments at each sampling day. Multivariate test was used for comparing the data from the different ages of the cheese samples made with 2 types of culture. The least significant differences were obtained using Duncan's multiple-comparison test ($P < 0.05$). Statistical analysis was performed using SPSS (version 10.0, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Identification Results of Lactic Acid Bacteria

Lactic acid bacteria that were used in starter culture combinations were phenotypically identified according to the reported scientific studies for *Lactobacillus* (Kandler and Weiss, 1986) and for *Lactococcus* (Bottazzi, 1993) species, and their technologically important properties were determined as shown in a previous study (Öner et al., 2006b). All lactobacilli were found to be facultative heterofermentative. Three of them were characterized up to the species level and the rest were identified only at the genus level. Cluster analysis with all available profiles of reference strains in the present lactic acid bacteria (LAB) database enabled 16H, 87A, 23, AK57A, and 1A to be identified as *L. plantarum*. The results also suggested that 58A probably belonged to *L. paraplantarum*. *Lactobacillus casei* and *L. plantarum* show highly similar phenotypes except for some carbohydrate fermentation properties

like mellibiose and raffinose (Kandler and Weiss, 1984). It is well known that biochemical identification results for LAB are often ubiquitous and unreliable, especially for strains that show atypical reactions; nevertheless, it is still an easy-to-use approach for identification of most common LAB species. One major reason for this mismatch might be ascribed to the loss or gain of plasmids because some carbohydrate fermentation capabilities are plasmid-encoded (Ahrné et al., 1989; Aquilanti et al., 2007). The lactococci strains used in the study were all identified as *Lc. lactis* ssp. *lactis* by traditional phenotypic tests.

Currently, species identification of lactic acid bacteria often relies on determination of the phylogenetic position using 16S rRNA gene sequence analysis and further genotypic or phenotypic comparison with nearest neighbors. With regard to molecular characterization, AFLP was found to be effective in both differentiation and characterization of isolates. Given the high economic importance of lactic acid bacteria, the availability of methodologies able to characterize univocally the single species to the strain level is important. The strains within different subspecies are well distributed to different clusters in dendrogram analysis (Busconi et al., 2008). *Lactobacillus plantarum* isolates were clearly differentiated at the strain level, with a minimum similarity level of 65% and a maximum of 85%. The isolate 58A was clustered in *L. paraplantarum* reference strains and found to be distantly related to other *L. plantarum* isolates at a level of 55%. *Lactococcus lactis* ssp. *lactis* isolates were also well separated, with a similarity level of 65%. Summaries of both methods are given in Table 1 and the dendrograms of the AFLP patterns are shown in Figures 1 and 2.

Similar studies were performed for the identification and classification of lactic acid bacteria isolated from fermented foods as well as from the human gastrointestinal tract (Gancheva et al., 1999; Kunene et al., 2000; Ventura and Zink, 2002; Busconi et al., 2008). In these studies, AFLP technique was also applied to evaluate the biodiversity and origin of lactic acid bacteria populations.

Figure 1. Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of amplified fragment length polymorphism analysis fingerprint profiles from reference strains of *Lactobacillus plantarum*, phylogenetically related species, and lactobacilli isolates.

Identification of lactic acid bacteria by phenotypic and especially genotypic methods and determination of their technologic properties were important for selection of starter cultures to produce Beyaz cheese as well as other fermented food products. Therefore, the studies on lactic acid bacteria concentrated on this subject. A similar study performed by Durlu-Özkaya et al. (2001) characterized isolates of lactic acid bacteria from Beyaz

cheese made from raw ewes' milk without any starter cultures and determined some of their technologically important properties. Lactic acid bacteria from Beyaz cheese consisted of lactococci, enterococci, and lactobacilli. Seventy-seven isolated lactic acid bacteria were classified with phenotypic criteria and SDS-PAGE of whole-cell proteins. It was also suggested that the technological properties of lactococci and lactobacilli that

Figure 2. Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of amplified fragment length polymorphism analysis fingerprint profiles from reference strains of *Lactococcus lactis* ssp. *lactis* and lactococci isolates.

may contribute to ripening characteristics of the cheese were important as well as identification results.

