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Microbiological changes throughout ripening of Keş cheese

Mikrobiološke promjene tijekom zrenja Keş sira

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Abstract

Microbiological changes in Keş cheese, traditional Turkish raw cow's milk cheese made without the addition of starter culture, were studied during 90 days of ripening. Physico-chemical parameters, pH, salt content and water activity (a_w), as important parameters for microbial growth, were also determined. Lactic acid bacteria were predominant microbial group. The time of ripening significantly influenced the count of total aerobic mesophilic bacteria, psychrophilic bacteria, mould ($P<0.05$), *Lactobacillus* spp. and *Lactococcus* spp. ($P<0.01$) which increased during ripening. *E. coli* and coagulase-positive staphylococci were not detected. The counts of *Enterococcus* spp. ($P<0.05$), *Staphylococcus-Micrococcus* spp., *Enterobactericeae* and coliform bacteria decreased throughout ripening of Keş cheese, which was connected to the physico-chemical parameters. Water activity significantly decreased ($P<0.05$), while the salt content increased ($P<0.01$) during ripening of Keş cheese. The obtained results will contribute to the establishment of the microbiological quality standards for Keş cheese and give necessary information for formation of autochthonous starter culture, which will help to understand ripening process of sour dried cheeses and improve the traditional farmhouse cheese production.

Keywords: Keş cheese, microbiological changes, physico-chemical parameters, ripening, traditional cheeses

Sažetak

Mikrobiološke promjene u Keş siru, tradicionalnom turskom siru proizvedenom iz sirovog kravljeg mlijeka bez dodatka mljekarske mikrobne kulture, praćene su tijekom 90 dana zrenja. Također su određeni fizikalno-kemijski parametri odgovorni za

mikrobni rast, pH vrijednost, udio soli i aktivitet vode (a_w). Bakterije mliječne kiseline bile su dominantna mikrobna skupina. Trajanje zrenja značajno je utjecalo na ukupan broj aerobnih mezofilnih bakterija, psihrofilnih bakterija, plijesni (P<0.05), *Lactobacillus* spp. i *Lactococcus* spp. (P<0.01) te se njihov broj povećavao tijekom zrenja Keş sira. U siru nisu detekrirani *E. coli* i koagulaza pozitivni stafilokoki. Broj *Enterococcus* spp. (P<0.05), *Staphylococcus-Micrococcus* spp., *Enterobactericeae* i koliformnih bakterija smanjivao se tijekom zrenja Keş sira što se može dovesti u vezu sa fizikalno-kemijskim parametrima sira. Aktivitet vode se značajno smanjivao tijekom zrenja Keş sira (P<0.05), dok se udio soli povećavao (P<0.01). Dobiveni rezultati pridonijeti će uspostavljanju mikrobioloških standarda kakvoće Keş sira, pružiti informacije potrebne za definiranje njegove autohtone mikrobne kulture, te pomoći u razumijevanju procesa zrenja kiselinskih sušenih sireva i poboljšanju tradicionalne proizvodnje ovih sireva na malim gospodarstvima.

Ključne riječi: fizikalno-kemijski parametri, Keş sir, mikrobiološke promjene, tradicionalni sirevi, zrenje

Introduction

Keş is traditional Turkish cheese, originally brought from Central Asia to Turkey. It belongs to the sour, dried, cone shaped hard cheese with spices, and has slightly sour and pungent taste. It could be consumed with thin bread or added to soups or pasta (Çakir et al., 2009; Dervisoglu et al., 2009). Keş cheese is an artisanal cheese produced from raw milk by family farms and small dairy plants in Anatolia, Black Sea and the Mediterranean regions of Turkey. In some regions of Turkey, it is also known as “Keşk”, “Keşük”, “Kis”, “Çökelek” or “Kurut”. It is produced mostly from cow’s milk, although in the past, it was produced from sheep’s and goat’s milk. Its manufacturing procedure is not standardised and the production method depends on the region where it is produced. Beside from milk, in some regions it is produced from semi-fat yoghurt. Cheese-making involves the use of traditional tools and manual production practices without starter culture addition, so the existing microbial population in cheese is autochthonous and originates from milk and environment (Çakir et al., 2009; Kirdar, 2012).

