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Research Article

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SOME PATHOGENIC BACTERIA ISOLATED AND IDENTIFIED FROM TRADITIONALLY PRODUCED TURKISH WHITE CHEESE

Emine MACIT^{1*}

¹Atatürk University, Faculty of Tourism, Department of Gastronomy and Culinary Arts, 25240, Erzurum, Türkiye

Abstract: In this study, some pathogens in the microbiota of traditionally produced White Cheese were determined by molecular methods and their phlogenetic similarities were analyzed. Eight different pathogenic species (*Citrobacter braakii, Hafnia paralvei, Klebsiella grimontii, Kosakonia sacchari, Raoultella ornithinolytica, Raoultella terrigena, Serratia liquefaciens, Serratia plymuthica*) were detected in the White Cheese, and *Klebsiella grimontii* was the dominant species. No study was found in the present studies in which *Klebsiella grimontii* was detected in cheese or dairy products. In addition, no study was found in which *Kosakonia sacchari,* another pathogenic bacterium we detected, was also detected in cheese. This study has revealed some pathogenic microflora in traditionally produced White Cheese.

Keywords: White Cheese, Pathogen microflora, Genotypic characterization, Klebsiella grimontii						
*Corresponding author: Atatürk University, Faculty of Tourism, Department of Gastronomy and Culinary Arts, 25240, Erzurum, Türkiye						
E mail: emine.macit@atauni.edu.tr (E. MACİT)						
Emine MACİT 🛛 🍈	https://orcid.org/0000-0001-6734-1633	Received: December 28, 2022				
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1. Introduction

White Cheese, also known as Tin Cheese, Pickled Cheese or Edirne Cheese, ranks first among local cheese varieties in Türkiye in terms of both production and consumption (Hayaloglu et al., 2002; Üçüncü, 2013). White Cheese production constitutes 60-80% of the total cheese production, and the average production amount in 2021 is 330.000 tons (TUIK, 2020).

This cheese is produced in many countries around the world and is known by different names. For example, it is called as Feta in the Mediterranean region, in Denmark and Greece, Bjalo Salamureno Sirene in Bulgaria, Domiati in Egypt, Brinza in Israel, Teleme in Romania, and Queso Blanco in the United States (Bintsis and Papademas, 2002; Hayaloglu et al., 2002). Although it is produced in every region in Türkiye, the majority of the production is carried out in the Thrace, Marmara, Aegean and Central Anatolia regions (Üçüncü, 2013).

White Cheese can be produced from pasteurized or unpasteurized milk without the use of starter culture, mostly in small dairy farms (Karakuş and Alperden, 1995; Hayaloglu et al., 2002). However, while high quality raw milk is mostly used for drinking purposes, the raw milk used for cheese making is generally of low quality. Especially in businesses where traditional and local type cheese are produced, cleaning and hygiene rules are not followed. and thus. properly microorganism contamination is generally highly possible in such environments. As a result of failing to comply with hygiene requirements during milking, transportation and storage, many microorganisms can be transmitted to milk. The sale of cheeses produced using these milks without enough maturation poses a health risk to consumers due to the potential of pathogenic microorganisms to survive (Çelik and Uysal 2009; Yerlikaya, 2018).

The aim of this study is to detect some pathogens in the microbiota of traditionally produced White Cheese.

2. Materials and Methods

2.1. White Cheese Production and Sampling

The cheese variety that ranks first in terms of production and consumption amount in Türkiye is White Cheese. Production is generally carried out in small dairy farms by using unpasteurized milk without the use of starter culture (Üçüncü, 2013). The milk is mostly handprocessed by the cheese master. The milk that will be used to make cheese is put through the clarifier, heated to the proper temperature for fermentation, and then transferred to the fermentation tank. Rennet is added to the extent that it will coagulate in 150 minutes. The clot is divided into pieces of 2 cm³ and allowed to rest for 5 to 10 minutes to release the whey. 100 kg of cheese milk are put under 20 kg of pressure until the output of whey stops. After the weights are removed, the cheese mass is cut into 7×7×7 cm³ blocks and placed in 14% brine for two hours. Salted blocks are rested in tin cans in rows for 24 hours. In this way, boxes are packed with 17 kg of cheese in 4 days. Each layer is salted, then the cans are filled with brine (10g/100 ml) and sealed (Hayaloglu et al., 2002; Öner et al., 2006).

In this study, 10 White Cheese samples obtained from production and sales points in 4 different provinces of

Türkiye were used.

