Preliminary Investigation of *Bacillus* Species in Meat and Meat Products

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Abstract

Microbial hazards seem to be the biggest food safety concern. The contamination of meat products with microbial hazards made them unsafe for human consumption and is considered global health. This study was conducted to determine the safety of some meat products in which one hundred and forty samples of processed meat product represented by sausage, minced meat, luncheon, beef meat, and liver that were randomly collected from different supermarkets and shops at Portsaid Egypt to evaluate the occurrence of *Bacillus cereus* group and *Bacillus subtilis* microorganisms as a biological hazard.The results revealed that (48) samples (34.2%) positive reactions to *B. cereus* groups which include(32) *B. cereus*(22.8%), 7 *B. mycoids*(5%) and 9 *B. thuringiensis*(6.4%)) and (11) samples (7.8 %) positive reaction to *B. subtilis* and with mean values of 1.65x104±1.01x104, 1.23x104±1.13x104, 1.96x104±6.79x103, 1.95x104±1.03x104 and 0.89x104±0.49x104 (cfu/g) of *B.cereus* in the evaluated samples, respectively. The results detect the presence of microbial hazards in meat products is unavoidable.

Keywords: *B.cereus,B.subtilis*, meat products, microbial hazards

Introduction

Meat products are gaining widespread popularity because of their ease of preparation and ability to address the issue of a lack of high-priced fresh meat that is out of reach for a large number of low-income families ***(Shawish and Tarabees, 2017)***. Consequently, while it is regarded as the finest choice for fixing human nutritional concerns, it may pose health risks due to the lengthy chain of preparation, processing, transportation, storage, and retailing. Biological,

chemical, and/or physical threats endanger the safety of meat products ***(Sofos, 2014).***

The most dangerous food-borne hazards are biological hazards, including pathogenic bacteria, viral pathogens, and parasites. Bacterial contamination is the most common cause of food-borne illness ***(WHO, 2007).*** Among the most common infections transmitted by meat products.

Bacillus cereus and *Bacillus subtilis* are extremely prevalent in the environment, owing to their spores' resistance. As a result, it should come as no surprise that *B. cereus* contaminates a variety of final food products ***(Ceuppens et al., 2010).*** The ability of *B. cereus* to produce thermoduric endospores, grow and live at chilled temperatures, and produce toxins are the primary factors contributing to its potential food processing issue ***(Okanlawon et al., 2010).*** It has been charged with causing two distinct types of food-borne illness, emetic syndrome and diarrheal syndrome ***(Drobniewski, 1993).***

Bacilli are a diverse collection of microorganisms classified into at least five separate categories. *Bacillus subtilis* is classified as a member of group 1 and is closely related to *Bacillus licheniformis* (which is frequently found on the cuticle of insects) and the group of animal pathogens comprised of *Bacillus thuringiensis*, *Bacillus cereus,* and *Bacillus anthracis*. *B. sphaericus* is a member of group 2, *B. polymyxa* is a member of group 3, and *B. stearothermophilus* is a member of group 5. ***(Hullo et al., 2001).***

Bacillus cereus is a genus of eight species: *B. pseudomycoide*, *B. mycoides*, *B. weihenstephanesis*, *B. cytotoxius*, *B. toyonensisi*, *B. cereus sensu stricto*, *B. anthracis*, and *B. thuringiensis* (***Pfrunder S et al., 2016***). These species are easily distinguished from other aerobic spore-forming bacteria due to their inability to ferment mannitol sugar and their synthesis of lecithinase. Nonetheless, they are difficult to distinguish from one another ***(Fritze Detal., 2004).*** As a result, the current study is aimed to investigate the prevalence of Bacillus species in different meat products, including sausage,minced meat,luncheon, beef meat and liver.

