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Original Research Article

Detection of Rope-Producing *Bacillus* in Bread and Identification of Isolates to Species Level by Vitek 2 System

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ABSTRACT

This study was conducted to detect the presence of rope-producing *Bacillus* spp. and the rope spore counts in one hundred samples of different types of bread obtained from several bakeries and supermarket stores in Bursa province and to identify the isolates at the species level. Rope spore counts determined using the Most Probable Number (MPN) technique varied between 30 and 90 MPN/g in eight samples while this count was 11 000 MPN/g in one sample. By VITEK 2 bacterial identification system, eight of *Bacillus* strains isolated were identified as *B. cereus* and one as *B. subtilis*. Additionally, it was observed the growth of yeast and mould in the counts varying between $1x10^2$ and $6.3x10^4$ cfu/g in the samples. The results indicated that breads analysed harboured *Bacillus* species which can be responsible for rope spoilage of bread or food poisoning in humans.

Key Words: *Bacillus*, bread, rope spoilage and rope-spore counts

Ekmeklerde Rop Üreten *Bacillus*'un Belirlenmesi ve İzolatların Vitek 2 Sistem İle Tür Düzeyinde İdentifikasyonu

ÖZET

Çalışma Bursa'da çeşitli fırın ve süpermarketlerden alınan 100 farklı ekmekte; rop üreten Bacillus spp.'nin varlığını ve rop sporu sayılarını belirlemek, ve izolatları tür düzeyinde identifiye etmek amacıyla gerçekleştirildi. En Muhtemel Sayı (EMS) tekniği kullanılarak belirlenen rop sporu sayıları; bir örnekte 11 000 EMS/g iken, sekiz örnekte 30 ve 90 EMS/g arasında değişti. VITEK 2 bakteri tanımlama sistemi ile, izole edilen Bacillus suşlarından sekizi B. cereus ve biri B. subtilis olarak identifiye edildi. Ayrıca, örneklerde 1x102 and 6.3x104 kob/g arasında değişen sayılarda maya ve küf gelişimi gözlendi. Sonuçlar; analiz edilen ekmeklerin, ekmeklerde rop bozulmasına veya insanlarda gıda zehirlenmelerine neden olabilen Bacillus türlerini içerdiğini gösterdi.

Anahtar Kelimeler: *Bacillus*, ekmek, rop bozulması ve rop-sporu sayıları

INTRODUCTION

Ropiness is bacterial spoilage of bread and generally caused by *Bacillus subtilis* (formerly referred to as *B. mesentericus*), but *B. licheniformis*, *B. megaterium*, *B. pumilus* and *B. cereus* can also be the causative agents (Erem et al. 2009, Thompson et al. 1998).

Members of *Bacillus* genus are aerobic or facultative anaerobic, Gram-positive or Gram-variable, spore-forming rods (Drobniewski 1993). These bacteria are common soil inhabitants and may contaminate bread through the raw materials and bakery equipments used (Fangio et al. 2010, Needham et al. 2005, Sorokulova et al. 2003). Previous studies (Aydin et al. 2009, Iurlina et al. 2006, Sorokulova et al. 2003) indicated that all types of flour were contaminated with *Bacillus* species, although flour is generally considered as a microbiologically safe product as it is low water activity commodity.

Bacterial spores cause major problems in the food industry due to their ubiquitous occurrence and their intrinsic high stress resistance characteristics (Brul et al. 2011). Spores that have retained their viability during baking can germinate and cause rope spoilage of bread if they are exposed to a warm and moist environment (Sorokulova et al. 2003). The water activity, pH and temperature during storage may also play important roles in

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spore germination and growth of vegetative cells of *Bacillus* species (Pepe et al. 2003). Rope in bread is initially characterized by a sweet fruity odour, similar to that of over-ripe melons or pineapples. This is followed by enzymatic degradation resulting in discoloration and the crumb eventually becomes soft and sticky to the touch because of the breakdown of starch and proteins by microbial amylases and proteases, and by the production of extracellular polysaccharides (Kornacki 2010, Mentes et al. 2007, Valerio et al. 2008).

