# A STUDY ON ISOLATION OF *BACILLUS* SPP., *YERSINIA* SPP., AND *SALMONELLA* SPP. FROM TURKISH HOME-MADE WHITE CHEESE MARKETTED IN BOLU

**by**
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# ABSTRACT

## A STUDY ON ISOLATION OF *BACILLUS* SPP., *YERSINIA* SPP., AND *SALMONELLA* SPP. FROM TURKISH HOME-MADE WHITE CHEESE MARKETTED IN BOLU

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Turkish white cheese is one of the most popular cheeses and actually the most consumed one in Turkey. It is not a rare case that raw milk or unpasteurized milk are used in production of Turkish home-made white cheese. That is why the possibility of the presence of pathogenic microorganisms is high in Turkish home-made white cheese. Infection and intoxication in humans cause by dairy products are closely related to the consumption of contaminated cheese with pathogenic microorganisms. Between March 2006 and September 2006, a total of 200 Turkish home-made white cheese samples were collected from various public bazaars in Bolu and analyzed according to *"The International Organization for Standardization"* methods in order to identify any possible presence of microorganisms such as *Bacillus* spp., *Yersinia* spp., and *Salmonella* spp. Out of 200 samples, the results were found to be positive for *Bacillus* spp. in

16 (8%), *Yersinia* spp. in 24 (12%) samples. Among *Bacillus* species, *B. cereus* was found to exist in eight (50%) samples, *B. stearothermophilus* in three (18.8 %), *B. brevis* in two (12.5%), *B. subtilis* in one (6.3%), *B. sphaericus* in one (6.3%) and *B. pasteurii* in one (6.3%) sample. Among *Yersinia* species, *Y. enterocolitica* was found to exist in 11 (45.8%), *Y. intermedia* in seven (29.2%), *Y. aldovae* in three (12.5%), *Y. pseudotuberculosis* in two (8.3%), and *Y. rohdei* in one (4.2%) sample. But *Salmonella* spp. were not isolated in any of the samples.

Keywords: White cheese, *Bacillus* spp., *Yersinia* spp., *Salmonella* spp., isolation, identification

# ÖZET

## BOLU İLİNDE TÜKETİME SUNULAN EV YAPIMI TÜRK BEYAZ PEYNİRLERİNDE *BACILLUS* SPP., *YERSINIA* SPP., VE *SALMONELLA* SPP. TÜRLERİNİN İZOLASYONU ÜZERİNE BİR ÇALIŞMA

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Türk beyaz peyniri Türkiye'de en çok sevilen peynir türlerinden biri ve gerçekte de en fazla tüketilen türdür. Ev yapımı Türk beyaz peynirinin üretiminde sıklıkla çiğ süt ya da pastörize edilmemiş süt kullanılmaktadır. Bu yüzden ev yapımı Türk beyaz peynirinde patojenik mikroorganizmaların bulunma ihtimali yüksektir. İnsanlarda görülen süt ürünleri kaynaklı enfeksiyon ve zehirlenme vakaları patojenik mikroorganizmaların kontamine ettiği peynirlerin tüketimi ile yakından bağlantılıdır. Bu çalışmada Mart 2006 ile Eylül 2006 ayları arasında Bolu ilindeki çeşitli halk pazarlarından toplam 200 adet ev yapımı Türk beyaz peyniri örneği toplanmış ve bu örnekler *Bacillus*, *Yersinia* ve *Salmonella* türleri gibi mikroorganizmaları ihtiva edip etmediklerini saptamak amacıyla 'Uluslar arası Standardizasyon Örgütü'nün yöntemleri doğrultusunda analize tabi tutulmuştur. Bu 200 örnekten 16'sında

(%8) *Bacillus* türlerine, 24'ünde ise (%12) *Yersinia* türlerine rastlanmıştır. İzole edilen 16 *Bacillus* türünden, 8'inde (%50) *B. cereus*, 3'ünde (%18,8) *B. stearothermophilus*, 2'sinde (%12,5) *B. brevis*, 1'inde (%6,3) *B. subtilis*, 1'inde (%6,3) *B. pasteurii* ve 1'inde (%6,3) *B. sphaericus* olarak bulunmuştur. *Yersinia* türleri arasında, 24 örnekten 11'inde (%45,8) *Y. enterocolitica*, 7'sinde (%29,2) *Y. intermedia,* 3'ünde (%12,5) *Y. aldovae,* 2'sinde (%8,3) *Y. pseudotuberculosis* ve 1'inde (%4,2) *Y. rohdei* görülmüştür. Ancak, *Salmonella* türleri örneklerin hiçbirinde izole edilememiştir.

Anahtar Kelimeler: Beyaz peynir, *Bacillus* türleri, *Yersinia* türleri, *Salmonella* türleri, izolasyon, tanımlama

**To my beloved husband, İhsan**

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# 1. INTRODUCTION

Cheese, whose history dates back to thousands of years ago, plays a quite important and extensive role in our nourishment having the widest variety among dairy products. Due to type of milk it is made from and differences in production methods, there exist today hundreds of various kinds of cheese (Atasoy and Akın 2004).

In Turkey, 40-50 types of cheese are known, but only three of them have national and economic value: Turkish White, Kasar and Tulum cheeses (Hayaloğlu *et a*l. 2002). Turkish White cheese is a semisoft brined cheese and a major dairy product in the Turkish market. Its consumption is rapidly increasing and it is produced in volumes of over 129.000 tons per year. It can be consumed while fresh, but it is mostly eaten after it has ripened in a brine solution (Erkmen 2000).

According to the Turkish Standards, white cheese is defined as the product obtained through pasteurization of raw milk or its mixtures, and addition of some additives as necessary in the production process depending on the manufacturing technology along with process of ripening (Türk Standartları). Turkish White cheese is usually produced under unmechanised or artisanal conditions and is processed at various stages of manufacture. Thus various types of microorganisms may contaminate the cheese during manufacture and subsequent handling (Turantaş *et al*. 1989).

For many years, traditionally-manufactured cheeses in particular have been regarded as being nutritious and safe. However, more recently, the dairy industry has had some unfortunate experiences with respect to the presence of pathogenic bacteria in these types of cheeses (Bintsis and

Papademas 2002). The rate of survival and/or growth of pathogenic bacteria in cheeses depends on the ecological conditions (water activity, pH, salt content, temperature of maturation) in which the cheese and/or brine are produced.

After ripening period, some hazardous food pathogens may still cause serious food safety problems for consumers in spite of added salt, the antimicrobial metabolites, presence of low pH and moisture levels (Öksüztepe *et al*. 2005).

The quality of the raw milk, heat treatment of the milk, activity of the starter culture and salting process together with the storage in brine are the most important control points for the prevention of growth/survival of undesirable microorganisms during the manufacture of white brined cheeses (Bintsis and Papademas 2002). The microbiological characteristics of cheese depends on the quality of raw milk, the conditions of production, the storage conditions, the producers and the personnel (Çolak 2006).

Microbial spoilage of foods and contamination by pathogenic microorganisms are of great concern to producers, retailers and consumers. Inactivation of either pathogenic and/or spoilage microorganisms is important to produce high quality foods with improved safety and shelf-life (Bozkurt and Erkmen 2001).

Milk is used in the manufacture of cheese. The features of milk may affect the quality of the final cheese. Milk is an excellent medium for microbial growth. Milk and dairy products, being rich in protein, Ca, P, Mg and other minerals, are an important part of the human diet; however, milk and dairy products are also perfect environments for microorganisms to live and

develop (Oktay and Heperkan 2006). Milk is produced in the alveoli of a healthy cow so that is free from microorganisms, but the farm environment makes it impossible to exclude bacteria during milking (Andersson *et al*. 1995).

Pathogenic bacteria in milk has been a major factor for public health concern since the early days of the dairy industry. Many diseases are transmissible via milk products (Ekici *et al*. 2004). Raw milk is that has not been heat-treated above 40℃ the term an “unpasteurized” usually applies to milk that has been heat-treated for a time/temperature below normal pasteurization conditions (De Buyser 2001). The bacterial load of raw milk ranges from $10^3$ CFU per millilitre when hygiene is good to $10^7$ CFU per millilitre when hygiene is poor (Rukure *et al*. 2001).

Milk used for cheese-making should be of a good bacteriological quality to avoid undesirable fermentation and enzymic reactions and without any substances that inhibit or interfere with the growth of the starter bacteria (Chapman and Sharpe 1981). The chemical composition of the milk affects the quality of the final cheese. To obtain a good quality Turkish White cheese, high-quality milk must be used in production (Hayaloğlu *et al*. 2002).

The main purpose of milk pasteurization is the elimination of pathogens, even though a post-pasteurization contamination may occur. However, milk pasteurization modifies the biochemistry and the microbiology of ripening, as well as the flavour and the texture of the cheese (Psoni *et al.* 2006).

Improved hygiene on the farm and at dairy plants, pasteurization of the cheesemilk, precautions to prevent post-pasteurization contamination

may help to improve the quality of Turkish White cheese (Hayaloğlu *et al*. 2002). It is a fact that cheeses made from raw or unpasteurized milk often contain pathogenic microorganisms that cause food-borne diseases such as *Bacillus* spp., *Yersinia* spp.*,* and *Salmonella* spp.

