PCR As A Confirmatory Method For Identification Of *Bacillus Cereus* Enterotoxigenic Strain Isolated From Flavored Milk

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ABSTRACT

Fifty random samples of flavored milk were collected from different supermarkets in Assiut city; these samples were examined for the presence of *Bacillus cereus* using surface plating technique on KG agar plates. *B.cereus* was isolated from 10 (20%) of the samples using conventional methods in identification. Presence of *hblA* gene in these 10 isolates was studied using polymerase chain reaction (PCR) assay and the results revealed that 5 isolates out of the 10 *B.cereus* strains were positive for the presence of such gene. It is well known that the hemolysin is a potent dermonecrotic /enterotoxic enterotoxin responsible for diarrheal food poisoning. The public health hazards and the recommended measures required to prevent contamination of such products by *B. cereus* were discussed.

INTRODUCTION

Flavored milk is a sweetened dairy drink made by adding sugar, coloring and artificial or natural flavorings to the milk, often pasteurized by using ultra-high temperature (UHT) treatment, which gives it a long shelf-life, sold in the refrigerated dairy case along side other milk products, it is very nutritious and has good taste. A myriad of studies support the benefits of children-drinking flavored milk and confirm its role in a healthful diet.

The occurrence of *B.cereus* in milk has been reported since, 1916 and this bacterium is a common contaminant of raw milk, sometimes can be found in large numbers in dairy products ***(1)***.

The role of *B.cereus* in outbreaks of foodborne illness is becoming well documented in 1950, the first report of confirmed cases of *B.cereus* food poisoning and the followed reports confirming the role of this organism in foodborne diseases ***(2-5).***

B.cereus spores have the ability to survive pasteurization and germinate ***(6)*** so it's not surprising that this organism considered a common contaminant of pasteurized milk, furthermore heat resistant strains of *B.cereus* may survive the UHT process if it was present in sufficient numbers initially ***(7)***. Elsewhere, its incidence in pasteurized milk is reported as being 2%in China ***(8)***, 25% in the Netherlands ***(9)*** and 33% in Australia ***(10)*** with levels generally being less than 1000 CFU/ml.

Bacillus cereus spores are very hydrophobic; such feature allows them to attach the surfaces of the pipelines of the dairy processing plant. Also the pasteurization heat is insufficient to kill the spores ***(11)***.

Two types of illness have been reported for *B.cereus*, diarrheal syndrome caused by at least 2 different types of enterotoxins during vegetative growth of *B.cereus* in food, as well as in the small intestine after consumption of contaminated food ***(12,13)***. While in emetic syndrome a quite number of vegetative cells may resporulated after heat treatment and produce the emetic toxin ***(14)***.

The food-borne diarrhea caused by *Bacillus cereus* mainly attributed to the production of heat-labile enterotoxins during the growth of vegetative cells in the small intestine ***(15)***.

B.cereus in dairy products is not only of concern as a public health hazard but also as a cause of economic losses through spoilage of contaminated products. Examples of such spoilage including bitty cream, sweet curdling of milk and off-flavors in various products ***(16,17)***.

The hemolysin Bl from *B.cereus* that consists of three components, binding protein B, lysine protein L_1 and L_2 is the enterotoxin responsible for the diarrheal form of gastroenteritis caused by food-borne strains of this pathogen *(18)*.The B component, encoded by the *hblA* gene, was cloned and sequenced *(19)*.

Hemolysin BL (HBL) is the best investigated enterotoxin, it has hemolytic, dermonecrotic, and vascular permeability activities and is considered the primary virulence factor in *B.cereus* diarrhea *(20)*.

Mäntynen and Lindström,*(21)* stated that all the enterotoxic strains of *Bacillus cereus* carry the *hblA* gene .

Pirttijarvi et al., *(22)* concluded that about half of *B.cereus* strains produce diarrheal enterotoxin. So the great majority of researches on the pathogenic nature of this organism have been toward identifying and characterizing of the diarrheal enterotoxin, evidence is accumulating that hemolysin BL is a major *B.cereus* virulence factor *(23)*.

Since its introduction in the Egypt or world, polymerase chain reaction (PCR) technology has proved to be a valuable method and the most precise analytical tool for detection of pathogens in food. PCR has now made the characterization of gene fragments from a large number of isolates feasible *(24)*.