Materials and methods

1 isolation and enumeration of isolates:

A total of 140 samples were obtained from various merchants and supermarkets in Port Said, Egypt, comprising sausage, minced meat, luncheon, beef meat, and liver (28 of each). After serial dilution, the generated samples were streaked onto

medium MYP(oxoid, Hampshire,UK) plates and incubated at 37°C for 24-48 hours. The isolates were counted after determining the existence of primary diagnosis characteristics associated with the *B. cereus* group. These traits resulted in hydrolyzed lecithin precipitation and an inability to metabolize mannitol. In 0.1 percent peptone water, a tenfold serial dilution of the obtained sample homogenate (10-1) was performed. Each sample was inoculated onto the surface of MYP(oxoid, Hampshire,UK)media using an aliquot of 0.1 ml of 10-1 and higher dilutions. For 24 hours, the plates were incubated at 37°C. The colonies of *B. cereus* and *B. cereus*-like isolates were counted using the usual plate method ***(Tallent et al., 2012)*** and then validated using a quick confirmatory staining procedure. Plates containing between 30-300 questionable colonies were chosen. If no initial colonies were visible after 24 hours, the plates were re-incubated at 37°C for a further 24 hours. The number of *B. cereus* and *B. cereus*-like isolates colonies per gram weight or milliliter of the studied samples was reported. The total viable count was expressed as Log 10 colony-forming units (CFU) per gram or milliliter of material. Each sample was subjected to three independent experiments.

2 Morphological characters

A flame fixed-film was prepared using one pure colony from the culture media stained with Gram's stain and then examined under oil immersion lens of the ordinary microscope to give a definitive characterization of *B. cereus group* isolates

Members of the *B. cereus* group are genetically identical and cannot be distinguished based on phenotypic or biochemical traits ***(Cardazzo et al., 2008)***. Suspected *B. cereus* pathogen group isolates were later identified using different tests and the reported results were indicated according to ***(Stenfors et al., 2008; Tallent et al., 2012 and SMIs)***

3 Detection of hemolysis

Isolates were streaked onto blood agar (oxoid, Hampshire,UK) plates and incubated at 30°C. A positive result is detected as β-haemolytic reaction surrounding the colonies. The hemolytic *Bacillus* species includes *B. cereus, B. thuringiensis* and *B. mycoids*. While *B .subtilis* gives α-hemolytic reaction surrounding the colonies.

4 Biochemical characterization

4.1 Lecithinase production (Nagler's reaction)

After inoculating the suspicious colonies onto MYP egg yolk agar plates and incubating the plates at

35-37°C for 18-24 hours. Lecithinase production was identified as a zone of egg yolk precipitation. Occasionally, *B. cereus* strains exhibit a strong Nagler's reaction, as described by ***(Quinn et al., 2002)***.

4.2 Catalase test (slide technique)

One colony from the culture medium was placed on a clean, dry sterile glass slide, and then it was mixed with one drop of H_2O_2 (3%). A positive reaction is indicated by gas bubbles formation, according to ***(Quinn et al., 2002)***.

4.3 Psychrotolerance

Isolates were streaked out onto two TSA plates and then the plates were incubated at 6°C for 28 days. None of *B. cereus* pathogen group can grow at 6°C, according to ***(Tallent et al., 2012)***.

4.4 Protein toxin crystal production

All identified strains were inoculated with a loopful of 24 h culture suspension onto nutrient agar slants. The slant was incubated for 24 hours at 30 °C and then at room temperature for 2-3 days. Smears were made on glass slides using sterile distilled water, air-dried, then intensely heat-fixed by passing the slides through a benzene burner's flam. After 30 seconds of menthol treatment, the stain was drained out and the slides were air-dried. The slides were stained with Zhiel-Neelsen stain with 0.5 %basic fuchsine or carbol fuchsine and gradually heated from underneath with a small benzene flame until steam appeared. After 1-2 minutes, the final process was repeated 3-5 times, and the slides were washed, dried, and inspected under an oil immersion lens for the presence of free spores and darkly stained tetragonal (diamond-shaped) toxin crystals. According to the authors, *B. thuringiensis* (insect pathogen) exhibits these toxin crystals as free or parasporal inclusions in three-day-old cultures (following lysis of the sporangium) ***(Tallent et al., 2012).***

4.5 Rhizoid growth

A loopful of 24 h culture suspension was inoculated by lightly touching the surface of nutrient agar at the middle of each plate. The plates were then incubated for 48-72 hours at 30°C. *B. mycoids* form long hair or root-like colonies as rhizoid growth that can reach several centimeters from the inoculation site, according to ***(Tallent et al., 2012).***