Chemical and Microbiological Properties of Cheeses

To obtain 2 different starter cultures and to evaluate their effects on the properties of Beyaz cheese, data from chemical and microbial analysis are presented separately for each combination in Tables 2 and 3. During ripening, the DM, fat, and fat in DM levels remained almost stable ($P > 0.05$) for all experimental cheeses; however, salt, salt in DM, total N, and WSN levels and RI changed significantly ($P < 0.05$). These results are in agreement with many studies (Karakus and Alperden, 1995; Tzanetakis et al., 1995; Shakeel-Ur-Rehman et al., 1999; Hayaloğlu et al., 2004). Combination 1 had an important effect ($P < 0.05$) on the development of acidity in comparison with the commercial starter culture, whereas combination 2 showed the same effect at the end of the ripening period. Acid development is a very important criterion in the manufacture of cheeses ripened in brine because of the inhibitory effect of lactic acid on undesirable microorganisms and curd stability during brining (Abd El-Salam et al., 1993; Hayaloğlu et al., 2005). Both combinations developed acidity faster than the commercial starter culture. According to TS 591 (TS, 1995), pH is required to be higher than 4.5. All samples were in accordance with Turkish Standards for Beyaz cheese.

As a result of salt diffusion into the cheese, salt contents of all cheese groups increased significantly with ripening time ($P < 0.05$). However, the salt contents of samples were higher than given in TS 591 (TS, 1995) after 30 d because cheese samples began to melt.

The level of WSN compounds and total N gives information on cheese maturity or quality (Hayaloğlu, 2007). For the 7-d-old cheeses, the WSN contents were 0.18 to 0.19 in experiment 1 and 0.13 to 0.19 in experiment 2. However, in experiments 1 and 2, these quantities reached 0.37 to 0.40 and 0.89 to 0.46 in 90-d-old samples. As ripening proceeded, a rapid increase in WSN was observed in the 90-d-old control sample of experiment 1. Depending on ripening throughout storage, WSN also increased ($P < 0.05$). At the end of ripening, WSN levels and RI were the highest in the control cheeses of experiment 2. These cheeses began to melt after 30 d and turned rancid at the end of storage period. Although the same commercial starter culture was used for both experiments, these differences could be because of milk quality. In experiment 2, combination 2 cheeses also melted slightly; however, they did not turn rancid and were consumable. The different starter culture combinations had no significant effect on the level of WSN in the cheeses at any stages of

ripening ($P < 0.05$). This indicates that the starter organisms in combinations do not make a direct contribution to the WSN content in cheese, as reported by Hayaloğlu et al. (2005).

The dynamics of the microbial groups were examined throughout ripening. As shown in Tables 2 and 3, lactobacilli, lactococci, and total mesophilic aerobic bacteria (log cfu/g) continued their presence in cheese. Lactobacilli levels displayed significant differences ($P < 0.05$) from control cheeses over the course of ripening. On d 7, *Lactobacillus* counts were highest in the cheeses to which the culture combinations had been added. *Lactobacillus* counts decreased 1 log unit in combination 1 cheeses during the first 30 d of ripening and then remained constant at around 10^7 cfu/g. Levels of lactobacilli in experiment 2 cheeses also remained unchanged during the ripening period (Table 3). As shown by other authors (Peterson and Marshall, 1990; McSweeney et al., 1994), in conventional cheesemaking, starter lactic acid bacteria grow rapidly in cheese milk and curd during manufacture, reaching 8.0 to 9.0 log cfu g/L, but subsequently decline at a rate dependent on the strain to approximately 1% of the maximum number within 1 mo of ripening. The decrease in starter cell numbers during cheese ripening is caused by the low adaptability to the hostile environment in the interior of the cheese, which has low pH and a high salt content, lacks a fermentable carbohydrate, and may contain bacteriocins (Fox et al., 1996; Cagno et al., 2006).

There were no significant differences in lactococcal levels among the batches on d 7 in experiment 1 (Table 2). Significant differences ($P < 0.05$) were apparent from d 30. Counts of lactococci in control cheeses decreased slightly over the ripening period; however, in combination 1, cheeses remained almost stable. In experiment 2, there were significant differences between control and combination 2 cheeses ($P < 0.05$); however, the cell counts of each batch did not vary throughout ripening. This finding indicates that the lactobacilli and lactococci selected for use in the culture combinations added in these experiments survived and adopted well in the cheeses.

Coliforms, yeasts, and molds were not detected in any of the cheeses throughout the ripening period. The initial contamination by Enterobacteriaceae and coliforms could be lower because of the winter season when the contamination count is thought to be lower than in summer. On the other hand, as expected, pasteurization reduced coliforms levels and contaminations were prevented by taking sanitary precautions. The microflora present in the environment in cheesemaking plants is an important factor, particularly in the case of cheeses made from pasteurized milks (Casalta and Corté, 1997).