Earlier studies of biochemical characteristics of Keş cheese were conducted on matured cheese ready for consumption (Çakir et al., 2009; Kirdar, 2012). Therefore, the aim of this study was to investigate changes in the distribution of the most representative microbial groups throughout ripening of Keş cheese as well as the most relevant physico-chemical parameters important for their growth. This information is expected to provide a scientific basis for understanding the microbiological characteristics and ripening process of sour dried variety of cheese.

Materials and methods

Cheese-making and sampling

Three batches of Keş cheese were produced from raw cow's milk. Each phase of cheese manufacturing was performed manually by traditional method. The sour coagulum for draining was obtained by lactic acid fermentation of raw cow's milk with no starter addition. The evening milk was left around 10-12 hours at room temperature (20 °C). After coagulation, the curd was put into cloth bags (a perforated, woven cloth made from 100% cotton) and allowed to drain (self-pressure) for 24 hours in a cool room (~15 °C), and then dry salted (cca 2%). The curd was kneaded manually for 5 minutes, afterwards black sesame (1%), capsicum (1%), black and red pepper (1%) were added. The curd was shaped into cubic and conical forms and dried 3-4 days under room temperature.

The Keş cheese was ripened 90 days at the temperature of 6±1 °C. Keş sampling was performed at the 1st, 30th, 60th and 90th days of ripening according to International Dairy Federation, IDF, Standard 122C (1996). The samples were transferred to the laboratory under refrigerated conditions (4 °C) and analysed immediately.

Microbiological analyses

Cheese samples (25 g) were homogenized using a Stomacher 400 with 225 ml of sterile buffered peptone water for at least 2 minutes. Decimal dilutions were prepared using the same diluent. Plate Count Agar (PCA) was used to determine total aerobic mesophilic bacteria and psychrophilic bacteria that had been incubated for 48 hours at 35 °C and 10 days at 7 °C, respectively (Peeler and Maturin, 1992). Baird-Parker agar was used for *Staphylococcus- Micrococcus* spp. at 35 °C for 48 hours. Koagulase-positive staphylococci on Baird-parker agar base supplemented with egg yolk tellurite emulsion, incubated at 37 °C for 48 h (AOAC, 2001). Violet Red Bile Dextrose Agar (VRBDA) was used for determination of *Enterobacteriaceae* which had been incubated at 37 °C for 48 hours. The count of the total number of coliforms was performed on standard Violet Red Bile agar (VRBA) that had been incubated at 35±1 °C for 24–48 hours (ICMSF, 1983; Marshall, 1992). Positive cultures were used to create sub-cultures on Eosin Methylene Blue Lactose Sucrose (EMBLS) agar. They were incubated at 35±1 °C for 24 hours. *Escherichia coli* isolates were biochemically characterized by IMVIC tests (MacFaddin, 1991). Potato Dextrose Agar (PDA) was used for determination of yeasts and moulds, which were incubated at 25 °C for 5 and 7 days, respectively (AOAC, 2001). Enterococci was grown on Slanetz-Bartley Agar plates (SBA) and incubated at 37 °C for 48 hours (Facklam and Sahm, 1995). De Man-Rogosa-Sharpe (MRS) agar was used for counting of *Lactobacillus* spp. that was incubated microaerobically in an anaerostat using Anaerocult A (Merck) at 37 °C for 48 h. *Lactobacillus* spp. was confirmed by biochemical tests (Dupont et al., 2000; Lopez-Diaz et al., 2000). M17 agar was used for counting *Lactococcus* spp. that was incubated at 30 °C for 48-72 hours (Terzaghi and Sandine, 1975).

Identification of the isolates was performed using the criteria of the Bacteriological Analytical Manual (AOAC, 2001). All of the media were obtained from Oxoid (Unipath

Ltd., Basingstoke, England). The analyses were carried out in triplicate in the Laboratory of Veterinary Faculty of Mehmet Akif Ersoy University.

