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Occurrence, spoilage characteristics and pathogenicity of *Bacillus* spp. and *Yersinia* spp. from cheeses

Vorkommen, Verderbnis-Eigenschaften und Pathogenität von Bacillus spp. und Yersinia spp. in Käse

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Summary

A total of 200 homemade white cheese samples collected from open-air markets were analyzed for the presence of *Bacillus* and *Yersinia* species. The overall incidence of *Bacillus* spp. in the cheeses was 8 %. *Bacillus cereus* was isolated from 8 (4 %) of the 200 samples. The rate of other *Bacillus* species was 4 %. *Yersinia* spp. were found in 24 (12 %) of the 200 cheeses. The incidence of *Yersinia enterocolitica* and other *Yersinia* spp. were 5.5 % and 6.5 %, respectively. The lipolytic and proteolytic activities of *Bacillus* spp. were also investigated. All *Bacillus* isolates were found to produce the enzymes of lipase and proteinase which are known as the food spoilage enzymes. The isolates of *Y. enterocolitica* were performed for biotyping and pathogenicity markers. Biotyping of *Y. enterocolitica* revealed that all corresponded to biovar 1A. As a result of virulence marker tests, the *Y. enterocolitica isolates* was regarded as avirulent, despite some biovar 1A strains may produce disease symptoms indistinguishable from that produced by known pathogenic biovars. Many varieties of cheese are made and consumed by humans around the world. The study implies homemade cheeses may harbor *B. cereus* and *Y. enterocolitica,* significant foodborne pathogens. Therefore, we presented this study considering the likely health threat of presence of those pathogens in cheeses traditionally made from raw milk or unpasteurized milk such as homemade white cheese, or due to the contamination of the equipment, people, and environment to consumers worldwide.

Keywords: *Bacillus* spp., *Yersinia* spp., cheese, lipase, proteinase, virulence marker

Zusammenfassung

Insgesamt 200 Proben von weißem Käse wurden auf Basaren genommen und auf das Vorkommen von *Bacillus* spp. und *Yersinia* spp. untersucht. Die Inzidenz von *Bacillus* spp. betrug 8 %. *Bacillus cereus* wurde bei 8 der 200 Proben (4 %) isoliert. Die Nachweisrate der anderen *Bacillus*-Spezies betrug 4 %. *Yersinia* spp. wurden in 24 der 200 (12 %) Käseproben nachgewiesen. Die Inzidenz von *Yersinia enterocolitica* und *Yersinia* spp. lagen bei 5,5 % beziehungsweise 6,5 %.

Die lipolytische und proteolytische Aktivität von *Bacillus* spp. wurde ebenfalls untersucht. Alle gewonnenen *Bacillus*-Isolate produzierten die Enzyme Lipase und Proteinase, die bekanntermaßen Verderbniserscheinungen verursachen können. Bei den Isolaten von *Y. enterocolitica* wurden eine Biotypisierung und ein Virulenzmarker-Test durchgeführt. Die Biotypisierung von *Y. enterocolitica* zeigte, dass alle Isolate dem Biotyp 1A zuzuordnen sind. Die Virulenzmarker-Tests ergaben, dass die *Y. enterocolitica*-Isolate als avirulent einzustufen sind, obwohl einige Biotyp 1A-Stämme Krankheitssymptome hervorrufen können.

Eine Vielzahl von Käsesorten wird weltweit hergestellt und konsumiert. Die Studie zeigt, dass hausgemachter Käse die Quelle von *B. cereus* und *Y. enterocolitica* sein kann. Ziel dieser Studie war es, das Gesundheitsrisiko von weißem Käse aufzuzeigen, der traditionell aus Rohmilch oder unpasteurisierter Milch hergestellt wird. Kontaminierte Anlagen, Personal und Umgebung spielen hierbei eine Rolle.

Schlüsselwörter: *Bacillus* spp, *Yersinia* spp, Käse, Lipase, Protease, Virulenzmarker

Introduction

Bacillus and *Yersinia* species include important pathogenic microorganisms that are being a common contaminant of dairy products because of their ubiquitous nature (Cosentino et al., 1997; Hamama et al., 1992). Consumption of raw milk, inadequately pasteurized milk and derived products such as cheese have been associated with foodborne infections (Soltan-Dallal et al., 2004).