Table 2. Chemical and microbiological characteristics of experiment 1 cheeses during storage period

Item ¹	Day 7		Day 30		Day 60		Day 90	
	Control	Combination 1	Control	Combination 1	Control	Combination 1	Control	Combination 1
pH	4.66 ± 0.09 ^c	4.36 ± 0.08 ^d	5.12 ± 0.03 ^a	4.89 ± 0.13 ^b	4.96 ± 0.01 ^{ab}	4.63 ± 0.16 ^c	4.88 ± 0.04 ^b	4.65 ± 0.19 ^c
DM (%)	35.98 ± 1.96 ^a	35.61 ± 2.15 ^a	35.89 ± 3.59 ^a	36.35 ± 3.12 ^a	35.70 ± 2.86 ^a	37.26 ± 2.80 ^a	34.93 ± 2.38 ^a	35.46 ± 2.22 ^a
Fat (%)	18.50 ± 2.82 ^a	18.50 ± 2.82 ^a	18.50 ± 2.82 ^a	18.50 ± 2.82 ^a	18.00 ± 2.82 ^a	19.25 ± 3.88 ^a	19.25 ± 3.18 ^a	19.00 ± 3.53 ^a
FDM (%)	51.26 ± 5.06 ^a	51.79 ± 4.80 ^a	51.40 ± 2.73 ^a	50.74 ± 3.41 ^a	50.25 ± 3.88 ^a	51.41 ± 6.57 ^a	54.91 ± 5.36 ^a	53.37 ± 6.63 ^a
Salt (%)	2.05 ± 0.04 ^d	2.07 ± 0.05 ^{cd}	2.18 ± 0.18 ^{cd}	2.34 ± 0.03 ^{cd}	2.45 ± 0.16 ^{cd}	3.04 ± 0.33 ^b	2.50 ± 0.41 ^c	3.74 ± 0.33 ^a
SDM (%)	5.69 ± 1.10 ^d	5.81 ± 1.06 ^d	6.07 ± 5.61 ^{cd}	6.43 ± 2.31 ^{cd}	6.86 ± 0.00 ^{bcd}	8.15 ± 0.39 ^b	10.02 ± 2.17 ^{bc}	10.54 ± 0.13 ^a
Total N (%)	1.95 ± 0.03 ^{ab}	1.96 ± 0.10 ^{ab}	2.00 ± 0.26 ^{ab}	2.08 ± 0.02 ^a	1.72 ± 0.06 ^{cd}	1.93 ± 0.03 ^{ab}	1.58 ± 0.04 ^d	1.80 ± 0.07 ^{bc}
WSN (%)	0.18 ± 0.02 ^c	0.19 ± 0.01 ^{de}	0.21 ± 0.09 ^d	0.27 ± 0.01 ^c	0.27 ± 0.16 ^c	0.35 ± 0.14 ^b	0.37 ± 0.14 ^b	0.40 ± 0.16 ^a
RI	8.95 ± 0.02 ^e	9.41 ± 0.05 ^e	10.47 ± 0.18 ^{de}	12.98 ± 0.02 ^d	15.65 ± 0.11 ^c	18.08 ± 0.08 ^b	23.41 ± 0.09 ^a	22.16 ± 0.12 ^a
Lactobacilli (log cfu/g)	5.90 ± 0.25 ^c	8.93 ± 0.55 ^a	5.71 ± 0.07 ^c	7.23 ± 0.65 ^b	5.85 ± 0.84 ^c	7.40 ± 0.78 ^b	6.65 ± 0.49 ^{bc}	7.53 ± 0.59 ^b
Lactococci (log cfu/g)	8.70 ± 0.48 ^{ba}	9.65 ± 0.13 ^a	7.62 ± 1.01 ^{cd}	9.16 ± 0.27 ^{ba}	8.51 ± 0.05 ^{bc}		8.35 ± 0.08 ^{bcd}	9.06 ± 0.02 ^{ba}
TMAB (log cfu/g)	9.00 ± 0.00 ^a	8.51 ± 0.21 ^a	6.85 ± 0.74 ^b	7.63 ± 0.68 ^{ab}	7.83 ± 0.12 ^{ab}	7.59 ± 0.63 ^{ab}	8.20 ± 1.18 ^a	7.56 ± 0.47 ^{ab}

^{a-c}Means in the same row with different superscript letters are significantly different ($P < 0.05$).

