ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Characterization of *Bacillus* spp. isolated from spices collected from Iraqi markets

Saad Sabah Fakhry, Ahmed Ibrahim Jessim, Samara Jafar Alwash
Ministry of science and Technology- food contaminated research center

Manuscript Info

Manuscript History:

Received: 15 January 2015
Final Accepted: 25 February 2015
Published Online: March 2015

Key words:
Characterization, *Bacillus* spp., spices, Iraqi and Ribosomal 16SrRNA

Corresponding Author

Saad Sabah Fakhry

Abstract

Aim of this work to study Bacillus spp. associated with common spices to understand if any harm they can cause to the consumers. A total 40 spores-formers Bacillus were isolated from different spices samples were characterized using Biochemical test, growth on Chromogenic media and further characterization by PCR technique using Ribosomal 16SrRNA. Results showed variation in biochemical test as well as the total count was higher with fennel spice **44.6×10^5** and lesser count with **Shawerma spice 0.21×10^5**. Seven isolates were identified with 16S rRNA gene-specific PCR. In conclusion it is clear that raw spices may be contaminated with microbial pathogens and spores forming Bacillus.

INTRODUCTION

Spices is a term generally means a group of substances of vegetable origin rich in aromatic and resinous principles and essential oils used as a condiment or, if necessary, in the pharmaceutical sector and cosmetics (1). At the time of collection, an inevitable contamination by the most different microorganisms originating in soil, in particular aerobic spore-forming bacteria and anaerobes (2). Most of them, moreover, are produced in tropical and subtropical countries where conditions general hygiene may be lacking, for which if not properly handled and stored, represent an important source of food contaminations to which they are added (3). Spices are dried and consequently have a low active water value, this means that the products are microbially stable (4). Because of their origin and the way of drying, they are highly microbially contaminated, after rehydrations by addition of moist ingredients, the end of products are susceptible to spoilage (5). In order to reduce the contamination of these products and the potential hazards arising from their use, the use of decontamination techniques, such as irradiation, is of fundamental importance. Testing of spices for pathogens is useful to screen for high rates of contamination entering a plant (6). Although spices have not been historically associated with outbreaks or product recalls due to some bacterial pathogens (e.g. *Listeria monocytogenes*, *Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus*, etc.), they have been associated with foodborne illness due to *E. coli* and *Salmonella* contamination (7). Bacteria in the spices such as *Bacillus* can cause a variety of foodborne illnesses especially when the spiced food are improperly refrigerated or when leftovers are improperly stored for several days (8; 9). The main impact being on public health risks is when using these spices and herbs as an addition to ready-to-eat foods (10). This is especially true for those that undergo little further processing after being added to these foods. Studies have shown that overall 3.0 % of herbs and spices contained high counts of *B. cereus* (11). For instance, issues have been raised concerning spices imported from India (12). Four unprocessed Indian spices sampled at the point of export were shown to be highly contaminated with bacteria. *B. cereus* detected in more samples of these spices than any other spore-forming aerobe (13). The present work was aimed to study *Bacillus spp.* associated with common spices to understand if any harm they can cause to the consumers.

Materials and Methods

Bacillus Isolation

15 different types of spices were collected from local Iraqi markets of Baghdad city .Samples were rehydrated in sterilized glass bottles by adding sterilized distilled water. By adding 500µl of spice suspension to 500 µl of Ethanol $2(CH_3)O$ (100%) and preparing serial dilutions after 30 minutes of $2(CH_3)O$ exposure, the sample was cultured on L.B agar.

Chromogenic media

Briefly, each sample of rehydrated spices mixed with an equal volume of 100%. Ethanol and incubated at room temperature for 30 min. one drop (100 µl) was then incubated onto HiCrom Bacillus Agar M1651 (Himedia), and the plates were incubated at 37°C under aerobic conditions for 24-48h.

Chemical and biochemical tests

Isolates were tested for catalase, motility, production of lecithinase, reduction of nitrate, the Voges – Proskauer reaction, tyrosinase activity, mannitol and arabinose utilization, anaerobic utilization of glucose, and starch and gelatin hydrolysis, according to standard protocols (14; 15; 16). Isolates were tested for hydrolysis of lecithin after incubation in egg-yolk nutrient agar, to detect production of phosphate acetylcholine hydrolase (17), and for haemolytic activity and production of a discontinuous haemolytic pattern on blood agar plates (18). When necessary, API 50 CHB strips (Biomerieux ux®) were used for further identification.