The aim of the study is to isolate and identify such pathogenic microorganisms in the Turkish home-made white cheeses produced by village people in Bolu. The current study may bear particular significance in identifiying the distribution of patterns of *Bacillus*, *Yersinia* and *Salmonella* species in Turkish home-made white cheeses sold in public bazaars in Bolu.

## 1.1. *Bacillus* spp.

### 1.1.1. History

The genus *Bacillus*, the type genus of the family *Bacillaceae,* holds a diversity of endospore-forming bacteria which range from obligate aerobes to strict anaerobes, from rods to cocci, from psychrophiles to thermophiles, and from alkaliphiles to acidophiles. Many of *Bacillus* species fall into several apparently distinct rRNA sequence groups such as the "*B. subtilis* group", the "*B. cereus* group", and the "*B. sphaericus* group" (Murray *et al*. 1999). *B. cereus* was first described by Frankland *et al*. (1887) in Larsen and Jorgensen (1997), following its isolation from air in cowshed.

### 1.1.2. Description of the Genera

The genus *Bacillus* is comprised of a diverse array of gram-positive, but sometimes gram-variable, aerobic and facultative anaerobic endospore-

forming rods (Drobniewski 1993). They are mostly catalase positive and may be motile by means of peritrichous flagella. Most species are mesophilic, but *Bacillus* contains some thermophiles and psychrophiles. *Bacillus* shows a wide range of sporangial morphologies and carbohydrate patterns (Murray *et al.* 1999). Sporangial morphology remains a valuable character in identification. The spore forming ability of *Bacillus* spp., may cause its survival during food processing treatments and the spores may then germinate if the foods are left at room temperature or even at refrigeration temperatures (Iurlina *et al.* 2006).

*Bacillus* species show a wide range of colonial morphologies both within and between species after 24 to 48 h. They vary from moist and glossy through granular to wrinkled; the shape varies from round to irregular, sometimes spreading with entire through undulate or crenate to fimbriate edges; sizes range from 1 to 5 mm; color commonly ranges from buff or creamy gray to off-white, but some strains may produce orange pigment, hemolysis may be absent, slight, or marked, partial, or complete. Despite this diversity, *Bacillus* colonies are not generally difficult to recognize (Murray *et al.* 1999). Colonies of *B. cereus* and its relatives are very variable but are readily recognized. They are characteristically large (2 to 7 mm in diameter) and vary in shape from circular to irregular, with entire to undulate, crenate, or fimbriate edges; they have matte or granular textures. The minimum temperature for growth is between 15°C and 20°C, and the maximum temperature for growth is between 40°C and 45°C, with the optimal being about 37°C. Several selective agars have been designed for the isolation, identification, enumeration of *B. cereus*. The essential ingredients of these

are egg-yolk, mannitol, and an indicator, exploiting the lecithin-hydrolyzing but mannitol negative nature of *B. cereus*. Pyruvate and Polymyxin may be included for further selectivity (Murray *et al*. 1999).

### 1.1.3. Natural Habitats

*Bacillus* spp. are distributed in a wide range of habitats and include species possesing environmental, industrial, and clinical significance (Drobniewski 1993). The reason *Bacillus* species are widely distributed in natural environment is mostly due to their physiological resistance with respect to pH, heat, and salinity. *Bacillus* are thermophilic and psychrotrophilic which can grow at temperatures as high as 75℃ or as low as 58℃ and can flourish at extremes of acidity and alkalinity, ranging from pH 2 to 10 (Koneman *et al*. 1997). The main habitats are soils of all kinds, ranging from acid to alkaline, hot to cold, fertile to desert, and the water columns and bottom deposits of fresh and marine waters (Murray *et al.* 1999).

*Bacillus* species are ubiquitous, inhabiting soil, water, airbone dust. Their spores readily survive in natural environments in a wide variety of habitats may frequently contaminate foods with spores, including dairy products, meats, infant-foods, rice dishes, vegetables, spices, milk powders and cereals (Iurlina *et al.* 2006).

### 1.1.4. Pathogenesis

*Bacillus* spp. may cause systemic, central nervous system, eye, soft tissue and muscle infections. *Bacillus* spp. infections can be serious and

even fatal especially when the patient has a major immune compromise such as neutropenia. Bacteremia from *Bacillus* spp. can be disseminated to other body sites such as the bones or eyes (Mandell *et al*. 2005).

Most *Bacillus* species are saprophytes widely distributed in the natural environment, but some species are oppurtunistic or obligate pathogens of animals, including humans, other mammals, and insects. Oppurtunistic infections with *Bacillus* species other than *B. anthracis* have been reported since the late 19$^{th}$ century. *B. cereus* is next in importance to *B. anthracis* as a pathogen of humans, causing food-borne illnesses and oppurtunistic infections, and its ubiquity ensures that cases are not uncommon (Murray *et al*. 1999).

The presence of *Bacillus* spp. in foods is their spoilage activity with consequent reduction of shelf-life and product acceptance. Public health issues also need to be considered as several species may cause two different forms of food-borne illnesses (Cosentino *et al.* 1997). The diarrheal toxin is heat-labile and can be reduced or eliminated by heating foods enough to kill the vegetative phase of the organism. The diarrheal syndrome is characterized by profuse diarrhea and cramping but rarely vomiting or fever. The onset is about 8 to 16 hours after ingestion of contaminated foods, is associated with a diversity of foods from meats and vegetable dishes to pastas, desserts, cakes, sauces, and milk. The emetic toxin is heat-stable and is associated with starchy foods such as rice. The emetic syndrome is characterized by nausea and vomiting 1 to 5 hours after eating the offending foods such as pasteurized cream, milk pudding, pastas. Heating or reheating foods may eliminate viable *Bacillus* spp. organisms and

the diarrheal toxin but not the emetic toxin. Strains of *Bacillus* may produce one toxin or the other, but they never produce both (Mandell *et al.* 2005).

*Bacillus* spp. are the main spoilage organisms in foods due to their heat resistant spores and the spores may allow its survival during food processing treatments; and the spores may then germinate, if the foods are left at room temperature or even at refrigeration temperatures (Christiansson *et al.* 1989). Also, *B. cereus*, *B. subtilis* and *B. licheniformis* have been associated with food poisoning (Iurlina *et al.* 2006).

*Bacillus cereus* was recognized to be the causative agent of food-borne illness in 1950. The diarrheal type of illness was described following the consumption of highly contaminated vanilla sauce. Haug (1955) isolated *B. cereus,* inoculated sterile vanilla sauce with 106 organisms per mL and consumed it. *B. cereus* is a well-established, food-borne pathogen. Due to its rapid sporulating capacity, the organism easily survives in the environment and during intestinal passage in cows. The organism contaminates raw milk via faeces and soil (Notermans *et al*. 1997).

Less related species of *Bacillus* that may be encountered less commonly in the human clinical microbiology laboratory are *B. subtilis*, *B. licheniformis*, *B. megaterium, B. pumilus* and *B. sphaericus* (Mandell *et al*. 2005).

*Bacillus cereus* is the most important of the spore-forming microorganisms because its spores are ubiquitous in raw milk, survive the pasteurization process, subsequently germinate, outgrow, and multiply. The spores of *B. cereus* survive milk pasteurization. Because of growth of psychrotropic strains, *B. cereus* is a limiting factor for the shelf-life of

pasteurized milk and cream stored >6 to 7℃. Psychrotrophic strains have been shown to be potential toxin producers and have been isolated from milk (Christiansson *et al.* 1999). *B. cereus* produce one or several enterotoxins may cause food poisoning (Mcguiggan *et al.* 2002, Valik *et al*. 2003). Several investigations Christiansson *et al.* (1999), Phillips and Grifftiths (1986), Te Giffel *et al.* (1995) reported that the maximum occurrence of psychrotrophic spores in milk is during summer and early autumn. Milk was more likely to become contaminated during summer for grazing cows than for cows housed during summer.

*Bacillus cereus* is now well recognized as an oppurtunistic pathogen like some other aerobic endospore formers, which can also, from time to time, exhibit an oppurtunistic characteristics (Murray *et al.* 1999). Low–level contamination of food-stuffs by aerobic endospore formers is common as is asymptomatic transient fecal carriage by human and animal populations. (Mcguiggan *et al.* 2002).

Strains of *B. mycoides* and *B. thuringiensis*, which are close relatives of *B. cereus*, may also produce the diarrheal toxin, *B. thuringiensis* has indeed been implicated in case of gastroenteritis. Reports of infections with non-*B. cereus* group species are comparatively rare, but the infections are very diverse. Other species like *B. brevis* may cause corneal infection and food-poisoning; transferred to the new genus *Brevibacillus* (Murray *et al.* 1999). A distinct form of food-poisoning has been associated with *B. subtilis*. This syndrome is characterized by a short incubation period (median 2.5 hours), vomiting, diarrhea, and various manifestations such as flushing, sweating, and headache in about 10 % patients (Mandell *et al*. 2005).

Therefore, in food-borne illness investigations, qualitative isolation tests are insufficient. The ideal criterion for proving that an aerobic endospore former is the aetiological agent is the isolation of significant numbers (>$10^5$ CFU/g) of the organism from the epidemiologically incriminiated foods (Murray *et al*. 1999). The diagnosis of *B. cereus* food-poisoning can be confirmed by demonstrating $10^5$ or more organisms per gram of food that has been implicated epidemiologically (Koneman *et al.* 1997).