The genus *Bacillus* is a Gram-positive, aerobic or facultatively anaerobic, and endospore-forming rod that is widely distributed in nature (Drobniewski, 1993). The spore-forming ability of *Bacillus* spp. may allow its survival during food processing treatments; and the spores may then germinate if the food is left at room temperature or even at refrigeration temperatures (Iurlina et al., 2006).

The presence of *Bacillus* spp. in foods is undesirable because a number of species have been implicated in foodborne disease. Among the *Bacillus* species, *B. cereus, Bacillus subtilis* and *Bacillus licheniformis* have been associated with food poisoning (Drobniewski, 1993). Within the genus *Bacillus, B. cereus* has been the main species investigated since it represents an important emerging foodborne pathogen. *B. cereus* is a causative agent in both gastrointestinal and non-gastrointestinal infections (Drobniewski, 1993; Ray, 2004).

Bacillus species are also known as spoilage organisms in food because of their versatile metabolism and heat resistant spores. They can produce enzymes, e.g. proteinases and lipases. Proteinases have been associated with spoilage of UHT-treated milk products, cheese and cultured dairy products. Lipases have been implicated in the development of both rancid and off-flavors in milk products. Proteinases and lipases are considered to be of major economic importance because of the spoilage potential of their dairy products (Ray, 2004). *Bacillus* spp., especially *B. cereus* are frequently isolated from a variety of foods such as milk, dairy products, cereals and food additives (Wong et al., 1988; Tham et al., 1990; Iurlina et al., 2006; Reyes et al., 2007; Bonerba et al., 2010).

The genus *Yersinia* is non-sporeforming, facultatively anaerobic, Gram-negative, rod-shaped or coccoid (Holt et al., 1994), and comprises 11 species, of which three *Yersinia pestis, Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are well-recognized human pathogens (Sulakvelidze, 2000). *Y. enterocolitica* is predominantly considered a foodborne agent, since it can multiply at refrigeration temperatures in food (Bottone, 1997).

Y. enterocolitica, an important foodborne pathogen, can cause a variety of intestinal and extraintestinal syndromes of various severities, ranging from mild gastroenteritis to mesenteric lymphadenitis and septicemia (Koneman et al., 1997). It is divided into six biotypes or biovars (1A, 1B, 2–5) according to biochemical activities. The biovar 1B and 2–5 are known as pathogenic biovars due to the presence of a 70-kb pYV (plasmid for *Yersinia* virulence) and certain chromosomal genes (Aleksic and Bockemühl, 1999). The *Y. enterocolitica* biovar 1A lacked both pYV plasmid and chromosomal virulence genes. Isolates belonging to biotype 1A are regarded as "avirulent" or "environmental", although they may be opportunistic pathogen (Bercovier et al., 1980; Bhagat and Virdi, 2007). The occurrence of *Y. enterocolitica* in various food samples (Tassinari et al., 1994; Soltan-Dallal et al., 2004) as well as in different types of cheeses (Hamama et al., 1992; Güven et al., 2010) has been investigated in several countries.

At present, many varieties of cheeses are made worldwide, which probably use more than 20 % of the total milk produced. Different cheeses are produced in small, medium and large companies and consumed by humans around the world (Ray, 2004). Cheeses may be contaminated with various types of microorganisms such as *Y. enterocolitica, Bacillus cereus, Salmonella* spp. and *Staphylococcus aureus* during manufacture and subsequent handling (Tham et al., 1990; Hamama et al., 1992; Güven et al., 2010). Survival of pathogenic bacteria in cheese is dependent on several factors, such as the level of contamination, the competitive microflora, the composition of the cheese and the conditions of manufacture (Bintsis and Papademas, 2002). Homemade white cheese is widely manufactured and consumed by the people of Turkey. It is a semisoft brined cheese and consumed while fresh, but it is mostly eaten after ripening. It is not a rare case that raw cow's milk or unpasteurized milk is used in the production of cheese (Erkmen, 2000).

The aim of the study was specifically to ascertain the occurrence of *Bacillus* spp. and *Yersinia* spp., which include foodborne pathogens, in cheeses from open-air bazaars, to investigate the lipolytic and proteolytic activity known as spoilage factors of *Bacillus* spp. and to determine the biotyping and pathogenic potential of *Y. enterocolitica* isolates, and to assess the potential health hazards for consumers.

Materials and methods

Cheese samples

A total of 200 homemade white cheese samples, which were made frequently from raw cow's milk without starter cultures in rural areas, were collected from open-air public bazaars in Bolu (Western Turkey). All samples were transported to the laboratory inside a portable cooler container (at 4 °C) and processed immediately.