2.2. Isolation of Bacteria

10 grams of cheese samples were weighed into sterile stomacher bags and homogenized by adding 90 ml of sterile physiological saline water (PSW). Then, dilution was prepared from the samples, and $10^{\text{-}4}$ and $10^{\text{-}5}$ dilutions were planted on Tryptic Soy Agar (TSA) (MERCK) media by spread plate method, and the petri dishes were incubated aerobically at 37 °C for 48 hours. At the end of the incubation process, morphologically different colonies that developed in the solid medium were selected, taken into the Tryptic Soy Broth (TSB) (MERCK) medium, and incubated at 37 °C for 48 hours. At the end of this period, inoculation was made from TSB medium again to TSA medium with the drawing method and after incubation at the same temperature and time, single colonies were transferred to TSB and incubated again under the same conditions. Stock solutions of bacteria isolates were prepared using 40% glycerol to be used in subsequent analyses and stored at -80 °C.

2.3. Genomic DNA Isolation

Bacterial cells were collected by centrifugation at 7000xg for 10 minutes by taking 1 ml of the culture grown overnight in the liquid culture medium into an Eppendorf tube. After removing the supernatant in the tube, 450 µl of TE (Tris EDTA) buffer was added to the collected cells, and the cells were suspended in the buffer with a gentle mixing. 50 µl of 10% SDS (Sodium dodecyl sulfate) and 2 μ l of Proteinase K were added to the suspension cells and incubated for one hour at 37 °C after being thoroughly vortexed. After the incubation, 0.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) mixture was added, the tubes were mixed thoroughly by turning them upside down and incubated for 5 minutes at room temperature. After the content was centrifuged at 7000xg for 10 minutes at 4 °C, the supernatant-like high-viscosity gel was collected using an automatic pipette and transferred to a new tube. The process was repeated once again with a mixture of phenol-chloroform-isoamyl alcohol, and the supernatant-like high viscosity gel formed was collected in a new tube. 50 µl of 5M sodium acetate was added to the content and mixed gently. It was gently mixed by inverting it after adding 1 ml of isopropanol until white strands of the precipitated DNA appeared. After the content was centrifuged at 3000xg for 10 minutes, the supernatant was removed, 0.5 ml of 70% ethanol was added to the obtained pellet, and after light mixing, and the content was centrifuged at 3000xg for 10 minutes. After removing the supernatant, the contents were kept at 37 °C for 5-10 minutes to remove the remaining ethanol, and then the DNA obtained was suspended by adding 100 μl of distilled water (İspirli, 2016).

2.4. Genotypic Characterization by RAPD-PCR

RAPD-PCR was carried out to perform preliminary discrimination from bacteria. PCR mixtures were prepared containing 5×PCR buffer for Taq polymerase (Promega), 2.5mM of dNTPs (Bioline), 1.5 U Taq polymerase (Promega) and 25 pMol of primer M13

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(GAGGGTGGCGGTTCT). PCR was performed with the following program: 35 cycles of 94 °C for 1 min, 40 °C for 20 s, then final step of 72 °C for 2 min. The PCR products were separated with electrophoresis on 1.6% (w/v) agarose gels at 90 V for 1.5 h and band patterns were visualised (Yuvaşen et al., 2018).

2.5. PCR Amplification of the 16S rRNA Region

After RAPD-PCR, 16S PCR was applied for colonies that were thought to be genotypically different. Universal primers AMP F (5'-GAGAGTTTGATYCTGGCTCAG - 3') and AMP R (3'- AAGGAGGTGATCCARCCGCA - 5') were used to amplify the 16S rRNA gene of the strains. PCR mixes were prepared by adding 1µl template DNA, 4 µl MgCl₂, 0.4 µl dNTPs, 1.0 µl 20 mM AMP-F primer, 1.0 µl 20 mM AMP-R primer, 0.25 µl Taq polymerase and up to 50 µl of sterile H₂O. PCR program was performed as 1 cycle at 95 °C for 2 min, 25 cycles at 95 °C for 30 s, at 55 °C for 20 s, at 72 °C for 30 s and at 72 °C for 5 min final extension.

PCR products were run on a gel to control amplification, and amplicons were sent to Medsantek (Istanbul) for sequencing.

2.6. Nucleotide Access Numbers and Phylogenetic Analysis

The 16S sequences of 20 pathogenic bacteria identified in this study were deposited in Genbank with different nucleotide access numbers. Phylogenetic trees were constructed using MEGA4.