The elimination of *B. cereus* spores from foods are not possible, the best practice to prevent this type of food-poisoning is to properly refrigerate foods to minimize the possibility of toxin production during storage.

## 1.2. *Yersinia* spp.

### 1.2.1. History

The first recognized reference to *Y. enterocolitica*, a gram-negative coccobacillus, was made in the United States in 1934 by McIver and Pike (McIver and Pike 1934, in Bottone 1997). In 1944, Van Loghem proposed the transfer of *Pasteurella pestis* and *P. pseudotuberculosis* to his newly defined genus *Yersinia*, named after Alexandre Yersin who had first isolated the plague bacillus during an epidemic in Hong Kong in 1894. The taxonomic status of *Y. ruckeri*, a fish pathogen, is still uncertain.

### 1.2.2. Description of the Genera

Members of the genus *Yersinia* are non-spore forming, facultatively anaerobic, gram-negative or gram-variable, rod-shaped or coccoid cells, 0.5

to 0.8 µm in width and 1 to 3 µm in length. Except for some *Y. ruckeri* strains and *Y. pestis*, which is always non-motile, the other species are motile at 22℃ to 30℃ but not at 37℃ (Holt *et al.* 1994). The genus of *Yersinia* include 11 species but only 3 of them *Y. pestis*, *Y. pseudotuberculosis*, and certain strains of *Y. enterocolitica* are of pathogenic importance for humans and certain warm-blooded animals. *Y. pseudotuberculosis* is differentiated from *Y. enterocolitica* through some biochemical tests such as ornithine decarboxylase, sucrose, and sorbitol tests. *Y. pseudotuberculosis* is negative for all three, whereas *Y. enterocolitica* is positive (Koneman *et al.* 1997). *Y. enterocolitica* grows slower than most other gram-negative bacteria found in foods and cannot compete in mixed populations at higher temperatures (Schiemann 1985).

*Yersiniae* grow under aerobic and anaerobic culture conditions between 0℃ and 45℃, with optimum at 25℃ to 28℃, on non-selective and certain selective media. Only *Y. pestis* has special nutritional requirements for L-valine, L-methionine, L-phenylalanine, and glycine or L-threonine. Glucose is fermentatively utilized with the formation of acid; gas and hydrogen sulfide are not produced. There is no growth in the prensence of KCN. Phenylalanine and tryptophan are not deaminated, gelatin is not liquefied, lysine is not decarboxylated, arginine is not dehydrolyzed. Nitrate is reduced to nitrite, catalase is produced, but oxidase is absent. Acetoin (Voges-Proskauer reaction) is produced at 25℃ to 28℃ (not at 37℃) by most strains of *Y. enterocolitica, Y. frederiksenii, Y. intermedia*, and *Y. aldovae* but not remaining *Yersinia* species. With the exception of *Y. pestis*,

urease is produced at 25℃ to 28℃. Ornithine is decarboxylated by all the species but *Y. pestis* and *Y. pseuodotuberculosis* (Holt *et al*. 1994).

### 1.2.3. Natural Habitats

The genus *Yersinia* are occur in a broad spectrum of habitats including humans, animals, especially rodents and birds, soil, water, dairy products and other foods (Holt *et al*. 1994). In a comprehensive surveillance study conducted in Brazil by Falcao (1991), several hundred *Yersinia* strains were isolated from raw and pasteurized milk, beef, pork, bovine and chicken liver chicken hearts, gizzards and lungs, sausage, hamburger, cheese, and lettuce.

*Yersinia enterocolitica* and *Y. pseudotuberculosis* are distributed worldwide, but occur mainly in the moderate and subtropical climatic areas of the Americas; Europe; north, central, and east Asia; South Africa; and Australia. The main reservoirs of *Y. pseudotuberculosis* are rodents, and wild birds. *Y. enterocolitica* has a wide distribution and can be found in humans, in all warm-blooded wild, domestic, and pet animals, reptiles, fish, and shell-fish. *Y. enterocolitica* has been reported from human feces, water, soil, and vegetables. The organisms are also isolated from foods, soil, and surface water. Pigs are important reservoirs for the human pathogenic serogroups O:3 and O:9; the organisms especially colonize the pigs' tonsils (Murray *et al.*1999). Because of such a wide and diverse reservoir in nature, food products such as dairy products, meats (e.g., pork and poultry), and vegetables would seem to be at greatest risk for contamination with *Y. enterocolitica* (Bottone 1997). *Y. enterocolitica* and related bacteria have

frequently been isolated from raw milk, but none of the isolates, with the possible exception of serotype O:5,27, are recognizable as pathogens. Some serotypes are always pathogenic: O:3, O:9, O:5,27, O:8, O:13. Under normal circumstances *Y. enterocolitica* does not survive pasteurization. If introduced into pasteurized milk, it can grow well at refrigeration temperatures (Schiemann 1985).

*Yersinia enterocolitica* is able to multiply in foods kept at refrigeration temperatures but several factors may preclude the more widespread overgrowth of *Y. enterocolitica* in foods. Epidemiological studies in food microbiology revealed that refrigerated foods stored over a long period pose an additional risk, because *Yersinia,* as a psychrotrophic microbe, is able to grow at temperatures as low as 0℃ .

*Yersinia frederiksenii* is most commonly recovered from fresh water, foods, and non-irrigated soil and has only infrequently been recovered from human specimens. *Y. intermedia* may also be recovered by cold enrichment from human fecal specimens. *Y. rohdei* has been isolated from dog feces, water, and human feces. *Y. mollaretii* has been isolated from human feces, drinking water, meat, and raw vegetables. (Koneman *et al*. 1997).

**Table 1.** Distribution of *Yersiniae* according to isolated specimens

| New Designation | Previous Designation | Characteristics |
|---|---|---|
| *Y. aldovae* | *Y. enterocolitica*-like group X2 | Biochemically similar to *Y. enterocolitica*, found in surface water, drinking water and fish. |
| *Y. bercovieri* | *Y. enterocolitica* biogroup 3B | Biochemically similar to *Y. enterocolitica*, isolated from human feces, water, soil, and raw vegetables. |
| *Y. frederiksenii* | Biogroup of *Y. enterocolitica* | Commonly isolated from water, sewage, and fish. Occasionally found in human feces, blood, and sputum. |
| *Y. intermedia* | Biogroup of *Y. enterocolitica* | Present in fresh water, sewage, and aquatic animals. In human blood, stool, urine, and wounds. |
| *Y. kristensenii* | Biogroup of *Y. enterocolitica* | Present in water, soil, and animals. |
| *Y. mollaretii* | *Y. enterocolitica* biogroup 3A | Isolated in human feces, drinking water, meat, and raw vegetables. |
| *Y. pestis* | *Pasteurella pestis* | Causative agent for plaque. |
| *Y. pseudotuberculosis* | | Cause infections in human. |
| *Y. rohdei* | | Found in dog feces, water, and human feces. |
| *Y. ruckeri* | “Red mouth bacterium” | Known as fish pathogen. |

### 1.2.4. Pathogenesis

Two outbreaks of yersiniosis have occurred in the United States that have aroused concerns about transmission of yersiniosis through milk. The first outbreak took place in 1977 in Oneida Country and involved a group of school children. The other outbreak of yersinosis occurred in the latter part of 1983 with hundreds of cases spread over several southern states. The source of the organism was identified as pasteurized milk from a Memphis diary (Schiemann 1985). The yersinioses are zoonotic infections of rodents, pigs, birds, and various other domestic and wild animals; humans are incidental hosts for infection (Mandell *et al*. 2005).

The genus *Yersinia* include pathogens *Y. pestis*, *Y. enterocolitica,* and *Y. pseudotuberculosis*. *Y. pestis* is the causative agent of plague, which is primarily a disease of wild rodents and may cause acute febrile lymphadenitis, called bubonic plaque*. Y. pestis,* a blood-borne organism, only recently evolved from *Y. pseudotuberculosis*, an enteric pathogen. *Y. pestis* is the cause of plaque, whose most common clinical form is acutefebrile lymphadenitis, called bubonic plaque. Less common forms include septicemic, pneumonic, pharyngeal, and meningeal plaque. Untreated, plaque has a high fatality; treatment in the early course of illness greatly reduces the occurrence of complications and death (Mandell *et al*. 2005).

*Yersinia enterocolitica* was first recognized during the 1960's as an important human enteropathogen. The species as later redefined includes both pathogenic and non-pathogenic forms (Schiemann 1985). Many strains of *Y. enterocolitica* will produce a heat-stable enterotoxin at temperatures

below 30℃ similar to that produced by enterotoxin similar to *Escherichia coli* that is measured by suckling mouse assay (Pai and Mors 1978). Both *Y. enterocolitica* and *Y. pseudotuberculosis* produce lipopolysaccharide endotoxin that has biologic properties similar to those of other gram-negative bacteria (Mandell *et al*. 2005). Intestinal yersinosis may present in three clinical forms: enteritis, terminal ileitis or mesenteric lymphadenitis causing "pseudoappendicitis", and septicemia (Murray *et al.* 1999).