Isolation and identification of *Bacillus* species

A 25 g of each cheese sample was taken aseptically and homogenized in 225 ml of sterile Buffered Peptone Water (BPW) (Merck, Darmstadt, Germany). Serial dilutions of all samples were made in sterile peptone saline solution (% 0.1 peptone and % 0.85 NaCl) until 1:1000. The dilutions (0.1 ml) were plated double on Cereus Selective agar (Fluka, Buchs, Switzerland) and incubated at 30 ºC for 48 h. Presumptive *Bacillus cereus* colonies appeared on Cereus Selective Agar with dry, rough surface, red-purple with a white precipitate and were picked up and cultured on Plate Count Agar (Merck) (24 h, 37 ºC) to obtain pure isolates. The various biochemical tests used for identification of the *Bacillus* species; Gram staining, catalase production, motility, starch hydrolysis, growth in triple sugar iron agar, citrate utilization, urease activity, indole production, Voges-Proskauer test, growth in 6.5 % NaCI, growth in anaerobic medium, nitrate utilization, hemolysis in blood agar, acid production from glucose, xylose, mannitol, lactose, sucrose and maltose, hydrolysis of casein and gelatin (Logan and Turnbull, 1999).

The protease and lipase activity of *Bacillus* spp. were tested. Protease activity was performed on Calcium Caseinate agar (Merck) at 35 °C for 48 h, lipase activity was evaluated in Tributyrin Agar (Merck) plates at 30 °C for 48 h. The reference strain was *B. cereus* RSKK 863 from culture collection of the Refik Saydam National Hygiene Center Ankara, Turkey.

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Isolation and identification of *Yersinia* species

A 25 g of homemade white cheese was aseptically added to 225 ml sterile 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 24 h. Then a loopful of each sample was streaked onto Cefsoludin-Irgasan-Novobiocin agar plates (Merck) (CIN) which inhibits the growth of competing flora and gives characteristics colony morphology. Each sample was plated double and incubated at 30 °C for 24 h. Suspect colonies of typical 'bulls-eye' appearance colonies from CIN Agar plates at least five colonies were picked up and cultured on Tryptic Soy Agar (Merck) (24 h, 37 ºC). Plates with presumptive positive *Yersinia* spp. were confirmed with following biochemical tests: Gram staining, oxidase and catalase tests, motility at 25 and 37 ºC, citrate utilization, acid reaction with methyl red, and acetoin production at 25 °C and 37 ºC, Kligler's iron agar reaction, urease, production of each of arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase, nitrate reduction, indole production, acid production from glucose, ribose, mannose, mannitol, trehalose, maltose, rhamnose, sucrose, melibiose and raffinose (Holt et al., 1994). *Y. enterocolitica* ATCC 1501 was used as the reference strain.

Biotyping of *Yersinia enterocolitica*

The isolates identified as *Y. enterocolitica* were biotyped as described by Aleksic and Bockemühl (1999), testing lipase test, esculin hydrolysis, indole production, fermentation of salicin, xylose and trehalose, nitrate reduction, DNase test, ß-D-Glucosidase, and pyrazinamidase activity.

Pathogenicity of *Yersinia enterocolitica*

Congo Red-Magnesium Oxalate test

The Congo red-magnessium oxalate (CR-MOX) test provides a simple method to screen for plasmid-containing pathogenic *Y. enterocolitica.* The test was performed as described by Riley and Toma (1989). Each colony grown on CR-MOX plates is examined for both calcium dependency and Congo red absorption, indicating the presence of virulence plasmid. Cultures were streaked onto CR-MOX plates and incubated for 24 h at 36 °C. Positive isolates always produced small red colonies and isolates were negative if only large colorless colonies were present.

Crystal violet binding test

The ability of *Y. enterocolitica* to bind crystal violet (CV) was tested as follows. Cultures were inoculated into Brain Heart Infusion (BHI) Broth (Merck) and incubated for 18 h at 25 °C with shaking. The cells were diluted to a concentration of 10^3 cells per ml and surface plated on BHI Agar (Merck). The plates were incubated at 25 °C for 30 h. Each plate were gently flooded with 8 ml of CV solution (85 µg/ml) for 2 min and decanted. The binding of CV to plasmid-positive colonies was observed by their dark violet appearance, while plasmid-negative colonies failed to bind the dye and remained white (Bhaduri et al., 1987).