Results

1 Characteristics of the recovered *B. cereus* groups and *B. subtilis* isolates from examined meat products

The typical colonies of *B. cereus* and *B. cereus* like isolates on Mannitol Egg Yolk Polymyxin medium are crenate, about 5 mm in diameter and have a distinctive pink color colony surrounded by a zone of precipitation without mannitol fermentation (Nagler's reaction), as shown in fig 1A. These features distinguish *B. cereus* from other *Bacillus* spp, except B. *thuringensis* and *B. mycoids*. They also grow on this media with low intensity, giving weak egg yolk precipitation and rhizoid colony, respectively. While *B .subtilis* show yellow color on this media as shown in fig 1*B. cereus* group appears as Gram-positive bacilli and about 1×3.5 μm in size. They arrange in pairs or chains with round corners, as shown in fig 2. They may have a single non-bulging endospore that may be central, terminal, ellipsoidal or cylindrical. All positive *B. cereus* group isolates streaked onto blood agar plates and showed β-haemolytic clear zone surrounding the colonies and *B. subtilis* exhibited α-hemolytic greenish clear zone surrounding the colonies.

3 The prevalence of *Bacillus cereus* in meat and meat products:

The bacteriological examination of samples revealed (48) samples (34.2%) positive reactions to *B. cereus* groups which include(32) *B. cereus*(22.8%), 7 *B. mycoids*(5%) and 9 *B. thuringiensis*(6.4%)) and (11) samples (7.8 %) positive reaction to *B. subtilis* **Fig (3).**

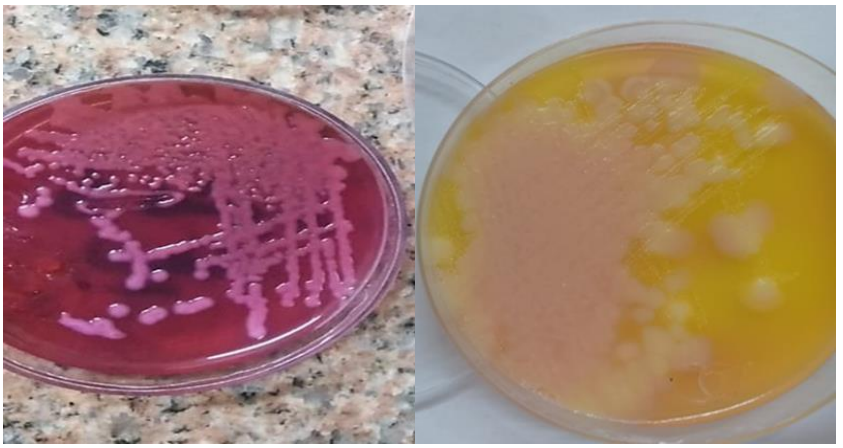
Statically, there is a significant difference in the prevalence of *B. cereus* group (F=4.812, P=0.021). The pairwise comparisons with adjusted P-value revealed a significant difference in the prevalence rate of *B. cereus* than *B. mycoids* (P=0.031) and *B. cereus* than *B.thuringiensis* (P=0.048).

There is a significant difference in the prevalence of *B. cereus* among examined meat products (t=3.128, P=0.020). Statically, there is a non-significant difference in the prevalence of *B. subtilis* among examined meat products(t=2.294, P=0.062).

4. Enumeration of *B. cereus* in the examined samples of meat products:

It is apparent from the results tabulated in a table (3) that *B. cereus* was isolated from the inspected meat products samples with mean values of 1.65x104±1.01x104, 1.23x104±1.13x104, 1.96x104±6.79x103, 1.95x104±1.03x104 and 0.89x104±0.49x104 (cfu/g) in sausage, minced meat, luncheon, beef meat and liver meat, respectively. Also, there was a significant difference between the

examined products based on *B. cereus* counts (P<0.05) by ANOVA analysis.



(A (B)

Figure (1). (A) Colonies of *Bacillus cereus* group grown on Mannitol Egg-Yolk Polymyxin agar plate (pink colonies surrounded by a zone of precipitation [lecithinase-positive], after overnight incubation at 30°C), (B) Colonies of *Bacillus subtilis* grown on Mannitol Egg-Yolk Polymyxin agar plate (yellow colonies).