*Yersinia enterocolitica* is a common human pathogen which causes a variety of intestinal and extraintestinal syndromes of various severities, ranging from mild gastroenteritis to mesenteric lymphadenitis and septicemia. Infections with *Y. enterocolitica* also are noted for post-infectious immunological sequale, including erythema nodosum, arthritis and glomerulonephritis (Koneman *et al*. 1997). *Y. enterocolitica* is widely distributed in lakes and reservoirs and epizootic outbreaks of diarrhea, lymphadenopathy, pneumonia, spontaneous abortions occur in various animals. *Y. frederiksenii* is believed to be part of the commensal flora and does not cause diarrhea. *Y. intermedia* may also be recovered by cold enrichment from human fecal specimens, but it is probably not related to intestinal disease. The pathogenic role of *Y. kristensenii* has not yet been determined (Koneman *et al*. 1997). Raw milk and inadequately pasteurized milk and milk products have also been implicated in transmission of *Y. enterocolitica* infections to humans. *Y. intermedia, Y. frederiksenii* and *Y. kristensenii* were the most common isolates after *Y. enterocolitica* (Sulakvelidze 2000).

An inoculum of $10^9$ organisms may be required to cause infection for *Y. enterocolitica*. After an incubation period of 4 to7 days, infection may result in mucosal ulcerations in the terminal ileum, necrotic lesions and enlargement of mesenteric lymph nodes. In severe cases, thrombosis of mesenteric blood vessels, intestinal necrosis, and hemorrhage may occur. The appendix has a normal histologic appearance or show mild inflammation. Septicemia may lead to focal abscesses in various organs (e.g., lung, liver, meninges) (Mandell *et al*. 2005).

*Yersinia pseudotuberculosis* is pathogenic for many animal species and occasionally humans, causing mesenteric adenitis, chronic diarrhea, and severe septicemia. *Y. enterocolitica and Y. pseudotuberculosis* typically produce enteric infection with fever, diarrhea, and abdominal pain that can mimic acute appendicitis (Mandell *et al.* 2005). Infections due to *Y. enterocolitica* and *Y. pseuodotuberculosis* may be acquired by ingestion of contaminated foods or water or, rarely, by direct person-to-person transmission. *Y. enterocolitica* is a common cause of human infection, whereas *Y. pseuodotuberculosis* is primarily an animal pathogen and infects humans only rarely (Holt *et al.* 1994).

*Yersinia pseudotuberculosis* is also endemic in a wide variety of animals, including fowl and is responsible for mesenteric lymphadenitis, particularly in children who manifest a clinical disease situmulating appendicitis (Koneman *et al*. 1997).

## 1.3. *Salmonella* spp.

### 1.3.1. History

*Salmonella* which were firstly isolated from porcine intestine called as *S. cholerasuis* contain a single genus and are named after the American microbiologists, Daniel Elmer Salmon, (Mandell *et al*. 2005). First isolated in 1880, *Salmonella* have been of significant interest to food microbiologists as a leading cause of food-borne bacterial infections (D'Aoust 2000).

### 1.3.2. Description of the Genera

*Salmonella* are gram-negative, non-spore forming, facultatively anaerobic bacilli that measure 2 to 3 by 0.4 to 0.6 µm in size. Like other *Enterobacteriaceae,* they produce acid on glucose fermentation, reduce nitrates, and do not produce cytochrome oxidase (Murray *et al.* 1999).

The genus *Salmonella* is divided into two species both of which have multiple subspecies and serotypes. The two species are *S. cholerasuis*, which has six subspecies (I, II, IIIa, IIIb, IV, and VI), and *S. bongori,* which was formerly subspecies V (Mandell *et al*. 2005).

The *Salmonellae* are the most complex of all the *Enterobacteriaceae* with more than 2200 serotypes described in the Kauffman-White schema. In this schema, the *Salmonellae* are grouped (A, B, C and so on) on the basis of somatic O antigens and are subdivided into serotypes (1, 2, and so on ) by their flagellar H antigens (i.e., A1, A2, B1, B2). Prior to July 1, 1983, three species of *Salmonella* were used to report positive results*: S. choleraesuis*, *S. typhi*, and *S. enteritidis*, with the most of the 2200 serotypes belonging to

the last species, *S. enteritidis*.. Presently, all former species and subgroups of *Salmonella* and *Arizona* are considered to be the same species, but can be separated into six distinct subgroups (Koneman *et al.* 1997).

**Table 2.** Classification of *Salmonella* spp.

| ***Salmonella* species: Includes Most Serotypes** |
|---|
| ***Salmonella* Subgroup 1** |
| *S. typhi* |
| *S. cholerasuis* |
| *S. paratyphi* |
| *S. gallinarum* |
| *S. pullorum* |
| ***Salmonella* Subgroup 2** |
| S. *salamae* |
| ***Salmonella* Subgroup 3a** |
| *S. arizonae* |
| ***Salmonella* Subgroup 3b** |
| *S. diarizonae* |
| ***Salmonella* Subgroup 4** |
| *S. houtenae* |
| ***Salmonella* Subgroup 5** |
| *S. bongori* |
| ***Salmonella* Subgroup 6** |
| *S. choleraesuis subsp. indica* |

### 1.3.3. Natural Habitats

*Salmonella* are found in humans, warm and cold-blooded animals, foods, and the environment. *Salmonella* are widely distributed in the environment and could be transmitted to dairy foods from various sources. *Salmonella* are primary pathogens of lower animals, (e.g. poultry, cows, pigs, pets, birds, sheep, seals, donkeys, lizards, snakes), which are the principal source of non-typhoidal salmonellosis in humans. Interestingly, humans are the only known reservoir for *S. tyhpi* (Koneman *et al*. 1997). Typhi and Paratyphi, are highly adapted to humans have no other known natural hosts. Others such as serotypes Typhimurium and Enteritidis, have a broad host range and can infect a wide variety of animals (Chiu *et al.* 2004). The main source of *S. typhimurium* have been reported to be cattle and pigs but it is also found in poultry, sheep, and goats (Wall *et al*. 1995 and Rabsch *et al.* 2002). Poultry and poultry products are important source of salmonellae. At the same time that poultry consumption is becoming greater, the reported incidence of human salmonellosis is increasing (Bryan *et al.* 1968). Salmonellosis has also been associated with direct or indirect contact with reptiles. Reptiles are the main natural reservoirs of *S. arizonea*, but humans, poultry, and other animals have also developed disease from this organism (Koneman *et al*. 1997). Some such as serotype Dublin (cattle) and serotype choleraesuis (swine) are most highly adapted to a specific animals (Chiu *et al.* 2004).

### 1.3.4. Pathogenesis

*Salmonella* strains can contain a wide variety of plasmid which encode virulence factors and antimicrobial resistance (Rotger and Casadesus 1999) and, therefore, play an important role in pathogenicity. Human infections with *Salmonellae* are most commonly caused by ingestion of foods, water, or milk contaminated by human and animal excrete (Konemann *et al*. 1997). *Salmonella* are recognized as a causative agent of food-borne diseases. Foods of animal origin, including meat, poultry, eggs, or dairy products can become contaminated with *Salmonella*. Eating uncooked or inadequately cooked foods or foods cross-contaminated with these products may lead to human infection. *Salmonella* infections begin with the ingestion of bacteria in contaminated foods or water, infective dose being approximately $10^6$ bacteria (Mandell *et al.* 2005). In particular, as *Salmonella* is widely distributed in the environment and could be transmitted to dairy foods from various sources. It caused speculation as to how long the organism could remain viable in contaminated milk products (Nassib 2003).

Especially raw or undercooked eggs, raw milk, meat and poultry are typical foods that have been implicated in salmonellosis outbreaks. Estimates of the infectious dose vary substantially and depend on the method of determination. Specific *Salmonella* serotypes most often produce characteristic clinical manifestations that have been given the syndrome designations such as gastroenteritis, enteric fever, bacteremia, and vascular infection, localized infections, and chronic carrier state (Mandell *et al*. 2005).

*Salmonella typhi* can cause typhoid fever, a severe systemic disease with a reported UK case-fatality rate of between 0.7% and 1.6%. *S. paratyphi*

is related bacterium that causes typically milder infection with a lower case-fatality rate and chronic carriage rate (Thomas *et al.* 2006). A syndrome similar to typhoid fever is caused by "paratyphoidal" strains of *Salmonella: Salmonella* serotypes Paratyphi A, Paratyphi B, and Paratyphi C (Murray *et al*. 1999). *Salmonella enterica* serovar Paratyphi A is the second most common cause of enteric fever after *S. Typhi*. Given global estimates of >21 million cases of typhoid fever in the year 2000, >5 million cases per year of *S. Paratyphi A* probably occur. Paratyphoid fever is a major clinical problem in India (Nair et al.2006).

*S. typhi* and *S. paratyphi* only colonize humans, and therefore, disease can only be acquired through close contact with a person who has had typhoid fever or is a chronic carrier. Most often, acquisition of infection occurs by ingestion of fecally contaminated food or water. Although direct person-to-person transmission is uncommon, person-to-person transmission of *S. typhi* including anal-oral transmission has been reported (Mandell *et al.* 2005).

*Salmonella* are ubiquitous in animal populations, and human illness is usually linked to foods of animal origin. Salmonellosis also is transmitted by direct contact with animals, by non-animal foods, by water, and occasionally, by human contact. Tyhpoid fever is a serious bloodstream infection common in the developing world. Typhoid fever typically has a low infectious dose (less than $10^3$) and a long, highly variable incubation period (1 to 6 weeks) (Murray *et al*. 1999).