3. Results and Discussion

As a result of genotypic characterization of 80 bacteria isolated from artisanal Turkish White Cheese, 20 pathogen bacteria belonging to 8 different species were determined (Table 1).

Citrobacter braakii, Hafnia paralvei, Kosakonia sacchari were detected in one sample, *Raoultella ornithinolytica, Raoultella terrigena, Serratia liquefaciens, Serratia plymuthica* in two samples and *Klebsiella grimontii* in six samples (Table 1). *Klebsiella grimontii* was the prominent pathogen bacteria in White Cheese samples (Figure 1). 1 species in each of samples 6 and 9, 2 species in each of 2 and 8, 3 species in each of 3 and 7, and 5 species of potentially pathogenic bacteria were detected in sample number 1. No pathogenic bacteria were detected in samples 4, 5 and 10.

2 isolates each of *Citrobacter braakii, Raoultella ornithinolytica, Raoultella terrigena, Serratia liquefaciens, Serratia plymuthica,* 1 each of *Hafnia paralvei, Kosakonia sacchari* and 8 isolates of *Klebsiella grimontii* were identified. Figure 2 shows phylogenetic relationships of 16S rRNA genes of different pathogenic bacterial strains isolated from Turkish White Cheese. According to Figure 2, it is seen that *Klebsiella grimontii, Kosakonia sacchari, Citrobacter braakii, Raoultella terrigena, Raoultella ornithinolytica, Serratia liquefaciens* clustered together, *Hafnia paralvei* and *Serratia plymuthica* separated from them and formed a different group.

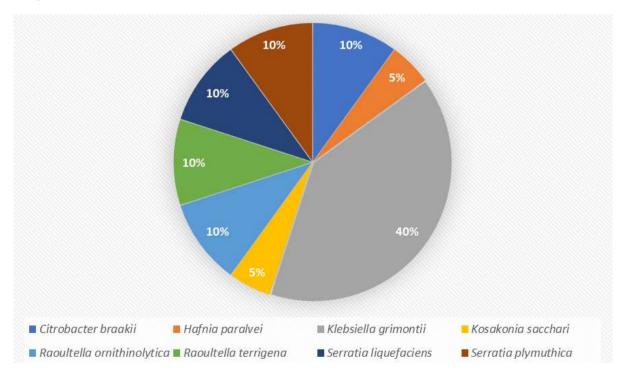
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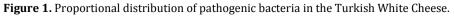
Isolate	GAN	Pathogenic bacteria	Codes of cheese samples									
code			1	2	3	4	5	6	7	8	9	10
TS-1	OP985117	Citrobacter braakii	+	-	-	-	-	-	-	-	-	
TS-3	OP985119	Citrobacter braakii	+	-	-	-	-	-	-	-	-	-
TS-7	OP985122	Hafnia paralvei	+	-	-	-	-	-	-	-	-	-
TS-4	OP985120	Klebsiella grimontii	+	-	-	-	-	-	-	-	-	-
TS-14	OP999616	Klebsiella grimontii	-	-	+	-	-	-	-	-	-	-
TS-31	0P985316	Klebsiella grimontii	-	-	-	-	-	-	+	-	-	-
TS-33	OP985319	Klebsiella grimontii	-	-	-	-	-	-	+	-	-	-
TS-34	OP985321	Klebsiella grimontii	-	-	-	-	-	-	-	+	-	-
TS-35	OP999353	Klebsiella grimontii	-	-	-	-	-	-	-	+	-	-
TS-52	OP999367	Klebsiella grimontii	-	-	-	-	-	+	-	-	-	-
TS-60	OP999613	Klebsiella grimontii	-	-	-	-	-	-	-	-	+	-
TS-2	OP985118	Kosakonia sacchari	+	-	-	-	-	-	-	-	-	-
TS-36	OP999354	Raoultella ornithinolytica	-	-	-	-	-	-	-	+	-	-
TS-54	OP999630	Raoultella ornithinolytica	-	-	-	-	-	-	+	-	-	-
TS-9	OP985123	Raoultella terrigena	-	+	-	-	-	-	-	-	-	-
TS-18	OP985300	Raoultella terrigena	-	-	+	-	-	-	-	-	-	-
TS-11	OP999365	Serratia liquefaciens	+	-	-	-	-	-	-	-	-	-
TS-56	OP999370	Serratia liquefaciens	-	-	-	-	-	-	+	-	-	-
TS-17	OP985318	Serratia plymuthica	-	-	+	-	-	-	-	-	-	-
TS-43	OP999366	Serratia plymuthica	-	+	-	-	-	-	-	-	-	-

Table 1. Distribution of pathogenic bacterial strains detected in traditionally produced Turkish White Cheese samples

 (+and - represent the presence of each species within the corresponding sample).

