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Identification and pathogenic potential of *Bacillus* species occurring in high numbers during Khamirat-Al laban spontaneous fermentations

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Introduction

Gergoush is a traditional Sudanese bread snack produced in three fermentation steps (Khamirat Al laban, adapted starter, final dough) followed by baking. The production of the primary starter Khamirat-Al laban is special because it is based on the spontaneous (acid) fermentation of milk and various legumes. The fermentation is known to be effected by lactic acid bacteria, *Bacillus* species and *Clostridium* spp. (Sherfi and Hamad, 2001). The studies performed on Gergoush fermentations so far have not identified the *Bacillus* to species level. The opportunistic human pathogen *Bacillus cereus* is often identified in African fermented foods, sometimes showing to be the dominant *Bacillus* spp. (Padonou et al., 2009). *Bacillus cereus* produces the toxin cereulide which is the cause of emesis, as well as cytotoxin CytK and two protein complexes; nonhemolytic enterotoxin (Nhe) and hemolysin BL (Hbl) involved in diarrheal food poisoning (Ehling-Schulz et al., 2004).

Main objectives

1) To sample four different milk/legume (chick pea, faba bean, lentil and white bean) based Khamirat-Al laban fermentations in order to identify the *Bacillus* present at species or group level. 2) To determine the pathogenic potential of identified *Bacillus cereus* species.

Methods and results

Bacillus species of Khamirat-Al laban

At the end of the primary starter fermentations at 24 h, the *Bacillus* spp. occurred in numbers of between 7.7 and 8.1 log₁₀CFU/g. At this time point the pH was between 4.8 and 5.0.

Randomly amplified polymorphic DNA PCR (RAPD-PCR) fingerprint profiles of the dominating *Bacillus* (198 isolates) were analyzed using the Bionumerics software, and 4 major clusters were obtained (Figure 1).

The dominating *Bacillus* species of the four different Khamirat-Al laban fermentations were; *B. cereus* sensu lato (RAPD group 1) occurring in the order of 50%, *B. licheniformis* (RAPD group 2), *B. sonorensis* (RAPD group 3), and *B. subtilis* (RAPD group 4).

The *B. subtilis* group species identifications were obtained by use of a combination of 16-23S rRNA internally transcribed spacer PCR (ITS-PCR), 16S rRNA gene sequencing and selected phenotypic tests.

Toxin gene profiles of the dominant *B. cereus* group species

The dominant *B. cereus* species isolates (110 isolates examined) from the four different Khamirat-Al laban were found to harbor at least one of the diarrheal enterotoxin encoding genes *hbl(A,D or C)*, *nhe(A,B or C)* or *cytK* as detected by duplex and multiplex PCR methods.

As shown in Table 1, only some of the isolates harbored the complete set of Hbl or Nhe encoding genes. None of the isolates harbored the cereulide synthetase gene *cesB* as detected by PCR.

TABLE 1: Percentage (%) occurrence of the food poisoning related toxin genes in *B. cereus* sensu lato isolates from Khamirat-Al laban made from chickpea (C), faba bean (F), lentil (L) and white beans (W). Numbers in parenthesis indicate the number of positive isolates.

Gene product	Hemolysin BL	Non hemolytic enterotoxin	Cytotoxin K	Cereulide synthetase <i>cesB</i>
Legume	<i>hbl(A,D,C)</i>	<i>nhe(A,B,C)</i>	<i>cytK</i>	<i>cesB</i>
C	67(12)	44(8)	39(7)	0
F	47(16)	32(11)	44(15)	0
L	59(16)	48(13)	37(10)	0
W	52(16)	65(20)	45(14)	0
Total	51(56)	47 (52)	42(46)	(0)0

hbl(A, D, C) and *nhe(A, B, C)* : Occurrence of all three genes encoding the complex of hemolysin BL and nonhemolytic enterotoxin respectively.

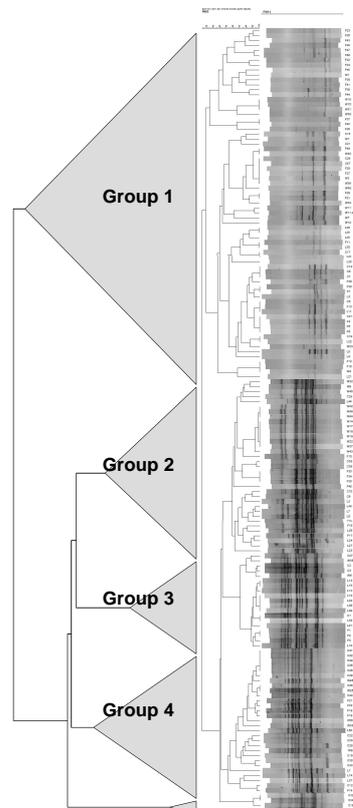


FIGURE 1. Dendrogram based on Dices coefficient of similarity with the Unweighted Pair Group Method with Arithmetic averages (UPGMA) of RAPD-PCR fingerprint patterns of the dominant *Bacillus* species of Khamirat Al laban. The *Escherichia coli* phage-M13 derived primer PM13, was used for the RAPD-PCR.

Conclusions

The present study is the first to identify the *Bacillus* of Khamirat-Al laban to species/group level. The *Bacillus* species present were; *B. cereus* sensu lato, *Bacillus subtilis*, *B. licheniformis* and *B. sonorensis*. It is the first time that *B. sonorensis* has been identified in a food product. The role of this bacterium is unknown.

Of concern was, that the opportunistic human pathogen *B. cereus* sensu lato was the most dominating *Bacillus* species representing approximately 50% of the *Bacillus* spp. in the four different Khamirat-Al laban fermentations.

The dominant *B. cereus* species showed to be potentially pathogenic, by harboring at least one of the *Hbl(A,D,C)*, *Nhe(A,B,C)* or *CytK* encoding genes related to diarrheal food poisoning

To evaluate the pathogenic potential of *B. cereus* as an indicator of the safety of Gergoush (the final product), investigations of the whole production process (Khamirat-Al laban, adapted starter, final dough, and after baking) are needed.

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