The potential to cause foodborne illness of *Bacillus* species increases the importance of preventing them from growth in bread (Leuschner et al. 1998). *B. cereus* produces at least two types of toxins that are important in the symptomatology of foodborne illness: diarrheic (heat-labile) and emetic (heat-stable) toxins (Souza and Abrantes 2011). Two heat labile enterotoxins have been associated with the diarrheal syndrome whereas a heat and acid stable peptide toxin has been associated with the emetic syndrome (Ankolekar 2009, Ehling-Schulz et al. 2004). There have been an increasing number of food poison outbreaks caused by this bacteria (Gaulin et al. 2002, Kim et al. 2010, Tay et al. 1982). Although less frequent, *Bacillus* species other than *B. cereus* also pose concern for human health (Apetroaie-Constantin et al. 2009). *B. licheniformis*, *B. subtilis* and *B. pumilus*, which comprise the subtilis group, have been associated with several human infections that cause a range of diseases and incidents of foodborne gastroenteritis (Salkinoja-Salonen et al. 1999, Veith et al. 2004). It is evident that bacilli causing foodborne illness or food spoilage present two kinds of problems to the food industry.

The aims of the present study were to detect the rope spore counts in different types of bread purchased from several bakery, market and supermarkets in Bursa province (Turkey) and to characterize the rope-producing *Bacillus* isolates at the phenotypic and species levels.

MATERIALS AND METHODS

Samples

One hundred samples of different types of bread including white, whole-wheat, whole-grain, rye and village breads were purchased from several bakeries or "bakery areas" of supermarkets in Bursa province of Turkey. Breads were provided as whole loaves or sliced; and transferred to the laboratory for microbial analysis.

The determination of rope spore counts

To determine the rope spore counts in breads, 11 g of each sample were aseptically weighed and homogenized with 99 ml sterile 0.1% peptone saline solution, using a Stomacher (Seward Lab-blender 400, London, UK) for 2 min. The suspensions were placed in a water bath and heated at 90-95ºC for 20 min to destroy vegetative bacteria. Ten-fold serial dilutions of the suspensions were prepared with same diluent, and a 1 ml aliquot of each dilution was inoculated into 3 tubes containing 9 ml Dextrose Tryptone Broth. After incubation for 3 days at 32ºC, the tubes were examined for rope formation. The development of a yellow membrane on the surface of tubes was regarded as positive, and rope spore counts were estimated using the Most Probable Number (MPN) table (Anonymous 1992).

The isolation and identification of Bacillus strains

For the isolation of *Bacillus* spp., a loopful from each of tubes observed rope-formation was streaked on Mannitol Egg Yolk Polymyxin (MEP, Hypet Media 0011) agar plates, and these were incubated under aerobic conditions for 48 h at 30ºC. Presumptive *Bacillus* colonies were selected from agar plates and further subcultured on Tryptone Soya Agar (TSA, Oxoid CM131). For each sample, randomly selected colonies isolated from TSA were examined for Gram reaction, the presence of spores by microscopy and catalase production. The stock cultures were maintained at -80ºC in nutrient broth containing 20% (v/v) glycerol. The confirmation and identification at species level of *Bacillus* isolates were achieved by biochemical reactions in the VITEK 2 bacterial identification system (bioMerieux, Marcy I'Etoile, France).

Determination of yeast and mould counts

Serial dilutions were plated on Potato Dextrose Agar (PDA, Merck 1.10130) (pH 3.5) with 10% tartaric acid for yeast and/or mould counts and the plates were incubated aerobically at 22ºC for 5 days (Anonymous 1990).

RESULTS

The results related to rope spore counts in breads examined in this study are summarised in Table 1. These counts were found at the levels ranging from 30 MPN/g to 90 MPN/g in eight samples while one of the sample contained rope spore in a level of 11 000 MPN/g. The isolates of rope-forming *Bacillus* were identified as *B. cereus* (eight strains) and *B. subtilis* (one strain) (Table 1). In addition, the yeast and mould counts determined in breads are shown in Table 2.