Autoagglutination test

The cultures of *Y. enterocolitica* were inoculated into two tubes containing Methyl red-Voges-Proskauer broth (Merck) (MR-VP). If the test is positive, parallel cultures in tubes containing MR-VP medium at 25 °C and at 37 °C show clearing of the medium with growth agglutinating at the bottom of the tube after 24 to 48 h at 37 °C, in contrast to a uniform turbidity at room temperature. Virulence plasmid-negative strains, on the other hand, grow with a uniform turbidity at both temperatures (Aleksic and Bockemühl, 1999).

Results and discussion

Bacillus spp.

In this study, out of the total 200 homemade white cheese samples tested, 16 (8 %) were found to be contaminated with *Bacillus* spp. *Bacillus cereus* was also found to be most prevalent with 4 % among *Bacillus* spp. isolated from cheeses. Overall, rates of incidence of *B. cereus* and other *Bacillus* spp. are presented in Table 1.

The occurrence of *Bacillus* spp. as a contaminant of various cheeses was previously reported. Iurlina et al. (2006) found 5 *Bacillus* spp. out of the 20 Quartirolo cheese samples and 15 *Bacillus* spp. out of the 30 Port Salut Argentino cheese samples. *B. cereus* was found in 15 out of the 30 Port Salut Argentino cheeses whereas no *B. cereus* isolate was found in the Quartirolo cheese samples. Our results showed that the incidence of *Bacillus* spp. was lower in Turkish homemade white cheeses than in both Port Salut Argentino and Quartirolo cheeses. The occurrence of *B. cereus* in Turkish homemade white cheeses was also lower than in Port Salut Argentino cheeses, but higher than in Quartirolo cheeses because of the absence of *B. cereus* isolated. A report from Sweden represented a 4 % incidence for *B. cereus* in semisoft goat cheeses (Tham et al., 1990). In the present study, the prevalence of *B. cereus* from semisoft Turkish homemade white cheese is similar to result obtained by Tham et al. (1990).

The occurrence of *B. cereus* was lower in this study than those in previous reports. A study conducted in Italy showed a high frequency of 18.6 % *B. cereus* positive in Sardinian dairy products including Ricotta cheese (Cosentino et al., 1997). *B. cereus* was detected in 11.1 % of the mozzarella samples which are Italian traditional stretched-curd cheeses (Bonerba et al., 2010). Molva et al. (2009) reported that the incidence rate of *B. cereus* was 6 % in cheese samples in Turkey.

Spores of the genus *Bacillus* are commonly found in raw milk. They show high resistance to the pasteurization temperature and are important contaminants penetrating from milk to pasteurized milk products (Pacova et al., 2003). Occurrence of *B. cereus* has been reported in different types of milk products. In a study by Wong et al. (1988)

TABLE 1: *Incidence of Bacillus spp. isolated from homemade cheese samples (n = 200).*

***Bacillus* species**	**No. of positive samples**	**(%) of positive samples**
B. cereus	8	4
B. stearothermophilus	3	1.5
B. brevis	2	1
B. pasteurii	1	0.5
B. subtilis	1	0.5
B. sphaericus	1	0.5
Total	**16**	**8**

n: number of samples tested

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 flavored milks were found to be contaminated with *B. cereus.* A previous study from Chile noted that of the dried milk products, 175 (45.9 %) contained *B. cereus* (Reyes et al., 2007). Uraz et al. (2001) isolated a high number of *Bacillus* spp. from 111 raw milk samples that include 19 isolates of *Bacillus.* However, they found only one isolate of *B. cereus* in raw milk samples.

Product quality is an important issue posed by *Bacillus* spp. in the dairy industry. Production of extracellular enzymes by *Bacillus* spp. such as proteinases and lipases in raw milk can cause spoilage of dairy products made from it. The enzymes are not inactivated by pasteurization and can cause proteolysis of casein and lipolysis of milk lipids to produce flavor defects (Ray, 2004).

In our study, all *Bacillus* isolates obtained from homemade cheeses showed positive proteolytic and lipolytic activities which may affect flavor and texture of cheeses negatively. Production of proteinases and lipases by *Bacillus* species can cause spoilage of cheeses, as reported in previous studies (Cosentino et al., 1997; Molva et al., 2009). Cosentino et al. (1997) found that nearly all isolates from dairy products showed strong proteolytic and lipolytic activity in their study. A report from Belgium also noted that *Bacillus* species isolated from raw milk showed proteolytic and lipolytic activity (De Jonghe et al., 2010).