Strains of non-typhoidal *Salmonella* usually cause intestinal infections such as diarrhea, fever, and abdominal cramps that often last 1

week or longer. Persons of all ages are affected; the incidence is highest in infants (Murray *et al*. 1999). In humans, non-typhoidal *Salmonella* infections are most often associated with food products and are the most frequently identified agent of food-borne disease outbreaks (Mandell *et al.* 2005).

*Salmonella* are effective commensals and pathogens that cause a spectrum of diseases in humans and animals including domesticated and wild mammals, reptiles, birds, and insects. *S. typhi*, *S. paratyphi*, and *S. sendai*, are highly adapted to humans and have no other known natural hosts, whereas others, such as *S. typhimurium*, have a broad host range and can infect a wide variety of animal hosts and humans (Mandell *et al.* 2005).

In 2000, *S. typhimurium* and *S. enteritidis* were the most common serotypes, together accounting for 42% of all laboratory-confirmed cases of human salmonellosis. (Mandell *et al.*2005). *S. typhimurium* is the second most common serotype after *S. enteritidis* in many countries (Herikstad *et al.* 2002).

*Salmonella choleraesuis* is host-adapted pathogen that causes swine paratyphoid. It is also highly pathogenic to humans, usually causing septicemic disease with little involvement of the intestinal tract (Chiu *et al.* 2004).

Salmonellosis is a food-borne infection caused by *Salmonella* species when the pathogen grows in the host's intestine and causes gastroenteritis, tyhpoid fever, enteric fever, and septicemia (Holt *et al*. 1994). Although it is one of the 'traditional' pathogens, *Salmonella* remains a leading etiological agent of food-borne infections (D'Aoust 2000). The worst *Salmonella* outbreak caused morbidity to over 16 000 persons who had

consumed low-fat pasteurized milk, and this incident directed interest towards milk products as vehicles for transmitting *Salmonella* (Guthrie 1992).

In 2002, the incidence rate of salmonellosis (17.7 per 100.000 population) was highest among 10 potentially food-borne diseases under surveillance and varied little by geographic region. The incidence of salmonellosis is highest during the rainy season in tropical climates and with the peak in food-borne outbreaks. Twenty-nine *Salmonella* outbreaks were identified, 14 were associated with milk, of which 6 with raw milk, 1 with cream from pasteurized milk, and 14 with cheese of which 8 were made from raw milk and 3 from unpasteurized milk. Additionally, there were 12 outbreaks associated with ice cream since 1984. However, they were not considered because contamination was most likely egg-borne: indeed the serotype involved for 11 of the 12 outbreaks was Enteritidis, which is mostly associated with eggs (De Buyser 2001). Many salmonellosis outbreaks caused by *Salmonella* serotype enteritidis (*S. enteritidis*) involving raw shell eggs. Foods containing partially cooked eggs have been reported in the United States in the past years (Ridzon *et al.* 1997). During the 1980s and 1990s, *S. enteritidis* associated with shell eggs emerged as the predominant Salmonella serotype and source of food-borne disease in the United States and some other countries. Outbreaks of *S. enteritidis* infection have been associated with ingestion of uncooked or lightly cooked eggs, egg-containing food products, and inadequately cooked poultry (Mandell *et al.* 2005).

In 1994, an outbreak of *S. enteritidis* infection was linked to a nationally distributed ice cream brand. Illnesses were documented in 41 states, and more than 200.000 persons were estimated to have been ill.

Consequently, foods containing raw or undercooked eggs pose a slight risk of infection with *S. enteritidis* ( Koneman *et al.* 1997).

*Salmonella* infection is one of the most commonly reported causes of food-borne disease in the Western World. Infection is often caused by poor food hygiene in homes or eating establishments, but can also originate from the site of food manufacture or from naturally contaminated foods, such as chickens and eggs. The severity of salmonellosis, which may result in death for some individuals, means that manufacturers need to detect contamination before foods are released for sale (Mcclelland *et al.* 1994).

The most cost- effective approach to the control and prevention of salmonellosis in food handlers is attention to good personal hygiene and maintenance of time-temperature standards for food handling (Mandell *et al*. 2005). *Salmonella* can be eliminated during the cooking, boiling, and frying processes and by pasteurization.

# 2. MATERIALS AND METHODS

## 2.1. Sample Collection

Turkish home-made white cheeses were examined that are produced with traditional methods and marketed by peasants. The samples of Turkish home-made white cheese were transported to the laboratory in a sterile containers and analysed the same day. A total of 200 samples of Turkish home-made white cheese were collected between March 2006 and September 2006 from public bazaars in Bolu.

## 2.2. Microbiological Analysis

### 2.2.1. Preparation of Dilutions

Tenfold serial dilutions of the samples were made by aseptically transferring 25 g of sample into 225 ml of sterile buffered pepton water (BPW) (MERCK) to give a $10^{-1}$ dilution (IDF 1992). Samples were homogenized for 3 minutes, further 10-fold dilutions of up to $10^{-8}$ were made by transferring 0.5 ml of successive serial dilutions into tubes containing 4.5 ml of sterile peptone saline solution: (%0.1 pepton and %0.85 sodium chloride (NaCl) (ISO).

### 2.2.2. Isolation and Identification of *Bacillus* spp.

Cereus Selective Agar (FLUKA) was used for the isolation of *Bacillus cereus.* The media were prepared according to the manufacturer's

instructions and poured into sterile petri dishes for the spread-plate technique. Plates for the spread-plate technique were prepared by pouring 12-15 ml of the relevant sterile culture media into sterile petri dishes. The plates were incubated at 37℃ in an incubator for contamination control. The dried plates were then inoculated with 0.1 ml of the homogenate dilutions of $10^{-4}$, $10^{-5}$, $10^{-6}$ and appropriately labelled. Each inoculum was spread over the surface of the dried plates using a sterile bent glass rod. The inoculated plates were incubated at 30℃ for 48h. The plates were examined for typical *B. cereus* colonies which were rough and dry with a pink to purple color surrounded by a ring of dense white precipitate. Typical colonies were subcultured on Plate Count Agar (PCA) (MERCK), and further confirmed by establishing the following morphological and biochemical properties as described in Murray *et al.* (1999): gram-positive rods with spores and positive for, Voges-Proskauer reaction, gelatine hydrolysis, nitrate reduction, motility, casein hydrolysis, starch hydrolysis, arginine dihydrolase, indole production, acid formation from; mannitol, salicin, and D-trehalose. As a result, 16 *Bacillus* strains were isolated in 200 Turkish home-made white cheese samples and the following species of *Bacillus* were identified: eight strains of *B. cereus*, one strain each of *B. subtilis*, *B. pasteurii, B. sphaericus,* two strains of *B. brevis*, three strains of *B. stearothermophilus*.

**Table 3.** Isolation of *Bacillus* spp. from Turkish home-made white cheese samples

| Tests (37℃) | *B. subtilis* | *B.cereus* | *B. stearothermophilus* | *B. pasteurii* | *B. sphaericus* | *B. brevis* |
|---|---|---|---|---|---|---|
| Motility | + | + | + | + | + | + |
| Methyl red | - | + | - | - | - | + |
| Voges-Proskauer | V | V | - | - | - | - |
| Casein hydrolysis | + | + | (+) | V | - | + |
| Nitrate reduction | + | + | V | + | - | + |
| Starch hydrolysis | + | + | + | - | V | V |
| Arginine dihydrolysis | - | V[-] | - | ND | V | + |
| Indole production | - | - | - | - | - | - |
| Gelatin hydrolysis | + | + | + | + | - | + |
| Mannitol, acid | + | - | - | ND | - | V |
| Salicin, acid | + | +[-] | (-) | ND | ND | ND |
| D-Trehalose | + | + | + | ND | - | - |

Notes: +, 90-100% positive; -, 0-10% positive; d, 11-89% of strains are positive; V, strains instability (not equivalent to “d”); [-], 11-25% positive; [+], 76-89% positive. ND; no data available

### 2.2.3. Isolation and Identification of *Yersinia* spp.

A 25 g of Turkish home-made white cheese was aseptically added to 225 ml sterile buffered peptone water (BPW) (MERCK) and homogenized. 0.1 ml was inoculated into tubes that contain 10 ml *Yersinia* Enrichment Broth (MERCK) and incubated at 30°C for 24h. Then a loopful of each sample was streaked onto Cefsoludin-Irgasan-Novobiocin (CIN) agar (MERCK) which inhibits the growth of competing flora and gives characteristics colony morphology. Each sample were plated double and incubated at 30°C for 24h. Suspect colonies of typical "bulls-eye" appearence were counted as *Yersinia* and subcultured on CIN agar. All isolates were examined for gram-staining, oxidase and catalase, motility at 37°C, kligler's iron agar reaction, urease, indole production, citrate utilization, acid reaction with methyl red, and acetoin production at 37°C and for production of each of arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase, nitrate reduction, and the ability to produce acid from each of mannitol, D-trehalose, L-rhamnose, sucrose, salicin, D-raffinose and D-melibiose, D-xylose (Holt *et al*. 1994). Twenty four *Yersinia* species were isolated in Turkish home made-white cheeses and the following species of *Yersinia* were identified: eleven strains of *Y. enterocolitica*, seven strains of *Y. intermedia*, three strains of *Y. aldovae*, two strains of *Y. pseudotuberculosis* and one strain of *Y. rohdei*.