Extraction of chromosomal DNA from Bacillus

A fresh colony of *Bacillus* spp. isolates was inoculated in 7 ml of LB with the specific antibiotic and incubated at 37C° overnight. After incubation, bacterial cells centrifuged at 7000 rpm for five minutes, the pellets was washed with five ml of Lysis Buffer consisting of : 50 mM EDTA and 0.1 M NaCL then centrifuged again and resuspended cells with 1 ml of Lysis Buffer to which added 0.250 ml of lysozyme, after that we incubate for 10 min. at 37C°. We added 0.075 ml of 20% Sarkosyl, stirred by vigorously and centrifuged at 1000 rpm 4C° for 1min. then moved to precipitation of DNA by adding 0.1 volume of sodium acetate and 2.5 volumes of $2(CH_3)O$. Subsequently, the precipitated DNA was washed with $2(CH_3)O$ 70%, and centrifuged again in same conditions above, the obtained pellets resuspended in 500 µl of sterilized deionized water. Analysis was performed by electrophoresis in a 1.6% agarose gel as previously described (19).

PCR amplification and analysis of 16S rRNA

Tow universal Primers derived from highly conserved areas of the 16S rRNA molecules U1 (5'CGTGCCAGCAGCCGCGGTAAT3';514 TO 534 *E.coli* numbering) and U2(5'AAGGAGGTGATCCAGCCGCA 3'; 1541 to 1522 *E.coli* numbering) described by (20) were used for PCR amplification of the 16S rRNA from *B. cereus* isolates. They were derived from conserved regions amplifying a DNA fragment of about 1.1 kb, of 16S rRNA genes from *Bacillus* spp. and closely related genera. PCR mixtures and amplifications were carried out as described previously (19).

Results and discussion

Bacterial morphology, chemical and biochemical tests

Morphological studies of vegetative cells and microscopic examination of spores also physiological and biochemical tests of the *Bacillus* spp. isolates are performed and the results summarized in Table1 .All isolates are Gram positive, catalase test was showed a positive reaction . All isolates hydrolyzed gelatin, and did not produced acid from arabinose. Variable results obtained for lecithinase activity, V–P reaction, mannitol utilization, starch hydrolysis, tyrosinase activity and reduction of nitrate to nitrites. Haemolytic activity shown by 95% of the isolates, of these, 11% produced a discontinuous haemolytic pattern, which usually correlated with the presence of haemolysin BL (18). All the isolates had ellipsoidal spores that occupied a central position without distention of the sporangium. None of the isolates presents a parasporal inclusions. A number of physiological and biochemical properties have been used to differentiate the *Bacillus* sp. have been recorded by other research groups (13). They found a variation in biochemical as well as fermentative reactions particularly with maltose and millibiose. However, distinct type of bimolecular being produced by them and perhaps in the present study, variation was observed with xylose and other sugars. This would clearly indicate that bacillus having specific phenotypic line

Table 1: Biochemical characterization of isolated spore forming isolates

Isolate No. / Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	-	-	-	- R	± y	-	+ y	- R	+ Y	- R	-	-	- O	- O	- O
2	- O	± y	+ y	+ y	-	- O	- O	+ Y	- O	- R	- O	- D.O	- O	- O	- O
3	+ y	+	+	+	+	+	+ Y	+ Y	+ Y	- R	+ Y	- R	+	+	+
4	W+ O	- P	+ y	- p	+ y	W+ O	+ Y	+ Y	+ Y	- R	+	W+ D.O	+	+	+
5	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+
6	-	-	-	-	-	-	- O	- R	- O	-Y	- Y	-R	-	-	-
7	-	+	-	+	-	+	+	+	+	+	+	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	-	-	-	-	-	-	-	-	-	-					
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	-	-	-	-	-	-	-	-	-	-					
18	-	-	-	-	-	-	-	-	-	-			-	-	-
19	+	-	-	+	-	+	-	-	-	-	+	-	+	+	+
20	-	+	- O	+	+ y	-	-	-	-	-	-	-	+	+	+
21	+	+	+	-	+	+	+	-	+	+	+	+			

*** O=Orange, R=Red, Y=yellow, P=pink, D.O =dark orange**

1-Acid from Xylose, 2- Acid from Arabinose, 3- Acid from Glucose, 4- Acid from Mannitol, 5- Gelatin, 6- Gas formation during Glucose Utilization, 7- Citrate utilization, 8- Growth at 8 ° C, 9- Growth at 30 ° C, 10- Growth in 2% NaCl, 11- Growth in 5% NaCl, 12- Growth in 6.5% NaCl, 13- Growth in 10% NaCl, 14 - Anaerobic growth, 15- Growth at pH 5.7 nutrient broth, 16- Growth at pH 6.8 nutrient broth, 17- pH 8 in V.P broth, 18 - pH 5.5 in V.P broth, 19- Growth on MR-VP medium, 20 – Starch, 21- Casein.