This study was planned to evaluate the occurrence of *B.cereus* in flavored milk package and the enterotoxigenic capability of the isolated *B.cereus* strains through detection of *hblA* gene in these isolates.

MATERIAL AND METHODS

Collection of samples

Fifty random samples of flavored milk were collected from different supermarkets in Assiut city, these samples were dispatched directly to the laboratory with a minimum of delay to be examined.

Each package of flavored milk was thoroughly mixed and aseptically opened. Eleven ml of each well mixed package were transferred to 99 ml of sterile 0.1% peptone water to make final dilution of 1:10 and 10 fold dilutions of the samples were prepared

Isolation of *B.cereus*

Ten fold serial dilutions of samples were prepared and 0.1 ml from each decimal dilution of samples under investigation was transferred carefully onto Kim-Goepfert (KG agar) plates and incubated by spreading evenly over a dry surface of the plates by a bent glass rode using surface plating technique.

KG agar plates were incubated for 24h at 30°C , after which examined for typical colonies which were dry, flat and surrounded by a wide cloudy zone. Colonies presumbed to be *B.cereus* were transferred to nutrient agar slants which were incubated at 30°C for 24h.

Identification of *B.cereus* isolates

Gram stain, motility test were done for each isolate *(25)*.

Biochemical identification of *B.cereus* based on hemolysis on sheep blood agar, methyl red reaction, Voges-Proskauer reaction, citrate utilization, were carried out *(23)*.

Identification of *B.cereus* by PCR technique

This part of the study has been done in Genetic Engineering and Molecular Biology Research center of Assiut University.

The ingredients necessary for PCR

1- Template DNA or chromosomal DNA: DNA was extracted from fresh cultures of *B. cereus (26)*.

2-Single stranded oligonucleotide primers (supplied by Biolegio, Netherlands): Two oligonucleotide primers were designed and synthesized according to the determined sequence of *hblA* gene encoding the B component of hemolysin BL from *B. cereus*. *(19)*.

Table 1. Showing specific primer sequences.

Primer	Primer sequences	Targete gene
Primer 1 (HBLA1):	5`-GTGCAGATGTTGATGCCGAT- 3` (Forward)	*hblA*
Primer 2 (HBLA2):	5`-ATGCCACTGCGTGGACATAT- 3` (Reverse)	

3. PCR beads (Ready- to- Go™ PCR Beads) (Amersham Pharmacia Biotech, Austria): PCR beads designed as pre-mixed predispensed reactions for performing PCR amplifications. Each bead contains all of the necessary reagents, except primer and template, for performing individual PCR reaction. Each reaction contained 1.5 units of Taq DNA Polymerase, 10 mM Tris-HCL (pH 9.0), 50 mM KCL, 1.5 mM MgCl2, 200 u M of each d NTP and stabilizers including BSA.

PCR reaction

PCR amplification was performed in a final volume of 25 µl as follow: 1.5µl of primer 1, 1.5µl of primer 2, 2.0µl of template DNA and 20.0µl of sterile dist. water. The reaction mixture was overlaid with a drop of sterile mineral oil to avoid evaporation of the reaction mixture and incubated in a programmable thermal cycler (Biometra) for amplification of DNA *(26)* as following: 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min.

The PCR products were analyzed by 1.5% agarose gel electrophoresis using 2% agarose gels in TBE buffer. Gels were run for 120 min. at 100 V and Molecular weight Marker (Amersham) was included. Migrating DNA bands were visualized by ethidium bromide staining and UV transilluminator (Biometra) and analyzed using Biodoc Analyse (Biometra).

RESULTS

Out of 50 UHT flavored milk samples examined in this study, *B.cereus* was successfully isolated from 10 samples. When these 10 samples examined for the presence of hblA gene, 5 were found to be positive as explained in table (2).