**Table 4.** Isolation of *Yersinia* spp. from Turkish home-made white cheese samples

| Tests (37℃) | *Y. aldovae* | *Y. enterocolitica* | *Y. intermedia* | *Y. pseudotuberculosis* | *Y. rohdei* |
|---|---|---|---|---|---|
| Indol production | - | d | + | - | - |
| Methyl red | + | + | + | + | d |
| Voges-Proskauer | - | - | - | - | - |
| Citrate, Simmons | - | - | - | - | - |
| $H_2S$ | - | - | - | - | - |
| Urease | [+] | [+] | [+] | + | d |
| Lysine decarboxylase | - | - | - | - | - |
| Ornithine decarboxylase | d | + | + | - | [-] |
| Motility | - | - | - | - | - |
| Melibiose | - | - | [+] | d | d |
| Raffinose,acid | - | - | d | [-] | d |
| L-Rhamnose, acid | - | - | + | d | - |
| Salicin, acid | - | [-] | + | [-] | - |
| Sucrose, acid | [-] | + | + | - | + |
| Mannitol, acid | + | + | + | + | + |
| D-Xylose | d | d | + | + | d |

Notes: +, 90-100% positive; -, 0-10% positive; d, 11-89% of strains are positive; V, strains instability (not equivalent to “d”); [-], 11-25% positive; [+], 76-89% positive.

### 2.2.4. Isolation and Identification of *Salmonella* spp.

The samples of Turkish home-made white cheese were weighed as 25 g and homogenized. Then each sample were aseptically added to 225 ml of buffered peptone water broth (BPW) (MERCK) as a pre-enrichment medium. After incubation for 16-20 h at 37°C, 0.1 ml of sterile buffered peptone water broth was transferred into 10 ml of a selective enrichment medium, Rappaport Vassiliadis (RV) enrichment broth (FLUKA) which was then incubated at 41.5°C for 24 h. Then a loopful of each sample were streaked onto Rambach agar (FLUKA). Each sample were plated double and incubated at 37°C for 24h. After incubation, three to five of suspect colonies were picked from each plate and restreaked on fresh plates of the Rambach agar for purification. The typical colonies were examined for oxidase and catalase, Voges-Proskauer and methyl red (VP-MR) reaction, indol production, citrate utilization, urease and for production of each of arginine dihydrolase, lysine and ornithine decarboxylase, nitrate reduction. Carbohydrates usually fermented include D-mannitol, D-mannose, D-trehalose, D-xylose, L-rhamnose, L-arabinose, maltose (Holt *et al*. 1994). Suspect colonies were also confirmed on Triple sugar iron agar (TSIA) (MERCK) and lysine iron agar (LIA) (MERCK) (Tahannassib 2003).

## 2.3. List of culture media used in isolation and identification of Bacteria

Buffered Peptone Water (BPW ) Broth……. (Merck 1.07228)

Cereus Selective Agar..... (Fluka 22310)

D-(-)-Raffinose……..…. (Merck 1.07419)

D-(-)-Mannitol……...…. (Merck 1.05982)

D-(-)-Ribose…….…… (Alfa Aesar 10060042)

D-(+)-Glucose monohydrate……..…… (Merck 1.08342)

D-(+)-Mannose……..…. (Fluka 63580)

D-(+)-Melibiose……... (Fluka 63630)

D-(-)-Salicin…….. (Sigma-Aldrich S0625)

D-(+)-Xylose……..…… (Merck 1.08689)

Griess' Reagent for Nitrite……..… (Fluka 03553)

Kligler's Iron Agar…….. (Merck 1.03913)

L(+)-Arabinose…… (Merck 1.01492)

L-(+)-Rhamnose Monohydrate…….. (Fluka 83650)

L-Arginine hydrochloride……..…… (Alfa Aesar 10101532)

L-Lysine monohydrochloride..... (Merck 1.05700)

L-Ornithine monohydrochloride……..…… (Merck 1.06906)

Lysine Iron Agar (LIA)..... (Merck 1.11640)

Maltose……. (Merck 1.05910)

Methyl-Red Voges-Proskauer Broth……. (Fluka 69150)

Plate Count Agar (PCA)..... (Merck 1.05463)

Rambach Agar..... (Fluka 84369)

Rappaport Vassiliadis (RV) enrichment broth……..... (Fluka 17173)

SIM Medium…… (Merck 1.05470)

SIMMONS' Citrate Agar……..…… (Merck 1.02501)

Sucrose…….. (Merck 1.07651)

Trehalose, Dihydrate……..…. (Calbiochem 655625)

Triple Sugar Iron Agar (TSIA)..... (Merck 1.03915)

Urea Broth…… (Fluka 51463)

Yersinia Agar (CIN) + CIN Supplement..... (Merck 1.16434 +1.16466)

Yersinia selective enrichment Broth ..... (Merck 1.16701)

# 3. RESULTS AND DISCUSSION

## 3.1. *Bacillus* species

Several studies indicated that food-borne infections were caused by consumption of contaminated foods. Of the 200 Turkish home-made white cheese samples, 16 (8%) were found to contain *Bacillus* spp. The occurrence of *Bacillus* isolates in the samples analyzed has been summarized in Table 5. In this research, 16 (8%) out of the 200 samples of Turkish home-made white cheese were found to be positive for *Bacillus* spp; eight (50%) out of 16 samples were *B. cereus*, three (18.8%) were *B. stearothermophilus*, two (12.5%) were *B. brevis,* one (6.3%) was *B. subtilis*, one (6.3%) was *B. pasteurii* and one (6.3%) was *B. sphaericus* .

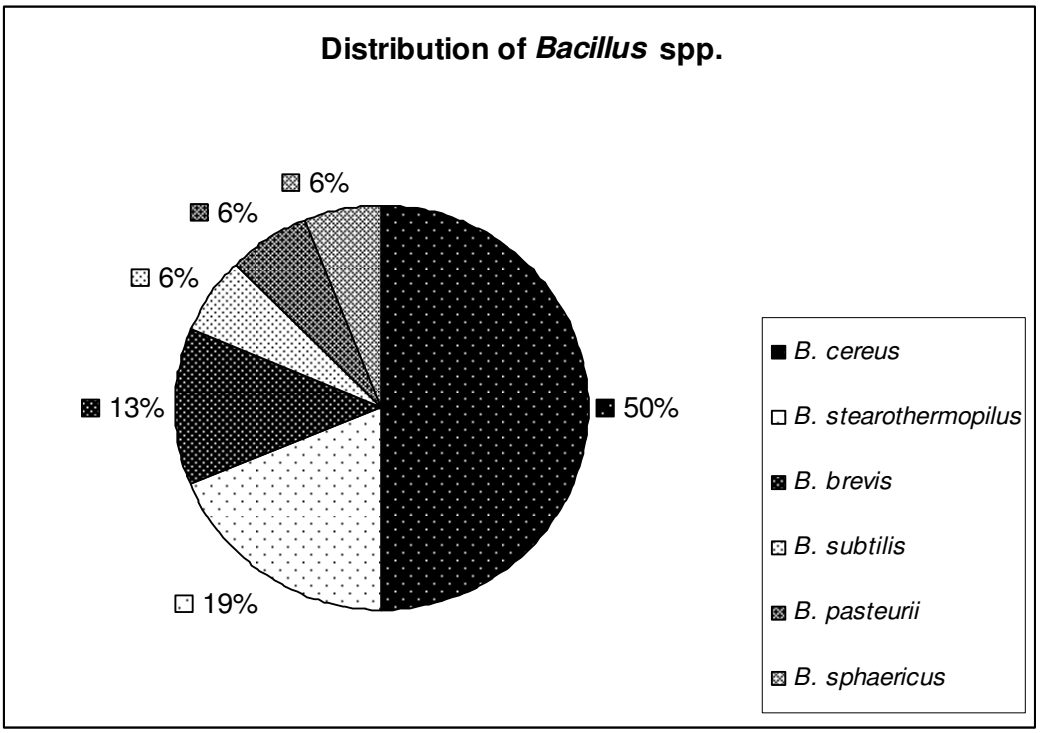


**Figure 1.** Distribution of *Bacillus* species according to percentage

**Table 5.** Distribution of the *Bacillus* species isolated from Turkish home-made white cheese samples

| Species Present | Number of Strains | No of Samples |
|---|---|---|
| *B. cereus* | 8 | 22, 56, 115, 149, 150, 160, 163, 209 |
| *B. stearothermopilus* | 3 | 22, 77, 128 |
| *B. brevis* | 2 | 97, 120 |
| *B. subtilis* | 1 | 169 |
| *B. pasteurii* | 1 | 84 |
| *B. sphaericus* | 1 | 199 |
| **Total** | **16** | |

In this study, the prevalence of *B. cereus* (50%), *B. subtilis* (6.3%), and *B. brevis* (12.5%) showed high incidence among the *Bacillus* strains. These three species, *B. cereus*, *B. subtilis*, and *B. brevis,* may be included in food-borne pathogens. The proportion of *Bacillus* species in Turkish home-made white cheese was found to be significant.