GAN= genbank accession number





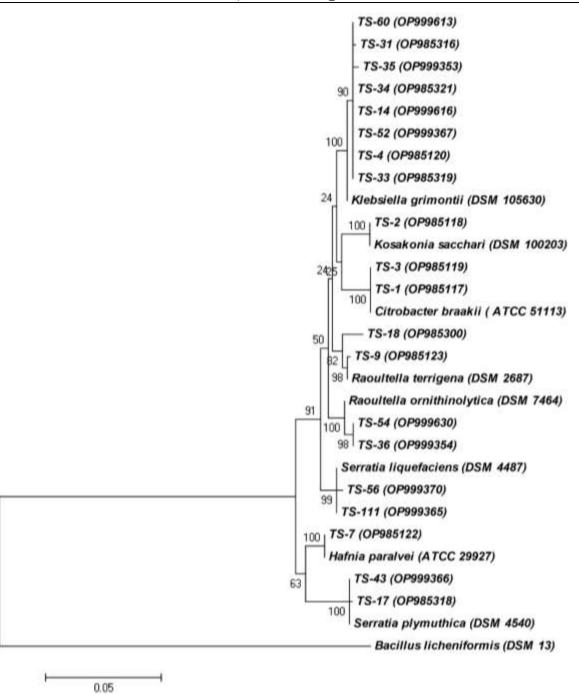


Figure 2. Dendrogram showing multiple sequence alignment of 16S rRNA gene sequences of pathogenic bacteria isolated from Turkish White Cheese.

Citrobacter braakii is a common species in nature and is a pathogen primarily found in the human urinary tract (Trivedi et al., 2015; Castellanos-Rozo et al., 2021). Gupta et al. (2003) reported that Citrobacter spp. are increasingly observed in infections in immunocompromised individuals. C. braakii was detected in artisanal Italian ewe's cheese by Chaves-López et al. (2006); Paipa Cheese by Castellanos-Rozo et al. (2021); May Bryndza Cheese, a traditional Slovak cheese produced from unpasteurized sheep's milk, by Pangallo et al. (2014); Bryndza Cheese by Kačániová et al. (2020). In a study examining the distribution of Enterobacteriaceae and some pathogenic microorganisms in unbranded

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White Cheese samples sold in Ankara, 1.16% of *C. braakii* was detected (Uraz et al., 2009). This species is considered to be among the species responsible for early swelling in soft and semi-hard sheep milk cheeses made from raw milk (Tabla et al., 2016).

Putrescine is one of the most common Biogenic amines (BAs) in foods. An excessive presence in foods can cause food poisoning due to increased toxic effects of other BAs and reduced food quality. This amine is potentially carcinogenic and *C. Braakii* was shown by Wunderlichová et al. (2014) among the microorganisms that form Putrescine in foods. Chaves-López et al. (2006) conducted a study on pasteurized skim milk and reported that *C.*

braakii that was isolated from artisanal Italian ewe's cheese produced biogenic amines such as Ethylamine, Tryptamine, Phenylethylamine, Putrescine, Cadaverine, Spermidine, Spermine, especially histamine.

Hafnia paralvei was another species detected in the White Cheese samples. Hafnia, a gram-negative enterobacterium genus, is frequently found in raw milk and raw milk cheeses. Although most gram-negative bacteria in cheese are known to cause spoilage and off-flavor, Hafnia species such as *H. alvei* and *H. paralvei* can improve cheese flavor by producing sulfur aromatic compounds (Yoon et al., 2016).

Characterization of autochthonal *Hafnia* spp. *strains isolated* from *Spanish soft raw* ewe's *milk PDO cheeses* for use as co-cultures resulted in the determination of *H. paralvei* as the dominant species among 17 different species (Merchán et al., 2022). *Hafnia paralvei* was among the species identified by Castellanos-Rozo et al. (2021) in cheeses produced by formal and informal micro-enterprises in Paipa, Colombia. The report stated that, for Paipa cheese, the focus should be on improving the hygienic quality of the milk from which it is made.