Table 2. incidence of *B.cereus* in the examined flavored milk samples

No. of examined samples	Positive samples by using conventional methods		Detection of *hblA* gene by using PCR assay	
	No.	%	No.	%
50	10	20	5	10

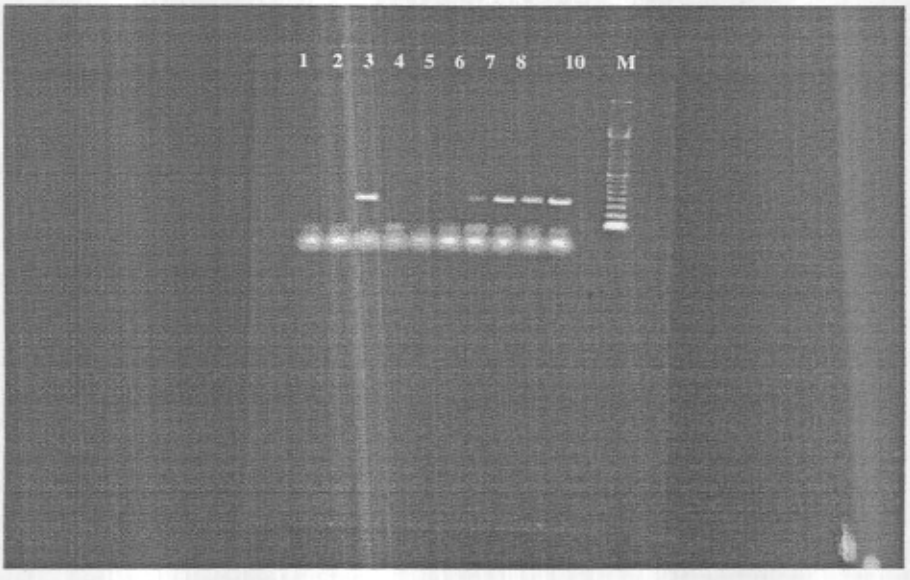

Fig 1. Electrophoretic analysis of 1.5% agarose gel stained with ethidium bromide of PCR amplification products of the tested samples
M : 100 base pair molecular weight marker
Lane : 3&7-10 are positive
Lane : 1,2& 4-6 are negative

DISCUSSION

In Egypt, ultra heat treated (UHT) milk has gained increase acceptance during the last few years to safeguard the milk consumers as well as to overcome the lack of cooling facilities needed by pasteurized milk at retail outlets. In spite of its subjection to high temperature during processing, several investigators *(27,28)* postulated that aerobic spore forming bacilli specially *B.cereus* are the organisms of greatest concern with the highest degree of heat resistance when the UHT process applied, that renders the product unfit for consumption and reflects something error in measures adapted during processing and post processing.

In Egypt, the consumption of flavored milk which is UHT milk, has been greatly increased in the last few years as a results of increased human welfare and its high palatability especially for children than plain milk.

No available data about incidence of *B.cereus* in flavored milk could be compared with the obtained results, so we will compare our results with the studies concerning UHT milk.

The results summarized in Table 2 revealed that out of 50 flavored milk samples, 10 (20%) were contaminated with *B.cereus* using conventional methods of identification. The obtained results were lower than that recorded in several previous studies ***(29-34)***.

On the other hand the freedom of examined UHT milk samples from *B.cereus* was recorded ***(35)***.

PCR technique was used to study the occurrence of *hblA* gene in these isolates; presence of such gene is an indicative that the strain which carries it is an enterotoxigenic strain. When these 10 *B.cereus* isolates were studied for the occurrence of the *hblA* gene, 5(50%) were found to be positive for the occurrence of such gene. These results indicate that these 5 isolates are enterotoxigenic strains. Our results are consistent with that which indicated that the *hblA* gene in was detected 52% of *B.cereus* strains and these strains are enterotoxin positive as revealed by test kit

from Oxoid. Also it has been reported *(36)* that 10 out of 23 (43%) of the *B.cereus* strains posses the *hblA* gene.

Hansen and Hendriksen *(26)* found that out of 22 *B.cereus* strains, 13(59%) were positive for the presence of *hblA* gene. In the other hand *hblA* gene was detected *(37)* in 66 out of 82(80.5%) *Bacillus cereus* strains isolated from milk and milk products. Also it is recommend the use of PCR-based technique for quicker detection of enterotoxigenic isolates from milk and milk products due to the very high correlation (97.5%) between results of PCR with the conventional phenotyping of toxigenic *B.cereus* using BCET-RPLA immunoassay kit (Oxoid).