Physico-chemical analyses

The pH of the samples was determined using a pH meter (Hanna instruments, Padova, Italy) and salt content was determined as suggested by the IDF (1979). The water activity (a_w) was determined using a water activity meter (TESTO-650). Duplicate measurements were conducted for each analysis.

Statistical analyses

All of the statistical analyses were performed using SPSS Statistical Software. The obtained values were presented as the mean ± standard error (SE). Evaluation of significance ($P<0.05$ and $P<0.01$) was performed using analysis of variance followed by Duncan's multiple range tests. Colony counts were converted to log CFU/g (Draper and Smith, 1998).

Results

Salt content, pH and a_w are important physico-chemical parameters which influence microbiological changes during the cheese ripening (Fox et al., 2000). The results of physico-chemical analyses are presented in Table 1. Acidification during coagulation induced the decrease of pH to an average value of 5.48 in the curd. The process of acidification continued during ripening, which except to the high production of lactic acid, is related to the low buffering capacity of the mass of the cheese. This is a consequence of the demineralization undergone during coagulation and the removal of the whey (Havranek et al., 2014). The value of a_w significantly ($P<0.05$) decreased during ripening of Keş cheese and it reached the final value of 0.89, while the salt content significantly ($P<0.01$) increased (Table 1).

Table 1. Physico-chemical properties of Keş cheese throughout ripening

Parameter	Ripening time (days)			
	1	30	60	90
a_w	$0.943\pm0.03^{*a}$	$0.935\pm0.05^{*ab}$	$0.916\pm0.05^{*ab}$	$0.889\pm0.02^{*b}$
pH	5.48 ± 0.33	5.11 ± 0.19	5 ± 0.41	4.72 ± 0.27
Salt (%)	$2.81\pm0.62^{**a}$	$2.87\pm0.54^{**b}$	$2.95\pm0.48^{**b}$	$3.05\pm0.41^{**b}$

[a, b]Different letters in the same row indicate significant statistical differences (Duncan test, *P<0.05, **P<0.01).

The development of the main groups of microorganisms involved in ripening of Keş cheese is shown in Table 2. The time of ripening had a significant effect ($P<0.05$) on the total aerobic mesophilic bacteria (TAMB) count. The average TAMB count increased up to 60th day of ripening, afterwards dropped by approximately 1.5 log unit (Table 2). The count of total aerobic mesophilic bacteria in Keş cheese was the highest throughout ripening, with the exception at 90th day when psychrophilic bacteria were predominant. The higher counts of TAMB can be connected to the raw milk, although TAMB count was approximately 3 log lesser than in other researches of traditional raw milk cheeses such as Portuguese São Jorge cheese (Kongo et al., 2009), Spanish Genestoso (Arenas et al., 2004) and San Simón cheese (Garcia Fontán et al., 2001). The time of ripening had significant effect ($P<0.05$) on the count of psychrophilic bacteria (Table 2) which increased throughout the ripening. This could be a consequence of the low ripening temperature (6 °C). Lactic acid bacteria (lactobacilli and lactococci) were the predominant group of bacteria during ripening of Keş cheese. Lactococci were the predominant lactic acid bacteria in the cheese curd. Other studies of raw milk cheeses have also reported the predominance of lactococci during the early stages of ripening (Nunez, 1978; Litopoulou-Tzanetaki and Tzanetakis, 1992; Centeno et al., 1996; Manolopoulou et al., 2003; Kongo et al., 2009). The time of ripening had significant effect ($P<0.01$) on the count of both, lactobacilli and lactoccoci. They reached a maximum value at the 60th day of ripening, 8.58 and 7.86, respectively. After that point, a slight decrease was noted at 90th day of ripening. Similar results were obtained by Anar et al. (2000), Xanthopoulos et al. (2000), Buffa et al. (2001) and Garcia Fontán et al. (2001). The increase of lactobacilli may be due to interactive associations among the microorganisms. Some species constitute the proper conditions for the degradation of proteins and carbohydrates by different microbial groups (Manolopoulou et al., 2003). The decrease of lactic acid bacteria at the end of ripening could be related to the weak possibility of competition with more acid-resistant microorganisms. The high decrease in the count of lactobacilli at the end of ripening time could be connected with the unfavourable conditions – very low pH, lower a_w and higher salt content.