¹FDM = fat in dry matter; SDM = salt in dry matter; WSN = water-soluble N; RI = ripening index; TMAB = total mesophilic aerobic bacteria.

Table 3. Chemical and microbiological characteristics of experiment 2 cheeses during storage period

Item ¹	Day 7		Day 30		Day 60		Day 90	
	Control	Combination 2	Control	Combination 2	Control	Combination 2	Control	Combination 2
pH	5.26 ± 0.08 ^a	5.25 ± 0.06 ^a	4.66 ± 0.09 ^b	4.65 ± 0.00 ^b	4.73 ± 0.08 ^b	4.67 ± 0.02 ^b	4.62 ± 0.00 ^b	4.50 ± 0.09 ^c
DM (%)	36.62 ± 1.49 ^b	39.95 ± 1.94 ^a	36.89 ± 0 ^b	38.16 ± 0.12 ^{ab}	37.74 ± 1.16 ^{ab}	38.78 ± 1.42 ^{ab}	38.00 ± 0.75 ^{ab}	36.61 ± 1.62 ^b
Fat (%)	20.37 ± 0.17 ^a	20.00 ± 2.47 ^{ab}	319.50 ± 0 ^{ab}	18.25 ± 0.35 ^b	19.50 ± 0 ^{ab}	18.75 ± 1.06 ^{ab}	19.50 ± 0 ^{ab}	18.75 ± 1.06 ^{ab}
FDM (%)	55.67 ± 1.78 ^a	49.96 ± 3.76 ^{bcd}	52.84 ± 0.00 ^{ab}	47.82 ± 0.77 ^d	51.68 ± 1.58 ^b	48.32 ± 0.96 ^{cd}	51.32 ± 1.02 ^{bc}	51.18 ± 0.62 ^{bc}
Salt (%)	2.98 ± 0.08 ^e	4.35 ± 0.04 ^{abc}	3.68 ± 0.41 ^d	4.14 ± 0.16 ^{bc}	3.69 ± 0.07 ^d	4.46 ± 0.12 ^{ba}	4.03 ± 0.24 ^{cd}	4.61 ± 0.33 ^a
SDM (%)	8.14 ± 0.78 ^d	10.88 ± 0.99 ^{bc}	9.99 ± 0.20 ^c	10.87 ± 0.14 ^{bc}	9.78 ± 0.61 ^c	11.52 ± 0.77 ^{ab}	10.62 ± 0.50 ^{bc}	12.60 ± 0.97 ^a
Total N (%)	2.57 ± 0.50 ^a	2.21 ± 0.13 ^{ab}	2.51 ± 0.52 ^{bc}	2.03 ± 0.25 ^{bc}	2.24 ± 0.50 ^{bc}	1.9 ± 0.33 ^{bc}	2.38 ± 0.01 ^{bc}	1.81 ± 0.26 ^{bc}
WSN (%)	0.16 ± 0.002 ^d	0.13 ± 0.04 ^e	0.18 ± 0.02 ^d	0.14 ± 0.001 ^e	0.44 ± 0.02 ^b	0.24 ± 0.01 ^c	0.67 ± 0.02 ^b	0.46 ± 0.02 ^b
RI	6.59 ± 0.25 ^d	5.73 ± 0.06 ^d	8.55 ± 0.05 ^d	6.69 ± 0.12 ^d	25.36 ± 0.045 ^b	12.63 ± 0.17 ^c	28.24 ± 0.01 ^b	25.69 ± 0.14 ^b
Lactobacilli (log cfu/g)	7.81 ± 0.13 ^b	8.45 ± 0.25 ^a	8.39 ± 0.71 ^a	8.68 ± 0.34 ^a	7.86 ± 0.00 ^b	8.68 ± 0.02 ^a	7.81 ± 0.07 ^b	8.84 ± 0.22 ^a
Lactococci (log cfu/g)	7.85 ± 0.20 ^b	8.85 ± 0.10 ^a	7.89 ± 0.01 ^b	8.91 ± 0.06 ^a	7.91 ± 0.11 ^b	8.90 ± 0.12 ^a	8.02 ± 0.25 ^b	8.91 ± 0.11 ^a
TMAB (log cfu/g)	7.90 ± 0.03 ^b	8.94 ± 0.06 ^a	7.89 ± 0.02 ^b	8.92 ± 0.06 ^a	7.96 ± 0.11 ^b	8.83 ± 0.02 ^a	7.96 ± 0.20 ^b	8.89 ± 0.05 ^a

^{a-c}Means in the same row with different superscript letters are significantly different ($P < 0.05$).