Yersinia spp.

Yersinia spp. was found in 24 (12 %) out of 200 homemade cheese samples. Out of these positive cheese samples, 11 were identified as *Yersinia enterocolitica* (5.5 %) and 13 as other *Yersinia* spp. (6.5 %). Results are presented in Table 2. Moreover, in this study, in no case *B. cereus* and *Y. enterocolitica* which are responsible for foodborne illnesses were detected in the same cheese sample.

The incidence rate of *Y. enterocolitica* was the highest among other *Yersinia* species in the present study. This finding agrees with other studies where *Y. enterocolitica* was the most common species in cheeses (Güven et al., 2010). It must be pointed out that *Y. enterocolitica* is the main species for concern in human infections. As we found in homemade cheeses, the presence of another species, *Y. intermedia,* in food might be due to the processing of that food and it should be considered a possible opportunistic pathogen for humans (Brenner et al., 1980). In the present study, *Y. pseudotuberculosis* was found to be in 2 out of 24 *Yersinia* isolates from cheeses. It is an important enteric pathogen for humans and particularly for children who manifest a clinical disease stimulating appendicitis (Koneman et al., 1997).

TABLE 2: *Incidence of Yersinia spp. isolated from homemade cheese samples (n = 200).*

Yersinia species	No. of positive samples	(%) of positive samples
Y. enterocolitica	11	5.5
Y. intermedia	7	3.5
Y. aldovae	3	1.5
Y. pseudotuberculosis	2	1
Y. rohdei	1	0.5
Total	**24**	**12**

n: number of samples tested

In a previous study, *Yersinia* spp. and *Y. enterocolitica* were found in 7.4 % and 2.1 % of the cheese samples obtained in Morocco, respectively (Hamama et al., 1992). The results of the present study showed that the incidence rates of *Yersinia* spp. and *Y. enterocolitica* were higher than those reported by Hamama et al. (1992). The occurrence of *Y. enterocolitica* in cheeses has been investigated in Turkey. Yücel and Ulusoy (2006) detected 14 (14 %) *Yersinia* spp. and 5 (5 %) *Y. enterocolitica* isolates in 100 cheeses. Güven et al. (2010) noted that *Y. enterocolitica* were found in 6 (4 %) out of 150 feta cheeses. The findings of the present study were consistent with those mentioned above.

Traditional cheeses made from raw milk have a significant responsibility for the high level of contamination with pathogens (Tham et al., 1990). In the previous reports, the level of raw milk contamination with *Y. enterocolitica* was variable: 81.4 % (Vidon and Delmas, 1981), 19.4 % (Tassinari et al., 1994) and 1.6 % (Soltan-Dallal et al., 2004). Under normal circumstances, *Y. enterocolitica* does not survive pasteurization. Survival of *Y. enterocolitica,* however, is possible due to substandard pasteurization or post-pasteurization contamination (Schiemann, 1987; Bottone, 1999).

In the present study, pathogenicity and virulence markers of *Y. enterocolitica* isolates were examined by means of the Congo red-magnesium oxalate test, crystal violet binding test and auto-agglutination test. *Y. enterocolitica* isolates tested showed negative results for virulence markers. As a result of biotyping, all of them were also not presumptively virulent. In other words, all *Y. enterocolitica* isolates were found to be biotype 1A (environmental isolates) in our study. Despite this, some biovar 1A strains produce disease symptoms indistinguishable from that produced by known pathogenic biovars (1B, 2–5) (Bhagat and Virdi, 2011). The results of Hamama et al. (1992) which exhibited the presence of *Y. enterocolitica* biotype 1A from traditional fresh cheese were similar to our result. The presence of *Y. enterocolitica* in cheese could be attributed to different factors such as use of raw milk and eventual contamination with human handlers, environment and water (Hamama et al., 1992).

In conclusion, the presence of *Bacillus* and *Yersinia* spp. in homemade cheeses may be considered remarkable. The present study revealed that the possible existence of *B. cereus* and *Y. enterocolitica* which are responsible for foodborne illnesses, in cheeses traditionally made from raw milk or unpasteurized milk indeed may pose a serious risk to consumers' health over the world. It is obvious that there is the need for application of effective cleaning, sanitation, and hygienic practices during manufacture, storage and sale of cheese.

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