Table 2. Microbiological changes of Keş cheese throughout ripening

log CFU/g	Ripening time (days)			
	1	30	60	90
Total aerobic mesophilic bacteria	6.88±0.41*^{a}	8.37±0.19*^{bc}	9.23±0.37*^{c}	7.69±0.88*^{ab}
Psychrophilic bacteria	5.88±0.46*^{a}	7.04±0.72*^{b}	7.47±0.56*^{b}	8.14±0.13*^{b}
Lactobacillus spp.	5.84±0.24**^{a}	8.05±0.06**^{b}	8.58±0.23**^{b}	7.16±0.53**^{ab}
Lactococcus spp.	6.51±0.22**^{a}	7.56±0.3**^{b}	7.86±0.16**^{b}	7.54±0.24**^{b}
Enterobactericiae	3.33±1.33	2.4±0.4	1.99±0	1.99±0
Coliform bacteria	3.28±1.13	2.33±0.34	1.99±0	1.99±0
Staphlococcus-Micrococcus	5.2±0.46	4±1.41	3.95±1.34	3.26±1.1
Enterococcus spp.	5.5±0.13*^{a}	3.08±1.09*^{b}	1.99±0*^{b}	1.99±0*^{b}
Moulds	4.76±0.09*^{a}	4.83±0.17*^{a}	5.59±0.34*^{b}	5.65±0.05*^{b}
Yeasts	5.47±0.22	6.64±0.67	7.20±0.63	6.64±0.82

$^{a, b, c}$Different letters in the same row indicate significant statistical differences (Duncan test, *P<0.05, **P<0.01).

Enterococcus spp. (P<0.5), *Staphylococcus-Micrococcus* spp., *Enterobactericeae* and coliform bacteria counts decreased throughout ripening (Table 2) which shows that physico-chemical parameters of Keş cheese create unfavourable conditions for their growth (Table 1). *Staphylococcus-Micrococcus* spp. count decreased from 5.198 at the 1st day to 3.257 log CFU/g at the 90th day. Coagulase-positive staphylococci were not detected in Keş cheese during ripening. The count of *Enterococcus* spp. decreased from 5.499 at the 1st day to 1.995 log CFU/g at the 90th day. According to the Dagdemir and Ozdemir (2008) lactobacilli, lactococci and enterococci are the most abundant microbial groups in the Turkish artisanal White pickled cheese. The numbers of *Enterococcus* and *Lactobacillus* isolates are higher than those of the other lactic acid bacteria. Enterococci also play an important role in the ripening of cheeses, most likely through proteolysis, lipolysis and citrate breakdown. Therefore, strains of Enterococci have been proposed as starters as they may improve the organoleptic characteristics of the final product (Arenas et al., 2004; Dagdemir and Ozdemir, 2008).

The presence of *Enterobaeteriaceae* is an important indicator of poor hygiene and it is related to the raw milk used for cheese production and/or contamination during production (Ardic et al., 2007). Table 2 shows that *Enterobactericeae* count decreased from 3.33 at the beginning of ripening to 1.99 log CFU/g at the end of ripening ($P>0.05$). These results are in agreement with the findings of Psoni et al. (2003) who reported 2.76 log CFU/g of *Enterobacteriaceae* in 90 days matured Batzos cheese. The fall in the *Enterobactericeae* count throughout ripening can be related to the low pH while pH less than 5 is required for its inhibition. As well as, $a_w<0.95$ restricts the growth of *Enterobactericeae* (Garcia Fontán et al., 2001). Arenas et al. (2004) stated that the presence of 2.3 log CFU/g at the end of ripening of Genestoso cheese may not be considered as significant pathogens although it could indicate a potential for contamination by more dangerous microorganisms.