¹FDM = fat in dry matter; SDM = salt in dry matter; WSN = water-soluble N; RI = ripening index; TMAB = total mesophilic aerobic bacteria.

Figure 3. Degradation products during ripening in experiment 1. Lane 1: protein marker; lanes 2 to 5: control cheeses after 7, 30, 60, and 90 d of ripening, respectively; lanes 6 to 9: combination 1 cheeses after 7, 30, 60, and 90 d of ripening, respectively; lane 10: α_S -CN marker. MW = molecular weight.

SDS-PAGE Patterns

The SDS-PAGE electrophoretogram of the experiment 1 cheeses during ripening is shown in Figure 3. Casein hydrolysis was observed from 7 to 90 d for both β - and α_S -caseins in cheese samples. At the beginning of the ripening period, no differences were seen between intensities of casein fractions of cheeses. Hydrolysis of the caseins accelerated, and β - and α_S -caseins especially began to degrade slightly after 30 d. α_S -Casein was observed with a high intensity at the beginning of ripening, and α_S -casein hydrolysis was observed as being lower in both cheese samples at the end of ripen-

ing. These results do not seem to be in agreement with other findings (Katsiari et al., 2002; Poveda et al., 2003; Hayaloğlu et al., 2005; Tarakçı and Tuncturk, 2008). It has been established that β -casein is more resistant to proteolysis than α_S -casein, especially in the cheese matrix, either by calf rennet or starter enzymes, owing to its structure and particularly its tendency to associate (McSweeney et al., 1993). However, different findings regarding α_S -casein degradation rate in the literature are present because of the differences in milk used, manufacturing procedure, and ripening conditions (Fox et al., 1993). Another possible reason for this pattern is the formation of hydrolysis products (β -II peptides)

Table 4. Sensory analyses¹ of experiment 1 cheeses ($\pm$ SD)

Item	Day	Appearance	Texture	Odor	Flavor	Total score
Combination 1	7	18.000 $\pm$ 2.000 ^a	32.000 $\pm$ 1.000 ^a	8.000 $\pm$ 2.000 ^a	30.000 $\pm$ 2.000 ^{ab}	88.000 $\pm$ 7.000 ^{ab}
	30	20.000 $\pm$ 0.000 ^a	32.333 $\pm$ 1.154 ^a	9.000 $\pm$ 0.000 ^a	33.000 $\pm$ 0.000 ^a	94.333 $\pm$ 1.154 ^a
	60	18.666 $\pm$ 1.154 ^a	31.666 $\pm$ 2.886 ^a	9.333 $\pm$ 0.577 ^a	31.666 $\pm$ 2.886 ^a	91.333 $\pm$ 7.505 ^a
	90	19.323 $\pm$ 1.723 ^a	27.000 $\pm$ 1.732 ^{ab}	9.333 $\pm$ 1.154 ^a	27.000 $\pm$ 5.196 ^{ab}	82.656 $\pm$ 4.650 ^{abc}
Control	7	17.000 $\pm$ 1.000 ^{ab}	21.000 $\pm$ 2.645 ^{bc}	8.000 $\pm$ 0.000 ^a	23.000 $\pm$ 0.000 ^b	69.000 $\pm$ 2.000 ^{cd}
	30	16.000 $\pm$ 0.000 ^{ab}	19.333 $\pm$ 4.041 ^{cd}	9.000 $\pm$ 0.000 ^a	28.333 $\pm$ 4.041 ^{ab}	72.666 $\pm$ 7.637 ^{bcd}
	60	11.000 $\pm$ 2.645 ^b	12.000 $\pm$ 3.464 ^d	9.000 $\pm$ 0.000 ^a	26.333 $\pm$ 2.309 ^{ab}	58.333 $\pm$ 8.386 ^{de}
	90	15.000 $\pm$ 6.000 ^{ab}	12.666 $\pm$ 4.618 ^d	8.666 $\pm$ 0.577 ^a	16.000 $\pm$ 0.000 ^c	52.333 $\pm$ 9.712 ^e

^{a-e}Means in the same row with different superscript letters are significantly different ($P < 0.05$).

¹Sensory analyses included analysis of appearance (0–20 scale), texture (0–35 scale), odor (0–10 scale), and flavor (0–35 scale).