The previous percentages support the theory that *B.cereus* was present in UHT milk, its presence may be attributed to poorly cleaned dairy processing , equipments, inadequate processing temperature , post UHT processing contamination or resistant surviving spores, neglected hygienic measures adopted during processing , failure to ensure asepsis during packaging, errors in storage and transportation,.

Bacillus cereus is one of the most important food-poisoning organisms as a result of its very widely distribution in nature. its presence in dairy products may be attributed to the environment or mastitic bovine sources *(38)*.

Wong et al. *(8)* stated that the high incidence of *B.cereus* in fruit- or nut-flavored milk mixes may be due to the carry-over of flavoring or coloring additives.

Detection of the hemolysin BL (*hblA*) indicates contamination of examined flavored milk packages with enterotoxigenic *B.cereus* *(18)*.

The explanation of the other 5 samples gave negative results in PCR is that these strains are not enterotoxigenic strains or they may be emetic strains. Several *B.cereus* strains don't contain *hbl* gene *(39)*, also none of the emetic isolates produce HBL, and didn't of them carry the *hbl* gene, whereas *hbl* production was frequently observed among isolates derived from diarrheal type food poisoning and from different food sources as well *(40)*. This may explain the low number of isolated strains of *B.cereus* by PCR assay in comparison with biochemical methods.

In addition to rapidness and accuracy of PCR technique there is another advantage of PCR-based method in comparison with conventional phenotyping of toxigenic *B.cereus* isolates that is amplified regions could be a basis for the rapid differentiation of strains that can't be identified by available classical tests *(37)*.

Therefore to improve the microbiological quality of market flavored milk and to safeguard the consumers from being infected, the following suggestions should be taken into consideration:

High quality raw materials must be used.

Efficient heat treatment (time-temperature) combination during processing must be applied including factory and equipment hygiene.

Licenses should not be given to establishment unless all equipment, facilities and hygienic conditions are fulfilled.

The ideal packaging material should be sterile and nearly impermeable to air and light.

Flavored milk should be produced under the modern quality assurance system, the hazard analysis and critical control point (HACCP) to ensure quality a safety of the product.

From the results obtained in this study we can stated that the risk of food-poisoning caused by *B.cereus* in flavored UHT milk must not be neglected.

REFERENCES

1. ***Kim, H.V. and Goepfert, J.M. (1971):*** Enumeration and identification of Bacillus cereus in food. Appl. Microbiol. 22: 581-587

2. ***Hauge, S. (1950):*** bacillus cereus as a cause of food poisoning. Nordisk Hyg. Tidskr. 31:

184-206 (cited after Kim and Goepfert, 1971)

3. ***Hauge, S. (1955):*** food poisoning caused by aerobic spore forming bacilli. J. Appl. Bacterial. 18: 591-595

4. ***Midura, T. M.; Wood, R and Leonard, A.R. (1970):*** Outbreak of food poisoning caused by Bacillus. Pub. Health Rep., 85: 45-48

5. ***Mossel, D.A.; Koopman, M.J. and Jongerius, E. (1967):*** Enumeration of Bacillus cereus in foods. J. Appl. Microbiol. 15: 650-653

6. ***Stadhouders, J.; Hup, G. and Langveld, L.P.M. (1980):*** Someobservations on the germination, heat resistance and outgrowth of fast germinating and slow germinating spores of bacillus cereus in pasteurized milk. Neth. Milk Dairy J. 34: 215-228

7. ***Westhoff, D.C. and Dougherty, S.L. (1981):*** Characterization of Bacillus spp isolated from spoiled ultra high temperature processed milk. J. Dairy Sci. 64 (4): 572-580

8. ***Wong, H.C.; Chang, M.H. and Fan, J.Y. (1988):*** Incidence and characterization of Bacillus cereus isolates contaminating dairy products. Appl. Environ. Microbiol. 54(3) : 699-702

9. ***Van Netten, P.; Van de Moosdijk, A.; Van Hoensel, P.; Mossel, D.A. and Perales,I. (1990):*** Psychotrophic strains of Bacillus cereus producing enterotoxin.J. Appl. Bacteriol. 69: 73-77

10. ***Rangasamy, P.N.; Lyer, M. and Roginski, H. (1993):*** Isolation and characterization of Bacillus cereus in milk and dairy products manufactured in Victoria. Aust. J. Dairy Technol. 48: 93-95