20 samples of Quartirolo cheese and 30 samples of Port Salut Argentino cheese were studied by Iurlina *et al*. (2006) who found 5 *Bacillus* spp. out of the 20 samples and 15 *Bacillus* spp. out of the 30 samples respectively. 15 samples of Port Salut Argentino cheese were found to be *B. cereus* positive whereas none of the samples of Quartirolo cheese were

found to be *B. cereus* positive. Our results show that the incidence of *Bacillus* spp. in Quartirolo cheese is lower than that in Turkish home-made white cheese, whereas the prevalence of *B. cereus* in Port Salut Argentino cheese is higher than that in Turkish home-made white cheese.

Previously, dairy products such as cheese were classified under "safe foods", but after the 1980s, infections and intoxications related to the consumption of contaminated cheese with pathogenic bacteria have been reported (Temelli *et al.* 2005). Cosentino *et al.* (1997) showed a high frequency of *B. cereus* and other *Bacillus* species in Sardinian dairy products including Ricotta cheese which was found to be 18.6% *B. cereus* positive. The prevalence of *B. cereus* was 50% in hard cheeses as stated by Helmy *et al*. (1984). In our study, the prevalence of *B. cereus* was 4% in Turkish home-made white cheeses.

It has been demonstrated that the most important factor leading to *B. cereus* disease outbreaks was improper storage or holding temperatures. Therefore, cheeses are very likely to be affected by the presence and the potential growth of these bacteria in dairy products. The maturation process in these cheeses would reduce the competitive flora, allowing spore-forming microorganisms to predominate. *B. cereus* is becoming an important food-poisoning organism because of its cosmopolitan distribution in nature. The relatively high hydrophobicity of the spores, the low spore surface charge and the spore morphology allow a strong adhesion to different surfaces and the survival through the processing line in the dairy industry (Andersson *et al*. 1995). Species of *Bacillus* are of special interest, as they can exist on the

surface of dirty processing equipment and cause spoilage of milk (Jackson 1990).

Occurrence of *B. cereus* in milk has been reported since 1916, and this bacterium is a common contaminant of raw milk produced on some dairy farms. It can also be found in large numbers in dairy products (Ahmed *et al.* 1983). In a survey by Wong *et al.* (1988) on dairy products, 52% of ice-creams, 35% of soft ice-creams, 29% of milk powders, 17% of fermented milks, and 2% of pasteurized milks and fruit flavoured milks were found to be contaminated with *B. cereus*. The 4% of *B. cereus* prevalence in Turkish home-made white cheese samples in this study is higher than 2% of *B. cereus* prevalence in pasteurized milks and fruit flavoured milks. The concern about the presence of *B. cereus* in dairy products is that it may cause certain defects in the products or produce toxins and thus cause food-poisoning (Rukure *et al.* 2001). This study also revealed high incidence of *B. cereus* in dairy products.

Uraz *et al*. (2001) reported that 19 strains of *Bacillus* were isolated from 111 raw milk samples and they found one strain of *B. cereus* in 111 raw milk samples. In this study, we isolated 8 strains of *B. cereus* in Turkish home-made white cheese which indicates that prevalence of *B. cereus* in Turkish home-made white cheese is fairly higher than that of *B. cereus* in raw milk. Although Uraz *et al*. (2001) isolated a high number of *Bacillus* spp., the number of *B. cereus* they isolated in raw milk is quite lower than that isolated in Turkish home-made white cheese in this study. Since home-made white cheese is consumed in larger amounts than raw milk, the results may indicate a significant threat to public health.

Spores of the genus *Bacillus* are commonly found in raw milk. They are important contaminants penetrating from milk to pasteurized milk products (Pacova *et al*. 2003). Improving microbial safety and extending the shelf-life of pasteurized milk and related products have always been an important concern to dairy industry. A major factor limiting realization of these goals is microorganisms surviving the pasteurization process and/or contributing to post-pasteurization contamination. *B. cereus*, *B. thuringiensis*, and *B. mycoides* are frequently found in pasteurized milk, causing spoilage because of the production of lipases and proteases (Schraft *et al.* 1996).

Larsen and Jorgensen (1997) reported that *B. cereus* was found in 257 (56%) of 458 samples of pasteurized milk and they also found that prevalence of *B. cereus* was higher during the summer than the winter period. The spores of *B. cereus* are ubiquitous in raw milk, survive the pasteurization process and of certain strains to multiply at low temperatures. (Valik *et al*. 2003). All of features that make *B. cereus* unique compared to other milk-borne pathogens.

*Bacillus cereus* has the capacity to grow and generate toxin at storage temperatures above 6℃ (Granum *et al.* 1993). A study of Christiansson *et al.* (1989) showed that the incidence of toxigenic *B. cereus* is high in milk and cream. Milk is a suitable medium for *B. cereus* to produce toxins even at 8℃. A food is contaminated with *B. cereus*, especially enterotoxigenic strains, it might represent a potential risk for the safety and health (Reyes *et al.* 2007).

## 3.2. *Yersinia* species

Several studies indicate that yersinosis in humans was caused by consumption of contaminated foods. In this study, *Yersinia* spp. were found in 24 (12%) out of 200 Turkish home-made white cheese samples. Data shown in Table 6. Out of these positive cheese samples, 11 samples were identified as *Y. enterocolitica* (45.8%), seven samples as *Y. intermedia* (29.2%), three samples as *Y. aldovae* (12.5%), two samples as *Y. pseudotubeculosis* (8.3%), and one sample as *Y. rohdei* (4.2%).

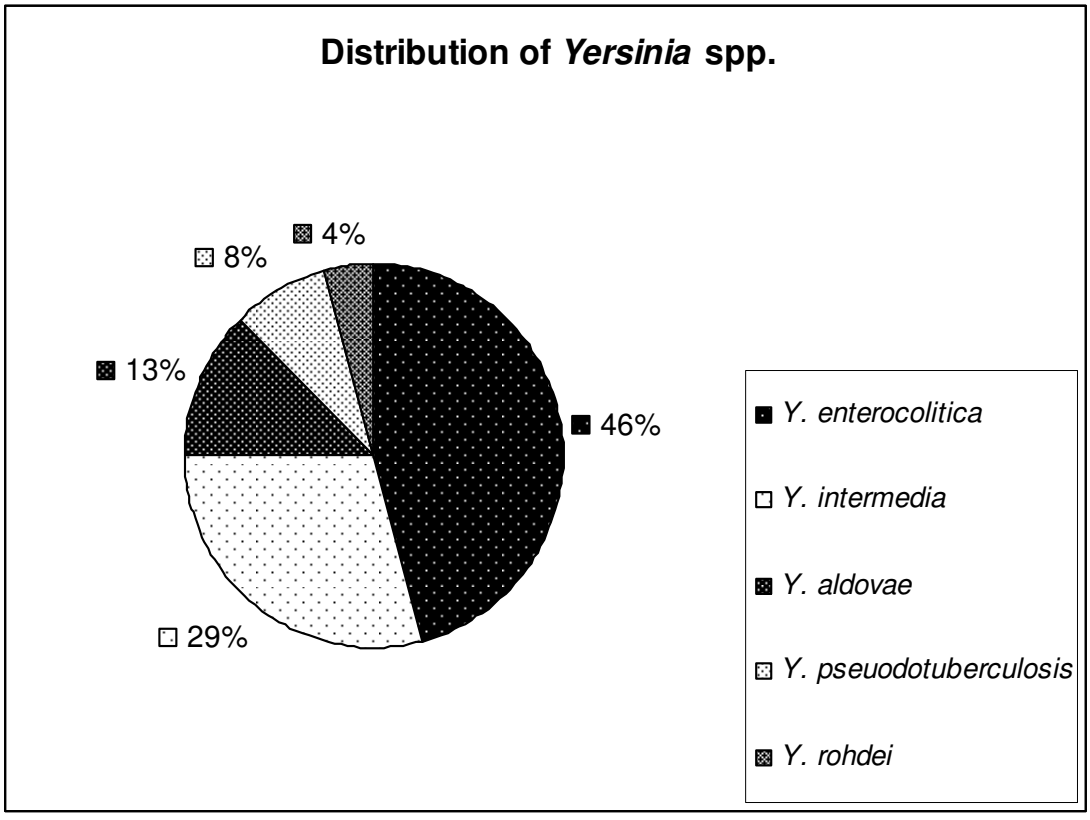


**Figure 2.** Distribution of *Yersinia* species according to percentage

**Table 6.** Distribution of *Yersinia* strains isolated from Turkish home-made white cheese samples

| Species Present | Number of Strains | No of Samples |
|---|---|---|
| *Y. enterocolitica* | 11 | 99, 115, 141, 144, 146, 147, 160, 169, 186, 193, 197 |
| *Y. intermedia* | 7 | 109, 116, 147, 170, 177, 188, 199 |
| *Y. aldovae* | 3 | 152, 161, 176 |
| *Y. pseuodotuberculosis* | 2 | 77, 128 |
| *Y. rohdei* | 1 | 129 |
| **Total** | **24** | |

In the present study, the *Yersinia* spp. were found in 12% of the Turkish home-made white cheese samples, which is higher than the incidence reported by Hamama *et al.* (1992) who found a 7.4% *Yersinia* spp. prevalence in the cheese samples obtained in Morocco. The incidence of *Y. enterocolitica* was found to be 5.5% in the Turkish home-made white cheese samples, which is higher than the incidence reported by Hamama *et al.* (1992) who found it to be 2.1%.

