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GOAT COLOSTRUM—SOURCE OF TOXIGENIC *BACILLUS CEREUS*

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ABSTRACT

The aim of this study was to evaluate the toxigenic potential of *Bacillus cereus* strains isolated from frozen goat colostrum. Of the 50 phenotypically suspected *B. cereus* isolates, 39 (78.0 %) were confirmed as *B. cereus* by the polymerase chain reaction (PCR) method based on the gyrB gene detection. In these isolates, genes encoding the production of haemolysin BL (Hbl), a complex of non-haemolytic enterotoxins (Nhe) and emetic toxin were detected by the PCR method. In 36 (92.3 %) confirmed *B. cereus* isolates, genes encoding at least one type of toxins of interest were detected. In all toxigenic isolates, we found the presence of genes for Nhe production, and in 16 (41.0 %) of the isolates, genes encoding both Nhe and haemolysin BL were shown. Eight (20.5 %) of the emetic strains of *B. cereus* were identified. The emetic toxin production gene was always detected simultaneously with genes encoding non-haemolytic enterotoxin production. The ability to produce BL haemolysin and non-haemolytic enterotoxins were confirmed by the immunochromatographic method. In summary, goat colostrum can be a significant source of toxigenic strains of *B. cereus*.

Key words: cereulide; haemolysin BL; non-haemolytic enterotoxin; PCR

INTRODUCTION

Bacillus cereus is a Gram-positive, facultative anaerobic, and spore-forming rod. As a soil associated ubiquitous organism, *B. cereus* is commonly found in food products where they can cause spoilage [6]. *B. cereus* is the causative agent of two different types of food-borne illnesses: the emetic syndrome, caused by ingestion of a preformed emetic toxin (cereulide) in the food, and the diarrheal syndrome, caused by different toxins that can be formed in the food but also in the small intestine [5, 8]. The occurrence of these diseases are mainly associated with the consumption of foods made from cereals and potatoes, as well as vegetable dishes, minced meat dishes, milk and rice [8].

The diarrheal syndrome has been associated with three toxins: a single protein named cytotoxin K, and two heat-labile complexes—haemolysin BL and non-haemolytic enterotoxin (both composed of three protein components). The emetic syndrome is caused by the heat-resistant toxin cereulide [6].

As evidenced by a number of studies, raw milk, dairy products and dairy farm environments are a common source of toxigenic *B. cereus* [4, 5, 6, 12, 13, 15]. Simultaneously, psychrotrophic *B. cereus* strains capable of growing at temperatures below 8 °C can also be isolated [16]. The incidence of *B. cereus* in raw milk can be dramatically affected by the housing conditions [4]. Milk and dairy products are more commonly associated with the occurrence of a diarrheal form of the disease [10]. Nevertheless, in comparison with other food matrices the risk of toxin-induced diarrheal illness through consumption of contaminated milk may be limited [5]. According to a study of M a et al. [13], *B. cereus* was the dominant bacterial species found in goat milk powder (42.22 % from 249 identified species). The high occurrence of *B. cereus* in raw goat milk has also been shown in a study by Z h a n g et al. [23].

The objective of our study was to evaluate the toxigenic potential of *B. cereus* strains isolated from frozen goat colostrum samples.

MATERIALS AND METHODS

Bacterial strains

A total of 186 samples of frozen goat colostrum obtained from a local producer of food supplements were examined for the presence of *Bacillus cereus*. The individual samples of goat colostrum were collected from goats from different herds (accurate data were not provided by the producer). The samples (25 g) were incubated at 37 °C for 24 h in 225 ml of Buffered Peptone Water (Oxoid Ltd, Basingstoke, Hampshire, UK). The enriched samples were inoculated onto Mannitol Yolk Polymyxin B agar (MYP; HiMedia Laboratories Pvt. Ltd., Mumbai, India) and incubated at 30 °C for 24 h. A total of 50 (26,9 %) phenotypically suspected *B. cereus* strains (appeared as colonies with typical morphology on MYP agar and complete haemolysis on blood agar) were obtained and included in the study.

DNA isolation

For DNA isolation, colonies grown on blood agar plates were used (after incubation at 30 °C for 24 h). The DNA isolation was carried out by means of heating the bacterial cells in 200 µl sterile saline at 100 °C for 20 min with consecutive centrifugations at 10 660 × g for 6 min. The supernatant was used as a template.