11. ***Andersson, A., Ronner, U. and Granum, P.E. (1995):*** What problems does the food industry have with the spore-forming pathogens Bacillus cereus and Clostridium perfringens?. Int J Food Microbiol 28: 145-55

12. ***Ombui, J.N.; Schminger, H.; Kagiko, M.M. and Arimi, S.M.(1997):*** Bacillus cereus may produce tow or more diarrheal enterotoxin. FE MS Microbiol.Letl., 149 (2): 245-248

13. ***Kotiranta, A.; Lounatmaa, K. and Haapasalo, M. (2000):*** Epidemiology and pathogenesis of Bacillus cereus. Infection J. Microbes Infect. 2(2) : 189-198

14. ***Eley, A.R.;(1992):*** Toxic bacterial food poisoning in A.R.Eley (ed), microbial food poisoning. 1st ed. Chapman, hall, London pp 37-55

15. ***Granum, P.E. (1994):*** Bacillus cereus and its toxins. J. Appl. Bacteriol. Suppl. 23: 61s-66s

16. ***Te Gifflel, M. C.; Beumer, R. R.; Leijendekkers, S and Rombbouts, F.M. (1996):*** Incidence of Bacillus cereus and Bacillus subtillis in food in the Netherlands. Food Microbiol. 13: 1096-1100

17. ***Pirltijarvi, T.S.; Andersson, M.A. and Salkinoja-Salonen, M.S. (2000):*** Properties of Bacillus cereus and other bacilli contaminating biomaterial-based industrial process. Int.J. Food Microbiol. 60: 231-239

18. ***Ombui, J.N.; Nduhiu, J.G. and Gicheru, M.M.(2008):*** Direct detection of Bacillus cereus enterotoxin genes in food by multiplex polymerase chain reaction. International Journal of Intergrative Biology, 2(3): 172-181

19. ***Heinrichs, J.H.; Beecher, D.J.; Macmillan, J.D. and Zilinskas, B.A.(1993):*** Molecular cloning and characterization of the hblA gene encoding the B component of hemolysin BL from *Bacillus cereus.* J. Bacteriol. 175: 6760-6766

20. ***Beecher, D.J.; Schoeni, J.L.; and Wong, A. C. L.(1995):*** Enterotoxin activity of hemolysin BL from Bacillus cereus. Infect. Immun. 63: 4423-4428

21. ***Mäntynen, V. and Lindström, K. (1998):*** A rapid PCR-based DNA test for enterotoxic Bacillus cereus. Appl. Environ. Microbiol. 64: 1634-1639

22. ***Pirttijärvi, T. S. M.; Graeffe, T. H. and Salkinoja-Salonen, M. S. (1996):*** Bacterial contaminants in liquid packaging boards:

assessment of potential for food spoilage. J. Appl. Bacteriol. 81:445-458

23.Beecher. D.J. and Wong, A.C.L. (1994): Identification of hemolysin BL-producing *Bacillus cereus* isolates by a discontinuous hemolytic pattern in Blood Agar. Applied and Environmental Microbiology 60:1646-1651.

24.Chang, Y. H.; Shangkuan, Y. H.; Lin, H. C. and Liu, H. W. (2003): PCR Assay of the groEL Gene for Detection and Differentiation of *Bacillus cereus* Group Cells. Appl. Environ. Microbiol. 69: 4502-4510

25.A.P.H.A. (1984): Compendium of methods for the microbiological examination of foods. 2 ed., Amirican Public Health Association, Washington, D.C.

26.Hansen, B. M. and Hendriksen, N. B. (2001): Detection of Enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* Strains by PCR Analysis. Applied and environmental microbiology, Jan. 2001, p. 185–189

27.Farahnik,S.(1982): A quality control program for recommendation for UHT milk processing and aseptic packaging of milk and milk by product. Dairy and Food San. 2 (11): 454-456

28.El-Talawy, Mona, F. (1998): Studies on thermoduric organisms in raw and heat – treated milks. M.V.Sc. thesis, Fac.Vet.Med., Moshtohor, Zagazig Univ., Egypt

29.El-Laboudy, Ahlam,A.(1985): Studies on spore forming foood poisoning organisms in milk and some milk products. M.V.Sc. thesis, Fac.Vet. Med., Alexandria Univ., Egypt