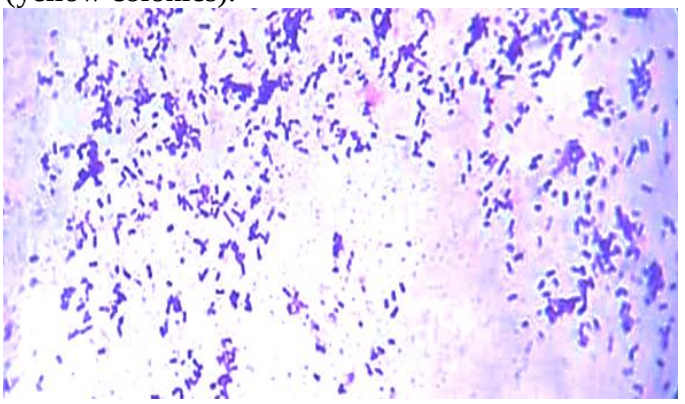


Fig 2 definitive character of *bacillus* under a microscope

3.2 Biochemical identification of *B. cereus* group and *B. subtilis* identification of *B. cereus* group and *B. subtilis* as shown in table (2)

Table (1): *Identification of B. cereus group and B. subtilis*

Species	Haemolysis	Rhizoid growth	Psychrotolelance	Protein toxin crystal production
B. cereus	+	-	-	-
B. mycoides	+	+	-	-
B.thuringiensis	+	-	-	+
B. subtilis	+	-	-	_

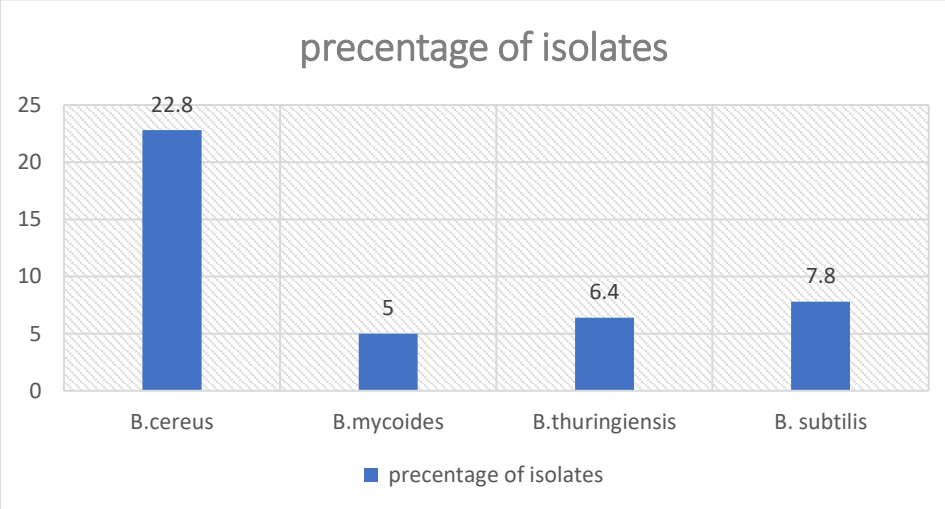


Fig 3 prevelance of *B.cereus* and *B.subtilits* among isolates

Table (2): *Statical analytical of B. cereus in the examined samples of meatproducts*

Samples	No.of collected samples	Min	Max	Mean± SE*
Sausage	28	$3.0x10^2$	$4.9x10^4$	$1.65x10^4 \pm 1.01x10^4$
Minced meat	28	$1.0x10^2$	$3.5x10^4$	$1.23x10^4 \pm 1.13x10^4$
Luncheon	28	$1.0x10^2$	$6.5x10^4$	$1.96x10^4 \pm 6.79x10^3$
Beef meat	28	$3.0x10^2$	$8.5x10^4$	$1.95x10^4 \pm 1.03x10^4$
Liver	28	$6.0x10^2$	$2.1x10^4$	$0.89x10^4 \pm 0.49x10^4$
Total	140			