Polymerase chain reaction

The polymerase chain reaction (PCR) was used for species confirmation and detection of genes encoding emetic toxin (cereulide), non-haemolytic enterotoxin (Nhe) and haemolysin BL (Hbl). A total of 4 multiplex polymerase chain reactions (mPCR) were carried out with specific primers to detect gyrB gene and toxicity determined by various *B. cereus* genes. The 16S rRNA gene was targeted as an internal control gene in the presence of bacterial DNA. The sequences of the primers used are given in the Table 1, the composition of the reaction mixture was adapted to PPP Master Mix use (Top-Bio, Ltd., Prague, Czech Republic). A total reaction volume of 25 µl was made up of 23 µl of the master mix and 2 µl of the template DNA. Amplification was performed on a PTC-200 thermocycler (MJ Research Watertown, Massachusetts, USA).

PCR 01: This was used for the detection of the gyrase B gene (*B. cereus* species confirmation), gene encoding the non-ribosomal peptide synthetase (which plays a role in the production of emetic toxin) and the highly conserved regions of the bacterial 16S rRNA (internal control). The PCR amplification involved: the initial denaturation step of 10 min at 95 °C, followed by 30 cycles, each including 1 min of denaturation at 94 °C, 1 min of annealing at 54 °C, 1 min of elongation at 72 °C, and the completion with a final elongation at 72 °C for 5 min.

PCR 02: This was used for the detection of the hblA and hblC genes encoding the production of haemolysin BL. The PCR amplification involved: the initial denaturation step of 4 min at 94 °C, followed by 36 cycles, each including 30 s of denaturation at 94 °C, 1 min of annealing at 62.5 °C, 1 min of elongation at 72 °C, and the completion with a final elongation at 72 °C for 7 min.

PCR 03: This was used for the detection of the nheA and nheC genes encoding production of the non-haemolytic enterotoxin. The PCR amplification involved: the initial denaturation step of 2 min at 94 °C, followed by

Table 1. PCR primers used in the study

Target gene	Primer	Primer sequence (5´– 3´)	Amplicon size [bp]	Reference	Annealing	PCR
gyrB	BC1	ATT GGT GAC ACC GAT CAA ACA	365	Yamada et al. (1999) [22]	54 °C	BC 01
	BC2	TCA TAC GTA TGG ATG TTA TTC				
NRPS*	CER1	ATC ATA AAG GTG CGA ACA AGA	188	Horwood et al. (2004) [11]	54 °C	BC 01
	CER2	AAG ATC AAC CGA ATG CAA CTG				
16Sr RNA	InKo1	GGA GGA AGG TGG GGA TGA CG	241	Martineau et al. (1996) [14]	54 °C	BC 01
	InKo2	ATG GTG TGA CGG GCG GTG TG				
hblA	HBLA1	GCT AAT GTA GTT TCA CCT GTA GCA AC	873	Rowan et al. (2003) [17]	62.5 °C	BC 02
	HBLA2	AAT CAT GCC ACT GCG TGG ACA TAT AA				
hblC	HBLC-N	AAT AGG TAC AGA TGG AAC AGG	399	Rowan et al. (2003) [17]	62.5 °C	BC 02
	HBLC-C	GGC TTT CAT CAG GTC ATA CTC				
hblD	HBLD-N	AAT CAA GAG CTG TCA CGA AT	439	Rowan et al. (2003) [17]	54 °C	BC 04
	HBLD-C	CAC CAA TTG ACC ATG CTA AT				
nheA	nheA344S	TAC GCT AAG GAG GGG CA	499	Ghelardi et al. (2002) [7]	54 °C	BC 03
	nheA843A	GTT TTT ATT GCT TCA TCG GCT				
nheB	NBF	TTT AGT AGT GGA TCT GTA CGC	743	Guinebretière et al. (2002) [9]	54 °C	BC 04
	NBR	TTA ATG TTC GTT AAT CCT GC				
nheC	NCF	TGG ATT CCA AGA TGT AAC G	683	Guinebretière et al. (2002) [9]	54 °C	BC 03
	NCR	ATT ACG ACT TCT GCT TGT GC				

* NRPS—non-ribosomal peptide synthetase

35 cycles, each including 1 min of denaturation at 94 °C, 1 min of annealing at 54 °C, 2 min of elongation at 72 °C, and the completion with a final elongation at 72 °C for 5 min.

PCR 04: This was used for the detection of the nheB gene (non-haemolytic enterotoxin) and hblD gene (haemolysin BL). The PCR amplification involved: the initial denaturation step of 2 min at 94 °C, followed by 35 cycles, each including 1 min of denaturation at 94 °C, 1 min of annealing at 54 °C, 2 min of elongation at 72 °C, and the completion with a final elongation at 72 °C for 5 min.

The amplified products were separated by electrophoresis on a 2 % agarose gel in 0.5× TBE buffer, followed by consecutive staining with ethidium bromide and visualization on a UV transilluminator.

Duopath® Cereus Enterotoxins GLISA

The ability of the strains to produce the diarrheal enterotoxins of Nhe and Hbl were verified by the use of the commercially available Duopath® Cereus Enterotoxins GLISA immunoassay kit (Merck KGaA, Darmstadt, Germany). The tests were performed according to the manufacturer's instructions. A strain was considered positive if the red lines appeared on both of the test and control zones.