30.Saad, Nagah, M.; El-Bassiony, T.A.; Ahmed, A H.A. and Moustafa, K.M. (1989): Microbiological quality of Egyptian ultra high temperature processed (UHT) milk. Assiut Vet.Med.J. 21(42): 63-69

31.Raju,V.V.R. and Kumar,M.K. (1989): Heat resistant aerobic spore forming bacteria in milk. Ind.J.Dairy Sci. 42: 71-74

32.El-Shinawy,Saadia,H.; Abdel-Aziz,Aida, M. and El-Hady, H.A. (1995): Microbiological quality of infant powdered milk. J. Egypt.Vet. Med. Assoc. 55(1,2): 147-154

33.Zahran,Seham,E.S.(1996): Assessment of the quality of UHT milk. M. V.Sc. thesis, Fac.Vet.Med., Moshtohor, Zagazig Univ. (Benha branch), Egypt.

34.Sobieh, Azza, M.K.; Al-Hawary,I.I. and Aman, I. (2002): Microbiological evaluation of milk and ice cream sold in Kafr El-Sheikh and Gharbia governorates. Minufyia Vet.J. 2(1): 79-89

35.Sabry,Y.M. (2001): Thermoduric psychotrophic bacteria in milk and some dairy products. M.V.Sc. thesis, Fac. Vet. Med., Assiut Univ., Egypt

36.Prüß, B.M.; Dietrich, R.; Nibler, B.; Märtlabauer, E.; and Scherer,S.(1999): The hemolytic enterotoxin HBL is broadly distributed among species of the Bacillus cereus group. Applied and Environmental Microbiology, 65(12) : 5436-5442

37.Torkar, K.G. and Možina, S.S. (2000): Differentiation of *Bacillus cereus* Isolates from Milk and Milk Products with Biochemical, Immunological,AP-PCR and PCR-RFLP Methods. Food technol. biotechnol. 38 (2) 135–142.

38.Davies, F.L. and Wilkinson, G. (1973): Bacillus cereus in milk and dairy products, p. 57-68 In B.C. Hobbs and J.H.B. Christian (ed.), The microbiological safety of food. Academic Press, Inc., New York

39.Okstad, O.A.; Gominet, M.; Purnelle, B.; Rose, M. ; Lereclus, D. and Kolstø, A. (1999): Sequence analysis of three *Bacillus cereus* loci carrying PlcR-regulated genes encoding degradative enzymes and enterotoxin. Microbiology, 145: 3129–3138.

40.Ehling-Schulz, M.; Svensson, B.; Guinebretiere, M.; Lindbäck, T.; Andersson, M. (2005): Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiology, 151: 183-197.

الملخص العربى

أختبار سلسلة تفاعل إنزيم البلمرة كوسيلة تأكيدية لميكروب الباسيلس سيريس المعزول من الألبان المنكهة

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تم فحص عدد ٥٠ عينة عشوائية من عبوات الألبان المنكهة والتى تم جمعها من محلات السوبرماركت المختلفة بمدينة أسيوط. وتم فحص العينات لمعرفة مدى تواجد ميكروب الباسيلس سيريس وذلك بإستخدام طريقة الفرد السطحى على مستنبت KG agar ، وقد تم عزل الميكروب من عدد ١٠ عينات (٢٠%) من عبوات الألبان المختبرة وذلك باستخدام الطرق البيوكيميائية التقليدية لتصنيف الميكروب. هذا وقد تم استخدام اختبار سلسلة تفاعل إنزيم البلمرة للكشف عن وجود الجين المسئول عن المكون (ب) للهيموليسين فى عترات الباسيلس سيريس المعزولة بالطرق التقليدية ووجد أن ٥ عترات من بين ١٠ عترات معزولة بالطرق التقليدية كانت ايجابية لوجود هذا الجين مما يدل على قدرة هذه العترات على افراز الهيموليسين الذى يعتبر الانتيروتوكسين الرئيسى لحدوث الاسهال المصاحب لحالات التسمم الغذائى.هذا وقد ناقش البحث الشروط الصحية لمنع تلوث الألبان المنكهة بميكروب الباسيلس سيريس وكذلك مدى خطورته على الصحة العامة.