Table 1. Rope-spore counts and rope-producing *Bacillus* in breads (n=100).

Type of bread	**Rope spore counts (MPN/g)**	***Bacillus* species**
White	90	*B. cereus*
White	70	*B. cereus*
White	30	*B. cereus*
White	40	*B. cereus*
White	40	*B. cereus*
White	40	*B. subtilis*
Whole wheat	70	*B. cereus*
Whole wheat	11 000	*B. cereus*
Whole wheat	40	*B. cereus*

Table 2. The yeast and mould counts (cfu/g) in retail breads (n=100).

Breads	**Type of contamination**	**Counts**
White	Mould	$1x10^2$
White	Yeast	$1x10^2$
White	Yeast	$2x10^2$
White	Mould	$2x10^2$
White	Yeast	$1.2x10^3$
White	Mould	$2x10^2$
White	Yeast	$1x10^2$
White	Yeast	$1x10^2$
White	Yeast	$1x10^3$
White	Yeast and mould	$1.6x10^4$
White	Yeast and mould	$2x10^2$
White	Yeast and mould	$9.5x10^3$
Village	Yeast	$1x10^2$
Village	Mould	$1x10^2$
Village	Yeast	$4.3x10^3$
Whole wheat	Yeast	$2x10^2$
Whole wheat	Yeast	$1.2x10^4$
Whole wheat	Yeast	$6.3x10^4$
Whole wheat	Yeast	$9x10^2$
Whole grain	Yeast	$3x10^2$
Whole wheat	Yeast	$1x10^3$
Whole wheat	Yeast	$3x10^2$
Whole wheat	Yeast	$1.6x10^3$
Whole wheat	Yeast	$6.7x10^3$
Whole wheat	Yeast	$1x10^3$

DISCUSSION

Microbial spoilage is the major problem causing deterioration of bread products (Needham et al. 2005). Rope spoilage is one of the frequent problems, occurring most frequently in baking industry and usually caused by *Bacillus* spp. (Mentes et al. 2007). Spoilage of bread by rope formation due to *Bacillus* species has been reported by several authors. Thompson et al. (1998) informed that of the 63 control slices of bread analysed, seven showed visible rope production after 96 h at 30ºC. Still, a report of Pepe et al. (2003) from Italy manifested that 30 out of the 56 white wheat bread developed rope spoilage within 5 days.

The objective of the present work was to investigate rope spore counts in breads obtained from bakeries and supermarkets found at various districts of Bursa province and to identify at the species level *Bacillus* strains isolated. Nine from one hundred bread samples analysed were observed the presence of rope spores which was characterized by a yellow membrane on the surface of tubes containing dextrose tryptone broth. Rope spore counts related to samples are summarised in Table 1. These counts ranged from 30 MPN/g to 90 MPN/g in eight samples while one sample had rope counts of 11 000 MPN/g. These values were consistent with legal limit (11 000 MPN/g) laid down in the Communique on Microbiological Criteria of the Turkish Food Codex (2011). A work performed by Certel et al. (2009) showed that *Bacillus* load (rope spore counts) in white and whole meal breads stored at 25ºC was between 0.696-8.728 cfu/g and 0.696-9.695 cfu/g, respectively. The survey authors also suggested that rope spore counts of the raw materials (yeast, whole wheat flour, white flour, water, sugar and salt) used for making bread in laboratory conditions were 93 MPN/g, 43 MPN/g, 23 MPN/g, 4.3 MPN/g, 3.6 MPN/g and <3 MPN/g, respectively.