In a study on the safety evaluation of Gram-negative bacteria associated with traditional French cheeses, it was stated that 4 species, including *H. paralvei*, were highly toxic to larvae, suggesting the presence of potential lethal factors in these strains. To the best of our knowledge, no foodborne poisoning or outbreak has been reported for any GNB (gram negative bacteria) of the genera/species related to the strains tested so far. The role of multiple dynamic interactions between cheese microbiota and GIT (gastrointestinal tract) barriers may be key factors explaining the safe consumption of the corresponding cheeses (Imran et al., 2019).

Klebsiella grimontii was the most prevalent species in the White Cheese samples examined in the present study. It was detected in six samples. Eight of the 20 isolates identified were *K. grimontii*. *K. grimontii* is a newly described species of the genus Klebsiella in the family Enterobacteriaceae. The genus Klebsiella contains important human and animal pathogens and is widely observed in the environment and animals (Passet and Brisse, 2018). *K. grimontii* is associated with human infections such as bacteremia and soft tissue infection and has been found in France, Germany, and South Africa (Liu et al., 2018). In the present studies, no study was found in which this species was detected in cheese or dairy products.

Another pathogenic bacterium detected in cheese samples was *Kosakonia sacchari. K. sacchari* was isolated from the surface sterilized stem of sugarcane cultivar in China in 1994 (Chen et al., 2014). It was isolated from surfacesterilized stem of a sweet potato by Shinjo et al. (2016). Abd-Elhafeez et al. (2018) reported that this strain leads to soft rot in potatoes. In the current studies, no study was found in which this species was detected in cheese.

Raoultella ornithinolytica was another species detected in two White Cheese samples. *R. ornithinolytica* has been

described by a number of researchers as a pathogenic species living in aquatic environments (Seng et al., 2016; Papadakis et al. 2021). This bacterium was detected in Montasio Cheese by Maifreni et al. (2013), in Bryndza Cheese by Kačániová et al. (2021a), in Parenica Cheese by Kačániová et al. (2021b), in Brazilian Minas Frescal Cheese by Teider et al. (2019), in soy cheese by Djogbe et al. (2019).

Raoultella terrigena was detected in two cheese samples. Two of the 20 isolates belonged to this species. *Raoultella terrigena* (*Klebsiella terrigena*) was previously classified in the genus Klebsiella (Podschun et al., 2000). This species is an opportunistic pathogen that is rare in nature and has a high mortality rate (up to 44%) (Lekhniuk et al., 2021).

R. terrigena was detected in raw milk by Kongo et al. (2008), in Bryndza Cheese by Pangallo et al. (2014), in the microbiota of Istrian Cheese by Fuka et al. (2010)

Serratia liquefaciens was detected in two cheese samples. *S. liquefaciens* is a psychrotrophic and highly mobile microorganism commonly found in water, soil, vegetation, and more specifically in dairy and raw milk. *S. liquefaciens* is a pathogenic microorganism that can cause infections in humans, especially in immunocompromised hosts. Pink color defects on the surface of some cheeses have been associated with this bacterium by some researchers (Martelli et al., 2020)

S. liquefaciens was the dominant species detected in the microflora of Spanish farmhouse goat's milk cheeses and Picante Cheese (a hard, very salty and spicy traditional cheese produced in Portugal from a mixture of goat's and sheep's milk) (Freitas et al., 1996; Martín-Platero et al., 2009).

Another species detected in the White Cheese samples was *Serratia plymuthica*. *S. plymuthica* is found in soil and has also been isolated from different types of food. Elshaer (2019) stated that *S. plymuthica* is rarely isolated from clinical specimens, but it should be considered as a serious multi-drug resistant pathogen, especially in immunocompromised patients. Hleba et al. (2021) reported that the isolates obtained from milk and dairy products samples were resistant to three antibiotics (Tetracyclines, Chloramphenicol, Ampicillin).

In the study where Uraz et al. (2009) examined the distribution of Enterobacteriaceae and some pathogenic microorganisms in unbranded White Cheese samples sold in Ankara, *Serratia plymuthica* was found to be 1.16%.

4. Conclusion

In conclusion, eight different pathogenic bacteria were isolated from the microbiota of traditionally produced Turkish White Cheese. Traditionally produced White Cheese may pose health risks to consumers. Therefore, it is crucial to reduce microbiological risk by taking certain measures such as making production out of pasteurized milk using starter culture, adhering more strictly to hygiene rules throughout the production, and conducting a proper maturation process.

Author Contributions

The percentage of the author contributions is present below. The author reviewed and approved final version of the manuscript.

	E.M.
С	100
D	100
S	100
DCP	100
DAI	100
L	100
W	100
CR	100
SR	100
PM	100
FA	100

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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