RESULTS

Out of the 50 phenotypically suspected *B. cereus* isolates included in the study, 39 (78 %) were confirmed by the PCR method as *B. cereus*. In 36 (92.3 %) of the iso-

lates, the genes encoding for the production of at least one type of the toxins of interest were detected. The detection of genes encoding Nhe, Hbl and emetic toxin (cereulide) production were performed only in the confirmed *B. cereus* isolates. The summary of the results is given in Fig. 1 and Table 2.

At the same time, genes encoding the production of all three toxins (cereulide, Hbl, Nhe) were not detected in any of the isolates. As shown in Fig. 1, the detection rate of non-haemolytic enterotoxin genes among all *B. cereus* positive isolates was 30.8 % (12 isolates). In 16 (41.0 %) of the *B. cereus* isolates, genes encoding both non-haemolytic enterotoxin (Nhe) and haemolysin BL (Hbl) production were shown.

In our study, all 3 genes (hblA, hblC, and hblD) encoding the individual components of this toxin were always detected simultaneously. However, there was observed a different variability of the nheA, nheB and nheC genes in the goat colostrum *B. cereus* isolates (Table 2). Genes encoding all three components of both haemolysin BL (hblACD) and non-haemolytic enterotoxin (nheABC) were detected simultaneously only in six *B. cereus* isolates (15.4 %). In all potentially enterotoxigenic strains of *B. cereus*, the immunochromatographic method confirmed the ability to produce the observed enterotoxins.

A total of 8 (20.5 %) emetic strains of *B. cereus* were detected (Table 2). The emetic toxin production gene was always detected simultaneously with genes encoding for the production of a complex of non-haemolytic enterotoxins (Figure 1).

DISCUSSION

The genes nhe encoding non-haemolytic enterotoxin are thought to be present in all *B. cereus* strains [20]. Also, other studies have confirmed the high proportion of nhe genes in toxigenic *B. cereus* isolated from food [4, 9, 19]. The results of our work confirmed these assumptions.

The Hbl enterotoxin complex consists of B, L1 and L2, and its enterotoxigenic activity appears when all 3 components of the Hbl complex are present [2, 18]. In our study, the simultaneous presence of all hblA, hblC, and hblD genes encoding the production of haemolysin BL was demonstrated in 41 % of the *B. cereus* isolates, always in combination with genes for non-haemolytic enterotoxin.

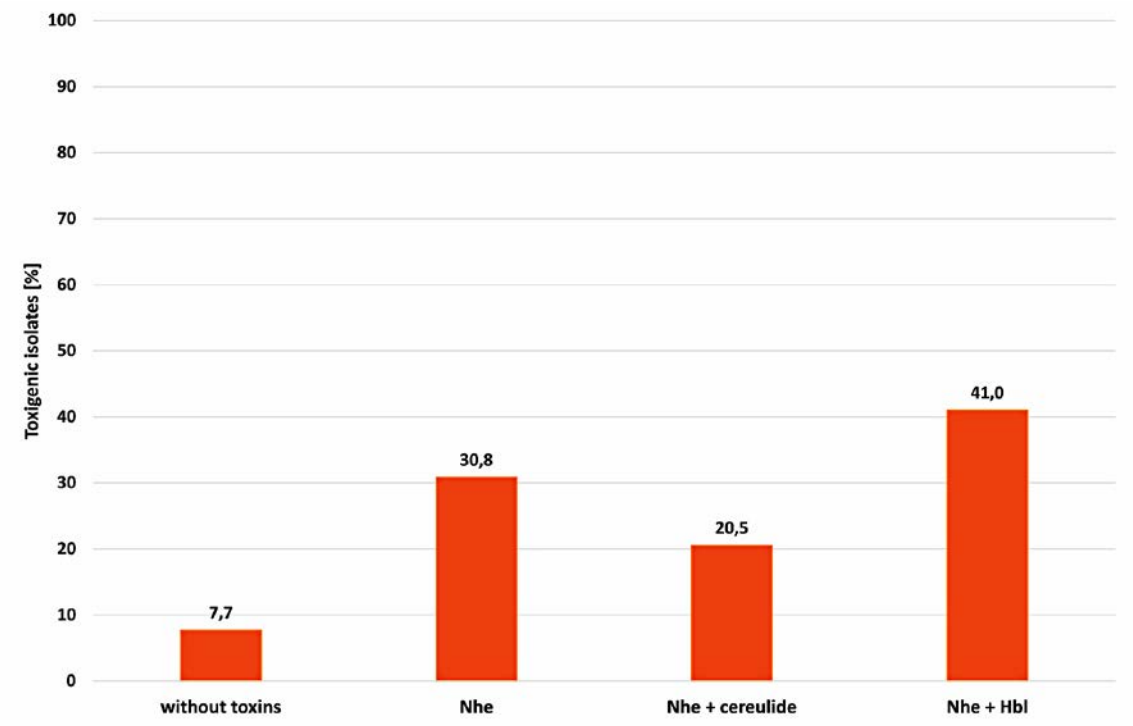

Fig. 1. Toxigenic potential of *B. cereus* isolated from goat colostrum (n = 39)
Nhe—non-haemolytic enterotoxin; Hbl—haemolysin BL; cereulide—emetic toxin