Table 5. Sensory analyses¹ of experiment 2 cheeses ($\pm$ SD)

Item	Day	Appearance	Texture	Odor	Flavor	Total score
Combination 2	7	20.000 $\pm$ 0.000 ^a	31.000 $\pm$ 6.928 ^a	9.000 $\pm$ 0.000 ^b	27.000 $\pm$ 1.732 ^a	87.000 $\pm$ 7.937 ^a
	30	19.333 $\pm$ 0.577 ^a	29.000 $\pm$ 5.196 ^a	10.000 $\pm$ 0.000 ^a	26.000 $\pm$ 0.000 ^{ab}	84.333 $\pm$ 5.773 ^a
	60	19.333 $\pm$ 0.577 ^a	29.000 $\pm$ 0.000 ^a	9.000 $\pm$ 0.000 ^b	26.000 $\pm$ 1.732 ^{ab}	83.333 $\pm$ 2.309 ^a
	90	16.000 $\pm$ 1.000 ^b	25.000 $\pm$ 0.000 ^a	10.000 $\pm$ 0.000 ^a	18.000 $\pm$ 5.000 ^c	69.000 $\pm$ 6.000 ^b
Control	7	19.000 $\pm$ 0.000 ^a	25.000 $\pm$ 1.732 ^a	9.333 $\pm$ 0.577 ^{ab}	28.000 $\pm$ 0.000 ^a	81.333 $\pm$ 1.154 ^a
	30	13.000 $\pm$ 0.000 ^c	10.000 $\pm$ 0.000 ^b	9.000 $\pm$ 0.000 ^b	20.666 $\pm$ 0.577 ^{bc}	52.666 $\pm$ 0.577 ^c
	60	11.000 $\pm$ 0.000 ^{cd}	8.666 $\pm$ 1.154 ^b	7.333 $\pm$ 0.577 ^c	23.333 $\pm$ 0.577 ^{abc}	50.333 $\pm$ 2.309 ^c
	90	10.333 $\pm$ 2.516 ^d	15.000 $\pm$ 0.000 ^b	9.000 $\pm$ 0.000 ^b	23.000 $\pm$ 3.000 ^{abc}	57.333 $\pm$ 5.507 ^c

^{a-d}Means in the same row with different superscript letters are significantly different ($P < 0.05$).

¹Sensory analyses included analysis of appearance (0–20 scale), texture (0–35 scale), odor (0–10 scale), and flavor (0–35 scale).

from β -casein with very similar electrophoretic mobilities to those of the α_S -casein bands and of densitometrical increase of the α_S -casein zone (Carretero et al., 1994; Trujillo et al., 2002). On the other hand, starter culture composition and salt content of Beyaz cheeses have an effect on β -casein degradation. In the white-brined cheeses, the principal proteolytic agents are the residual coagulant and enzymes from starters or the indigenous microflora. Tuncturk et al. (2008) found that breakdown of β -caseins in cheeses with different starter cultures (yogurt culture and helveticus culture) was higher than in the other experimental cheeses at the end of the ripening period. In another study, it was determined that accelerated salt concentration ($>5\%$) prevented hydrolysis of β -casein by chymosine (Topçu, 2004). In our study, salt concentration of cheeses was less than 5%; therefore, degradation of casein fractions may change.

Sensory Analysis

The mean sensory scores of the experimental cheeses are shown in Tables 4 and 5. No significant differences were determined between our starter culture combinations (1 and 2) and control cheeses at the first week except for the texture of the control cheeses in experiment 1. However, the graders reported that combination 1 cheeses had more flavor or flavor intensity throughout ripening period. At the end of ripening, bitter flavor and deterioration of texture were detected in the control cheeses in experiment 2. Control cheeses in both experiments received considerably lower total scores than the other cheeses after 30 d. A soft texture was determined in experiment 2 cheeses after 60 d of ripening; this may be linked to the degradation of α_{S1} -casein (Fenelon et al., 1999). Therefore, combination 1 and 2 cheeses revealed similar sensory scores and were significantly different ($P < 0.05$) from control cheeses. Both starter culture combinations were found to be suitable for Beyaz cheese manufacture.

CONCLUSIONS

The results clearly showed that the starter cultures affected the chemical and microbiological properties of Beyaz cheese during ripening. Starter culture combinations supported some important Beyaz cheese properties such as ripening characteristics, pH, and lactobacilli and lactococci counts. The RI of all groups was found to be similar at the end of the ripening period. β -Casein in experimental cheeses degraded and its amount decreased during ripening. All analyses showed that when their quality attributes were comparable to commercial starter culture, Beyaz cheeses with these combinations were satisfactory. Moreover, the sensory characteristics of these cheeses were rated higher than those of the control cheeses.

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