The Turkish Food Codex (2015) states that cheese should not contain more than 100 CFU/g of coliform bacteria and no *E. coli*. Kirdar (2012) reported that the average count of coliform bacteria in 64 Keş samples taken from the market is 2.44 log CFU/g. In this research, the count of coliform bacteria decreased during ripening from 3.28 at the 1st day of ripening to the 1.99 at 90th day of ripening ($P>0.01$). Lactic acid production by lactic acid bacteria is probably the cause of the coliform reduction due to its acid sensitivity (Nunez et al., 1985). The presence of coliform bacteria in cheese is the indicator of unsanitary conditions and contamination during cheese-making (Trmčić et al., 2016). Coliform contamination is undesirable because it creates structural defects in cheese (Donnelly, 2007). As well as, coliform groups can be used to measure hygiene status during the processing and packaging of dairy products produced from pasteurized milk, because they are thermosensitive. Hence, any coliform found in the product indicates contamination after pasteurization (Birollo et al., 2001). Kilic et al. (1997) and Tuncturk and Coskun (2002) reported high counts of coliform microorganisms at the beginning of cheese ripening, particularly in raw milk cheeses, which number decrease during the ripening period.

A low pH (4.2-4.6) is not the optimal medium for the growth of the most spoilage bacteria (Robinson et al., 2006), but it can favour the growth of mould and yeasts, which can negatively affect the appearance and flavour of cheese (Robinson et al., 2002). As well as yeasts and moulds might represent a potential health risk through producing different toxins with carcinogenic, teratogenic and mutagenic effect (Godič Torkar and Venguš, 2008). At the 1st day of Keş ripening the count of yeast was 5.47 log CFU/g. Their counts increased during ripening, till the 60th day (7.2 log CFU/g) and after that decreased (6.64 log CFU/g). The time of ripening had a significant effect ($P<0.05$) on the mould count, which increased through the 90 days of ripening. The results obtained, showed that the yeast and mould counts in Keş cheese were higher than the value of 10^2 CFU/g noted in Turkish Food Codex (2015). High count of yeasts and moulds could be connected to the low hygienic standards of raw milk and cheese manufacturing. Further studies have to include research of predominant species of yeasts and moulds and their potential pathogenic characteristics, as well as possible presence of mycotoxins in Keş cheese. If predominant species of yeasts and moulds are not pathogenic, in that case yeasts and moulds of Keş cheese could be treated as a natural microflora, which contributes to the desirable traditional sensory properties of Keş cheese. Moreover, previous research (Kirdar, 2012) showed the presence of yeasts and moulds in Keş cheese (5.68 log CFU/g). The

source of yeasts and moulds contamination could be found in the phase of drying of Keş cheese 3-4 days at the room temperature and in adding the spices. Similar mould counts were observed in artisanal and industrial products, and this may be related to the environmental contamination of the production area and manufacturing tools that are in contact with the milk and cheese during cheese production (Bonetta et al., 2008). As well as, Valkaj et al. (2013) determined the high yeasts and mould counts in the Prgica cheese, Croatian traditional sour dried cheese, probably due to contamination by dry red pepper used in its production. Contrary, the yeasts and mould counts of the mozzarella cheese with added spices garnet, mint, cumin and fennel decrease during the storage period of 28 days ($P<0.05$), probably due to their antimicrobial properties (Akarca et al., 2016).

Conclusions

Environmental conditions such as temperature, origin of the milk, processing and sanitary conditions are believed to exert a significant influence on the microbial characteristics of traditionally-made dairy products. Microorganism counts of Keş cheese samples were high, particularly the TAMB, psychrophilic bacteria, LAB, yeasts and moulds, which is not surprising, due to the fact that raw milk and traditional manufacturing methods were used, although *E. coli* and coagulase-positive staphylococci were not detected.

Recent studies have shown that homemade cheeses have different and typical microbial populations that are related to the traditional manufacturing processes and geographic origin. The present study is expected to encourage additional research of the microbial diversity of traditional Keş cheese and give necessary information for formation of autochthonous starter culture, which will help to understand ripening process of sour dried cheeses and improve the traditional farmhouse cheese production. Moreover, the obtained results will contribute to the establishment of the microbiological quality standards for Keş cheese.

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