The isolation of *Bacillus* from samples observed rope formation was carried out by the surface plating method with *Bacillus* specific medium, mannitol egg yolk polymyxin agar. *Bacillus* isolated were initially identified by phenotypic characteristics, and further identified to the species level using VITEK 2 system. Eight from rope forming nine *Bacillus* strains belonged to *B. cereus* and one to *B. subtilis* (Table 1). Nazir and Islam (2007) informed the presence of *Bacillus* in 84.6% of bakery food samples. Sorokulova et al. (2003) detected *Bacillus* in fresh baked bread from different samples of flour and ropy bread obtained from bakers, and identified as *B. subtilis* and *B. licheniformis* the isolates obtained from these samples. In an Italian survey (Pepe et al. 2003), sixty-one *Bacillus* isolates were determined from ropy breads and identified as *B. subtilis* all isolates. In a survey of Rosenkvist and Hansen (1995) *Bacillus* isolated from white and whole meal wheat loaves were characterized as *B. subtilis* (70%), *B. licheniformis* (24%), *B. pumilus* (2%) and *B. cereus* (2%). In comparison to the results of these studies, predominant *Bacillus* species identified in our study was *B. cereus* with an incidence rate of 8%. *B. cereus* has been recognised as a causative agent of food poisoning with this species connected to foodborne emetic and diarrhoeal syndromes (Ehling-Schulz et al. 2004, Fernandez-No et al. 2011) and thus our results also provide the evidence that bread consumption can pose a serious health risk for consumer. In Turkey, a work (Erem et al. 2009) showed that *Bacillus* species resulted from the rope spoilage in white and whole meal breads baked at laboratory conditions. The isolates from white bread were defined as *B. subtilis, B. megaterium, B. licheniformis, B. coagulans* and *B. pumilus*, and as *B. subtilis, B. megaterium* and *B. licheniformis* the isolates from whole meal bread. Still, Leuschner et al. (1998) noticed the isolation of *B. pumilus, B. subtilis* and *B. licheniformis* from brown soda bread.

Bread may be contaminated with *Bacillus* through the raw material, especially flour, and bakery equipment used (Fangio et al. 2010, Viedma et al. 2011). The contamination with *Bacillus* of bakery ingredients and equipments has been reported by different workers. Bailey and von Holy (1993) demonstrated the presence of *Bacillus* in raw materials, dough, food contact surface and bread samples obtained from bread making line. The isolates from this study were characterized as *B. licheniformis, B. subtilis, B. megaterium, B. pumilus* and *B. laterosporus*. A previous study (Sorokulova et al. 2003) indicated that *Bacillus* strains including *B. subtilis* and *B. licheniformis*, isolated from wheat flour samples in Ukraine survived during baking and produced rope in baked bread. Fangio et al. (2010) notified that wheat flour samples were contaminated with *B. cereus, B. licheniformis, B. pumilus* and *B. subtilis*. A similar observation has been made by Iurlina et al. (2006) showing *Bacillus* spp. was recovered in 100% of rye and wheat flour samples in Argentina. Aydin et al. (2009) also

recorded a 4.2% incidence of *B. cereus* in wheat flour samples from Thrace region of Turkey. Again, a study conducted by Demir (2006) revealed that five out of 100 samples of wheat flour were contaminated with *Bacillus* spp. at levels ranging from $2x10^2$ to $1.6x10^4$ cfu/g.

Table 2 shows the results of yeast and mould counts in breads analysed. According to this, 25 out of 100 bread samples were observed the growth of yeasts and moulds, and the counts were found between $1x10^2$ and $6.3x10^4$ cfu/g. In Turkey, the Communique on Microbiological Criteria of the Turkish Food Codex (2011) established a guideline with maximum level of 10^3 cfu/g for yeast and mould. With respect to the this guideline, the counts ($1.2x10^3$ - $6.3x10^4$ cfu/g) obtained from eight bread samples were higher than the allowable value in the Turkish legislation and thus regarded as being of unsatisfactory quality. However, Certel et al. (2009) reported that mould growth was not observed on white and whole meal breads baked at laboratory conditions and stored at 4, 25, 37 and 45ºC for 7 days.

CONCLUSIONS

The results of this study showed the contamination with rope-forming *Bacillus* species of breads sold in Bursa province. *Bacillus* species, including *B. cereus* and *B. subtilis*, identified in our work are potentially causative agents of foodborne illnesses caused by primarily *B. cereus* and occasionally other species. Therefore, to prevent the rope spoilage in bread and to eliminate the possible public health concerns arising from *Bacillus*, special attention should be given to the baking temperature and good sanitary conditions during bread manufacturing.

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