***SE**=standard error of the mean

Discussion

In general, the presence of microbial hazards in meat products is unavoidable due to microorganisms on animals and in their environment ***(Maricica et al., 2014).*** Additionally, exposure to microorganisms from multiple sources during preparation and processing differed according to the manufacturing method and the quality of non-meat added ingredients ***(Xavier et al., 2014).*** In this study, the bacteriological examination of 140 samples, including sausage, minced meat, luncheon, beef meat and liver meat, were collected from various retailers and supermarkets throughout Port Said, Egypt, revealed 48 (34.3%) positive reactions to *Bacillus* group. The majority revealed (32) of the isolates were *B. cereus* with percentage (22.8%) and the others were (7) *B. mycoids* and (9) *B. thuringiesis* with 5% and 6.4%, respectively. However, 11 (7.8%) revealed in positive reaction to *B. subtilis*. The positive prevalence in meat and meat products consumed in Turkey was 22.4 % ***(Tiwari et al., 2015),*** close to our results. In general, the presence of *B. cereus* group and *B.subtilius* in meat products is most likely owing to the heat resistance of *Bacillus* spores, which enable this bacterium to persist in hard environments, in addition to incorrect storage conditions and cooking ***(Rather et al., 2011)*** Additionally, it was discovered that components such as spices, seasonings, and protein supplements added during processing included *B. cereus* ***(TeGiffel et al., 1999).***

In addition, the results in a table (3) show that *B. cereus* was isolated from the inspected meat products samples with mean values of 1.65x104±1.01x104, 1.23x104±1.13x104, 1.96x104±6.79x103, 1.95x104±1.03x104 and 0.89x104±0.49x104 (cfu/g) in sausage, minced meat, luncheon, beef meat and liver, respectively. These results exceeded those obtained by ***(Ibrahim-Hemmat et al. 2014)***in sausage samples. Generally, the absence or lack of hygienic precautions during processing, handling, and storage, as well as the misuse of storage temperature and inappropriate cooking, allow the spore of *B. cereus* to germinate and multiply, which is considered the probable reason for the increased *B. cereus* count ***(Bashir et al., 2017).*** This microorganism causes two types of illness syndromes: emetic syndrome, caused by the ingestion of a toxin called cellulite that is pre-formed within the food during *B. cereus* growth. The emetic

syndrome has a relatively short incubation period, with symptoms of nausea, vomiting, and abdominal cramping occurring between 1-5 hours after intake and typically resolving within 6-24 hours.

Additionally, the diarrheal syndrome is caused by enterotoxins produced by B. cereus inside the body and typically lasts 12-24 hours but can last for many days, accompanied by nausea, abdominal cramps, and watery diarrhea ***(Senesi and Ghelardi, 2010).*** Additionally, as indicated in the table, ANOVA analysis demonstrated that the differences between the studied meat products samples were statistically significant ($p<0.05$) (3). This could be due to the products' disparate components and processing methods ***(Hefnawy and Youssef, 1984).***

5. Conclusion

These findings reveal that the presence of microbial hazards such as *B. cereus* ,*B. mycoids* , *B. thuringiensis* and *B. subtilis* in meat products are significant sources of food poisoning and major hazardous diseases. Overall, the high prevalence of *Bacillus cereus,B. mycoids, B. thuringiensis* and *B. subtilis* in the analyzed meat products, indicating that meat products may constitute a public health danger. Therefore, efforts to ensure the quality of raw materials and environmental and hygienic conditions during processing should be implemented to ensure the provision of reasonably safe products.

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الكشف عن بعض المخاطر الميكروبية في اللحوم ومنتجات اللحوم

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الملخص العربي

الميكروبات و أخطارها تعد من اكبر المخاطر التي يجب ان تؤخذ في الاعتبار. تلوث منتجات اللحوم بالميكروبات يجعلها غير امنه للاستهلاك الادمي. في هذه الدراسه استخدمنا 140 عينه من منتجات اللحوم ممثله في السجق واللحم المفروم واللانشون والبيف والكبد ولقد جمعت بشكل عشوائي من مختلف تجار التجزئه ومحلات الجزاره في محافظه بورسعيد لدراسه وجود ميكروب الباسيلس سيريس والباسيلس ساتلس وكانت النتائج وجود 48عينه بنسبه (34.2%) موجبه لمجموعه الباسيلس سيريس متضمنه 32 عينه للباسيلس سيريس بنسبه (22.8%) و 7 عينات للباسيلس ميكويدس (5%) و 9 عينات للباسيلس ثيورنجينسس (6.4%) و 11 عينه للباسيلس ساتلس (7.8%) بمتوسط قيم 1.65 ,1.23x104±1.13x104 ,x104±1.01x104 1.96x104±6.79x103, 1.95x104±1.03x104 and 0.89x104±0.49x104 (cfu/g) للباسيلس سيريس في هذه العينات بالترتيب . وتلك النتائج تحدد وجود خطر ميكروبي في عينات منتجات اللحوم .