Spices are known to be heavily contaminated with microorganisms. Fennel and Kabab spices may carry as many as $44.6x10^5$and $39.2x10^5$ bacteria / g. Curry, to a lesser extent, may also be infected (2, 5, 6, 11). most bacteria present in spices are aerobic spore-former. In the present study we have obtained results quite similar with four species of *Bacillus* being identified as shown in (Figure 1).. Among the species, we isolated only few of them have been associated with food poisoning. Indeed, *B. cerus* recognized as the etiological agent of food poisoning outbreaks in Europe as far back as 1960 (8). Black peeper contaminated with aerobic spore formers enormously increased the microbial count of meat like sausage. Aerobic spore formers also detected in Black peeper powder (9).

Table 2. Number of bacterial counts

Common name	Number of bacteria Cfu/mg
Cubeb	2.0×10^5
Cloves	0.42×10^5
Nutmeg	1.4×10^5
Black pepper	14.0×10^5
Cinnamon	1.2×10^5
Fennel	44.6×10^5
Cumin	12.1×10^5
Chili pepper	11.3×10^5
Turmeric	2.7×10^5
Curry	22.5×10^5
Biryani spices	0.22×10^5
Shawerma spices	0.21×10^5
Kabab spices	39.2×10^5
Bastirma spices	0.38×10^5
Dolma spices	0.6×10^5

The chromogenic plating medium provides selectivity for members of the Bacillus spp., the sensitivity and speed of detection depend upon the samples treatment that may contain mixed flora. In the present study, we show that Hi Crom *Bacillus* Agar M1651, now commercially available can also enhance the growth of *Bacillus* spp. spores. A chromogenic culture medium enables rapid isolation of *Bacillus* spp. recent development in synthetic enzymatic substrates has allowed improved detection and identification of microorganisms in various specimens. Chromogenic culture media contain multiple substrates that allow bacteria to form colored colonies depending on their enzymatic activity. This feature facilitates the differentiation of target pathogens with polymicrobial cultures with high specificity (16, 17). In our study, the proportion of *Bacillus* spp. showing a high extent of growth and was greater on HiCrom Bacillus Agar, although the colors were not significantly different on days 1-2 (Figure 1). The chromogenic enzymatic substrates and suitable germinate in HiCrom Bacillus Agar may allow better production of *Bacillus spp.* colonies on this medium than other conventional medium.

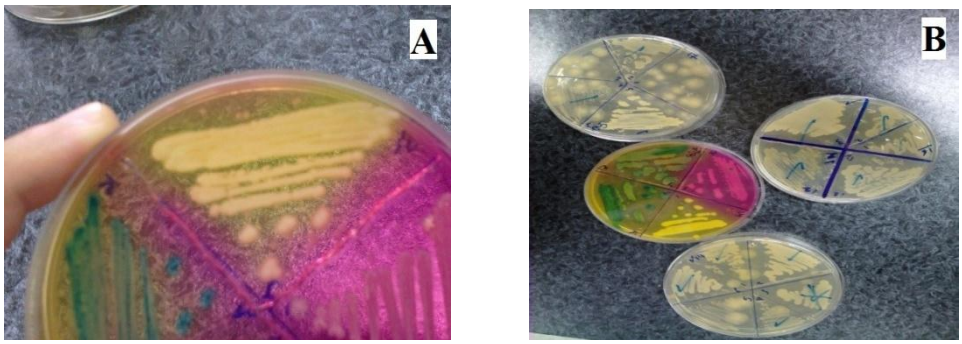

Figure 1. comparison of the extent of the growth by culture for Bacillus spp. on HiCrom Bacillus Agar (A), and L.B agar (B).

In the present study we have focused on identification of Bacillus spp. using a specific fragment of 16S-Rrna. The universal primer set used in this study generated 1.1- Kb amplicon after PCR amplification. Each isolates is identified with Ribosomal 16S-rRNA gene-specific PCR allowed for seven strains (indicated in Figure- 2) to be identified subsequently as positive. Of the 40 isolates, 15 isolates were chosen for molecular characterization. Ten isolates were further characterized by PCR using U1 and U2 primer to identify up to species level. A characteristic banding pattern compare with molecular marker determined as the *Bacillus cereus*. As many researchers have

developed a lot of different Bacillus specific primers i.e. (21), synthesized a Bacillus specific primer pair which was used to amplify a 257bp sequence near the 5' end of 16S rDNA gene and this sequence was very specific for identification and classification of Bacillus strains (22) developed a Bacillus specific primer pair (Bac F and Bac R). Specificity of both primers was checked independently and some species of Bacillus have shown 100% similarity with primer pair with 6 different *Bacillus spp*. Vardan et al (23) developed variable region in 16S rDNA gene of Bacillus.