Table 2. Detection of toxin-encoding genes in B. cereus isolated from goat colostrum (n = 39)

Isolate	Cereulide	Haemolysin BL			Non-haaemolytic enterotoxin		
	cer	hblA	hblC	hblD	nheA	nheB	nheC
BC 505	–	+	+	+	+	+	+
BC 506	+	–	–	–	+	+	+
BC 507	–	+	+	+	+	+	+
BC 508	+	–	–	–	+	–	+
BC 511	–	–	–	–	–	+	+
BC 512	+	–	–	–	+	+	+
BC 513	–	–	–	–	–	–	–
BC 515	–	–	–	–	–	–	–
BC 516	–	–	–	–	+	–	+
BC 518	+	–	–	–	+	+	+
BC 519	–	+	+	+	+	+	–
BC 520	–	+	+	+	+	+	–
BC 521	+	–	–	–	+	–	+
BC 522	–	+	+	+	+	–	+
BC 523	–	+	+	+	+	+	–
BC 524	–	+	+	+	+	–	+
BC 525	–	–	–	–	–	+	+
BC 526	–	+	+	+	+	–	+
BC 527	–	+	+	+	+	–	+
BC 531	+	–	–	–	+	+	+
BC 533	–	+	+	+	+	+	+
BC 534	+	–	–	–	+	+	+
BC 536	–	–	–	–	–	+	+
BC 537	–	+	+	+	+	+	+
BC 539	–	+	+	+	+	+	–
BC 540	–	–	–	–	+	+	+
BC 541	–	–	–	–	+	+	+
BC 542	+	–	–	–	+	+	+
BC 543	–	–	–	–	–	–	–
BC 544	–	+	+	+	+	+	+
BC 546	–	–	–	–	+	–	+
BC 549	–	+	+	+	+	–	+
BC 550	–	+	+	+	+	+	+
BC 552	–	–	–	–	+	+	+
BC 553	–	–	–	–	+	+	+
BC 554	–	–	–	–	–	+	–
BC 556	–	–	–	–	–	+	–
BC 557	–	–	–	–	–	+	+
BC 558	–	+	+	+	+	–	+
Total (%)	8 (20.5)	16 (41.0)	16 (41.0)	16 (41.0)	30 (76.9)	26 (66.7)	30 (76.9)

Similar results were obtained by Cui et al. [4], who detected 55 % Hbl-positive *B. cereus* strains isolated from raw milk samples. Interestingly, the toxigenic strains of *B. cereus* have not yet been shown to produce Hbl as a separate enterotoxin [3].

The occurrence of *B. cereus* emetic syndrome is mainly associated with the consumption of rice and rice dishes, but also with foods of higher starch content, such as pasta or noodles [20]. Cressey et al. [3] reported that most of the *B. cereus* strains are capable of producing either diarrheal or emetic toxins. In contrast, Beattie and Williams [1] detected 36 % of *B. cereus* strains capable of producing both toxins simultaneously. In our study, we detected 20.5 % of emetic strains that also produced non-haemolytic enterotoxin. From this point of view, goat colostrum can be considered as a significant source of emetic *B. cereus*. Conversely, a relatively low proportion of emetic strains have been shown by the results of a study conducted in Sweden to monitor the occurrence of emetic toxin in *B. cereus* isolates from raw cow's milk samples from different farms. Of the 722 isolates, only 11 (1.5 %) emetic strains were detected [21]. The low incidence of emetic *B. cereus* strains was also shown in a study by Seong et al. [19], who detected 5 (7 %) emetic strains among 71 food isolates.

CONCLUSIONS

Recently, the popularity of goat's milk and its products has increased. Goat colostrum is increasingly used as a part of dietary supplements. *Bacillus cereus* spores very well withstand adverse environmental conditions as well as many technological operations used in food production. The results of our study confirm that goat colostrum may be a significant reservoir of toxigenic *B. cereus* strains, which may be able to produce not only different types of enterotoxins but also emetic toxins under appropriate conditions. From this point of view, the occurrence of toxigenic *Bacillus cereus* in goat colostrum could be a risk for consumers.

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