The incidence of *Y. enterocolitica* in cheese curd samples *as* stated by Schiemann (1978) is higher (9.2%) than in Turkish home-made white cheese samples (5.5%) as indicated by the current study.

According to our results, the presence of *Y. enterocolitica* in Turkish home-made white cheese could be attributed to different factors such as use of raw milk and eventual contamination from human handlers, environment and water. Also storage conditions could also increase contamination (Hamama *et al.* 1992). In the traditional or artisanal manufacture of Turkish White cheese, starter culture is not added to the cheesemilk. The milk may or may not be pasteurized and the curd is handled extensively by the cheese maker (Turantaş *et al.* 1989, Erkmen 1995, Hayaloğlu *et al.* 2002).

The presence of *Y. pseudotuberculosis* in Turkish home-made white cheeses may have significance since two samples were isolated as *Y. pseudotuberculosis* which is an important enteric pathogen for humans and particularly for children who manifest a clinical disease stimulating appendicitis (Koneman *et al*. 1997).

In Turkish home-made white cheeses, seven samples were isolated as *Y. intermedia* which showed high incidence after *Y. enterocolitica*. *Y. intermedia* is one of the most common isolate after *Y. enterocolitica* (Sulakvelidze 2000).

*Yersinia enterocolitica* has been isolated from raw milk in many countries. The level of raw milk contamination with *Y. enterocolitica* varies; 1.6% in Northern Iran (Soltan Dallal *et al*. 2004), 18.2% in Southern Ontario (Schiemann 1978), 19.4 % in Sao Paulo, Brazil (Tassinari *et al.* 1994), 32.1% in Southern Ontoria (Schiemann and Toma 1978), 36.6% in Morocco (Hamama *et al*. 1992), and 81.4% in Alsace, France (Vidon and Delmas 1981). Our result showed that the incidence of *Y. enterocolitica* (5.5%) in

Turkish home-made white cheese samples was higher than in raw milk samples (1.6%) as indicated by Soltan Dallal *et al*.

Pathogenic bacteria in milk has been a major factor for public health concern since the early days of the dairy industry. Many diseases are transmissible via milk products (Ekici *et al.* 2004). If Turkish White cheese made from raw or heat-treated milk contains high numbers of pathogens, viable pathogens may survive after ripening at 4°C for 90 days in the white cheese (Erkmen 2000).

*Y. enterocolitica* is a psychrotroph, the only enteropathogenic bacterium of this nature, and can multiply to infective doses in refrigerated milk (Schiemann 1985).

The presence of *Y. enterocolitica* in raw milk puts the possibility of transmission of this pathogen by cheese manufactured from unpasteurized milk. Pasteurization will destroy *Y. enterocolitica* and the presence of this organism in pasteurized dairy products is rare. *Y. enterocolitica* can however, become established in pasteurized milk holding vats if disinfection is inadequate (Schiemann 1985).

A study of Greenwood and Hooper (1990) emphasized that *Y. enterocolitica* biotype 1, serotype O.10K was isolated from 19 patients, the same serotype of *Y. enterocolitica* was also isolated from pasteurized milk. The presence of *Yersinia* spp. in pasteurized milk may also indicate poor hygienic conditions at dairy plants. It is also important to consider that *Yersinia* strains can multiply at refrigeration temperatures, and if there is a pathogenic strain in the milk, a food-borne outbreak may occur (Tassinari *et al.* 1994). *Yersinia enterocolitica* present in raw milk will not survive the aging

period required for Cheddar cheese manufactured from raw milk (Schiemann 1978).

Research showed that *Y. enterocolitica* could be found in raw milk due to unfavorable conditions related to milking hygiene and thus it survived in cheeses made from this milk. Kept at 4°C, the samples of Cheddar and Italian cheeses made from unpasteurized milk were found to be Y. enterocolitica positive after 4 weeks, and *Y. enterocolitica* negative after 8 weeks (Schiemann 1978). *Y. enterocolitica* is not normally harbored by milk cows, and therefore, its presence in raw milk must occur through contamination from other sources, which could be human or swine carriers or contaminated water (Schiemann 1985).

## 3.3. *Salmonella* species

*Salmonella* infection is often caused by poor food hygiene in homes or eating establishments but can also originate from the site of food manufacture or from naturally contaminated foods. The presence of *Salmonella* in milk products remains a public health hazard.

Of 29 *Salmonella* outbreaks implicating milk and milk products reported in several countries, 14 were associated with raw or pasteurized milk, and 14 with cheeses mostly from raw or unpasteurized milk. Although several cheese types were involved, soft cheese were the most frequently implicated in outbreaks. Typhimurium was the predominant serotype, isolated in 12 outbreaks from several countries, and associated with 10 deaths in total. *S. dublin* was responsible for three outbreaks associated with cheeses made from raw or unpasteurized milk in England and in France (De Buyser

2001). In England 42 people were infected with *S. dublin* in 1989 after eating an Irish soft cheese which made from unpasteurized cows' milk.

In this study *Salmonella* spp. were not isolated in 200 samples of Turkish home-made white cheese.

*Salmonella* spp. were not found in 26 Tulum cheese samples by Özalp *et al.* (1978), in 20 Tulum cheese samples by Kıvanç (1989), in 38 White cheese samples by Turantaş *et al.* (1989), in 80 White cheese and 40 Çeçil cheese samples by Gülmez and Güven (2001) and in 50 Carra cheese samples by Aygün *et al.* (2005). Our findings showed similarity with these results.

In countries other than Turkey, related results were established on the presence of *Salmonella* spp. such as in 24 Tetilla cheese which is produced from raw cow's milk cheese in Galicia, Spain. *Salmonella* spp. were not isolated in any samples of Tetilla cheese (Menendez *et al*. 2001). These results are similar to results of Olarte *et al*. (1999) who could not isolate *Salmonella* spp. in any samples of Cameros cheese. In another study conducted by Carvalho *et al.* (2007) *Salmonella* spp. were not isolated in 93 Minas Frescal cheese in the city of Campinas-Sao-Paulo. Our findings are similar to the results obtained in the study by Angelidis *et al.* (2006). *Salmonella* spp. were not detected in 14 semi-hard traditional cheeses in Greece.

A study of Oktay and Heperkan (2006) stated that thirteen samples including; 7 cheese, 5 butter and 1 paked potato were identified as *Salmonella* spp according to the ISO. Cheeses showed that high incidence in potential presence of *Salmonella* species. In our country, the incidence of

*Salmonella* spp. in different types of traditional cheeses has been reported in various studies by a number of researchers. However the isolation of *Salmonella* spp. was reported by Tekinşen and Özdemir (2006) in 50 unripened Van otlu cheese samples obtained in Van and Hakkari markets. Three (6%) of the 50 samples were found to be positive for *Salmonella* spp. Van otlu cheese is of interest from a public health point of view since it is produced from raw milk, mainly in small primitive dairies under poor hygienic practices and marketted while 9fresh or after ripening (Tekinşen and Özdemir 2006).

The presence of *Salmonella* may be possible due to Van otlu cheeses are produced from raw milk. Raw milk is perfect environment for microorganisms to live and develop. *Salmonella* appears as the second most common pathogen associated with cheeses from raw or unpasteurized milk in France (De Buyser 2001). The presence of *Salmonella* spp. in foods may cause serious illnesses and death. For this reason, manufacturers need to detect contamination and use pasteurized milk in producing cheese and other milk products before foods are released for sale. It is pleasing to note the absence of *Salmonella* spp. in all of the Turkish home-made white cheese samples sold in Bolu.

# 4. CONCLUSION

The results of the study indicate that the Turkish home-made white cheese creates a potential hazard to consumers and this fact seems to be closely related to use of raw milk and inadequate processing and storage conditions. Taking into consideration any potential presence of various pathogenic bacteria in the Turkish home-made white cheese, attempts should made for improvement of production techniques (e.g. adequate heat treatment of cheese-milk and use of appropriate starter culture) and hygienic practices during manufacturing and storage heeding the ripening period for the cheese prior to sale.

Out of 200 Turkish home-made white cheese samples, the results were found to be positive for *Bacillus* spp. in 16 (8%), *Yersinia* spp. in 24 (12%) samples. Isolated species showed a wide distribution. Six different types of *Bacillus* species and five different types of *Yersinia* species were isolated. The incidence of *Bacillus* spp. and *Yersinia* spp. may be considered remarkable.

The current study has indicated that the presence in the unpasteurized Turkish home-made white cheeses of *B. cereus and Y. enterocolitica*, which are responsible for food-borne illnesses, indeed poses a serious risk to consumers' health. Therefore, a need emerges for proper manufacturing practices and quality check-up systems as well as high safety standards such as raw milk quality, effective pasteurization process, hygienic production and storage conditions, proper cleaning and sanitation processes in production places in dairy industry.

Public authorities should guide consumers to gain an awareness as to not to consume raw milk products, especially those traditional cheeses made from raw or unpasteurized milk, which are sold in uncontrolled conditions in public bazaars by village people.

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