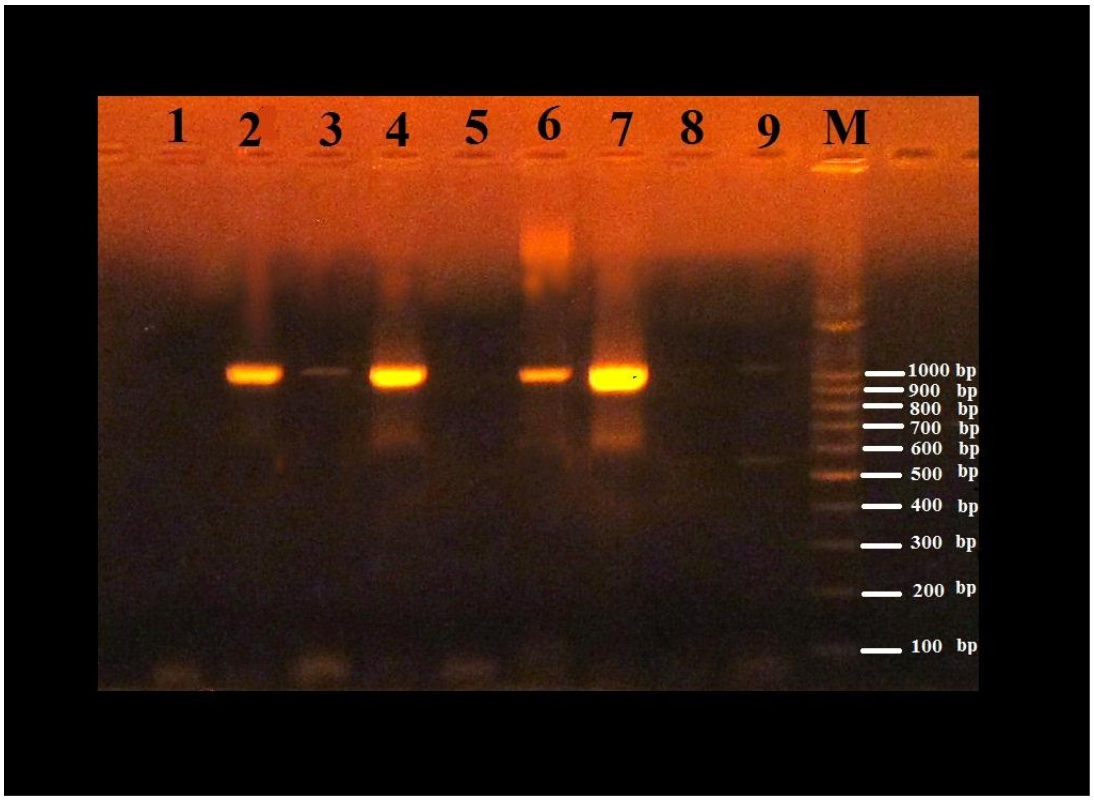

Figure (2): Representative PCR products showing amplicons of six *B. cereus* isolates. Lanes molecular size marker 100 .

Conclusions

Although it is clear that raw spices may be contaminated with microbial pathogens, there are treatment and testing options available to ensure food safety and minimize risk. Comprehensive food safety plans must always include knowledge of ingredients, controlling the supply chain, auditing suppliers, and planning for supply chain interruptions. Food manufacturers must then consider these programs when determining if use of a treated or untreated spice. All of this is taken into account to design a sound sampling and testing regime can help to minimize food safety risk.

References

1. Brackett, R. E. (2007). Microbiological consequences of minimally processed fruits and vegetables. J. Food Qual. 10(3): 195-206.
2. FAO. (2008a) Microbiological Hazards in fresh vegetables and herbs. Accessed December 3 2012.
3. Sagoo, S. K. Little, C. L. Greenwood, M. Mithani, V. Grant, K. A. McLauchlin, J. de Pinna, E. Threlfall, E. J. (2009). Assessment of the microbiological safty of dried spices and herbs from production and retail premises in the United Kingdom. Food Microbiol. 26(1): 39-43.
4. De Freitas Araújo, M. G. and Maria Bauab, *T.* Microbial Quality of Medicinal Plant Materials. Chapter 4 p 67 -77. In Akyar, I. (2012). Latest Research into Quality Control. Hand book. ISBN 978-953-51-0868-9, 514 pages, Publisher: InTech, Chapters published December 12, 2012 under CC BY 3.0 license. DOI: 10.5772/45955.

5. Guarino, P. A. (1972) Microbiology Of spices, herbs and related material. Proceeding 7th Annual symposium West New York State Institute of Food Technology,16-18,
6. Mousumi, B. P. (1972). Microbiological quality of some retail spices in India Food Res. Int. 36: 469 – 474.
7. Vij, V. Ailes, E. Wolyniak, C. Angulo, F. J. and Klontz, K. C. (2006). Recalls of spices due to bacterial contamination monitored by the U. S. Food and Drug Administration: The predominance of Salmonellae. J. Food Prot. 69(1): 233-237.
8. Jenson I, Moir CJ (2003) *Bacillus cereus* and other *Bacillus* species. Ch 14 In: Hocking AD(ed) Foodborne microorganisms of public health significance. 6th ed, Australian Institute ofFood Science and Technology (NSW Branch), Sydney, p. 445-478.
9. Agata N, Ohta M, Yokoma K (2002) Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. International Journal of Food Microbiology 73:23–27.
10. Jacobs Jr, D. R. and Steffen L. M. (2003). Nutrients, foods, and dietary patterns as exposures in research: a framework for food synergy[1–3]. American Society for Clinical Nutrition. ;78 (suppl):508S–13S.
11. Granum, P. E. and Lund, T. (2006) Bacillus cereus and its food poisoning toxins. FEMS Microbiology Letters. 157: 223-228.
12. Nokwanda, M. and Ijabadeniyi, O. A. (2013). Food borne bacteria isolated from spices and fate of *Cronobacter sakazakii* ATCC 29544 in black pepper exposed to drying and various temperature conditions. African Journal of Microbiology Research 7(12):990-995.
13. Seenappa, M. and Kempton, A. G. (2008). A Note on the Occurrence of *Bacillus cereus* and Other Species of *Bacillus* in Indian Spices of Export Quality. Journal of Applied Bacteriology 50(2):225-228.
14. Gordon, R. E. (1981). One hundred and seven years of the genus Bacillus. In R. C. Berkeley, and M. Goodfellow (ed). The aerobic endosporeforming bacteria. Academic Press. London. p. 1-15.
15. Lancette, G.A., Harmon, S.M., (1980). Enumeration and confirmation of Bacillus cereus in foods: collaborative study. Journal of the Association of Official Analytical Chemists 63, 581 –586.
16. Pirttijärvi, T. S. M., M. A. Andersson, A. C. Scoging, and M. S. Salkinoja-Salonen (1999) Evaluation of methods for recognizing strains of the Bacillus cereus group with food poisoning potential among industrial and environmental contaminants. Syst. Appl. Microbiol, 22: pp.133-144.
17. van Netten, P., and J. M. Kramer. (1992). Media for the detection and enumeration of Bacillus cereus in foods: A review. Int. J. Food Microbiol. 17:85–99.
18. Beecher, D.J. and A.C.L. Wong. 1994. Identification of Hemolysin BL-producing Bacillus cereus isolates by a discontinuous hemolytic pattern in blood agar. Appl. Environ. Microbiol, 74:1646-1651
19. Alippi, A.M., Lopez, A.C., Aguilar, O.M., 2002. Differentiation of Paenibacillus larvae subsp. larvae, the cause of American Foulbrood of honeybees, by using PCR and restriction fragment analysis of genes encoding 16S rRNA. Applied and Environmental Microbiology 68, 3655–3660.
20. Ash, C., Priest, F. G. and Collins, M. D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus Paenibacillus. Antonie Leeuwenhoek J. Microbiol. 64: 253-260.
22. Goto K, Omura T, Hara Y, Sadaie Y (2000) Application of the partial 16S rDNA sequence as an index for rapid identification of the species in the genus Bacillus. J Gen Appl Microbiol 46:1 –8
23. Garbeva P, van Veen JA, van Elsas JD (2003) Predominant Bacillus spp. in Agricultural Soil under Different Management Regimes Detected via PCR-DGGE. Microb Ecol 4 5 (3): 30 2–316.
24. Vardhan S, Kaushik R, Saxena AK, Arora DK (2011) Restriction analysis and partial sequencing of the 16S rRNA gene as index for rapid dentification of Bacillus species. Antonie Van Leeuwenhoek 99:283–296