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METHODS EVALUATION TO DIFFERENTIATE PRESUMPTIVE *BACILLUS CEREUS* ON BUTTERHEAD LETTUCE

Background and research objective

Plant protection is nowadays increasingly based on biological control. An often used microbial control agent is *B. thuringiensis*, a naturally occurring, soil-dwelling bacterium which is closely related to the human pathogen *B. cereus*. Although there is a long history of safe use, there is still a lack of knowledge in some issues. One of these is the detection of *B. cereus* and *B. thuringiensis* in foodstuffs. *B. thuringiensis* and *B. cereus* cannot be differentiated using classical cultural detection or 16S rDNA sequencing. The objective was therefore to evaluate different analytical techniques for identification and differentiation of presumptive *B. cereus* on butterhead lettuce.

Methods

Thirteen butterhead lettuce samples (purchased in supermarkets in Ghent, Belgium) were plated on Mannitol Egg Yolk Polymyxin (MYP) agar, after which presumptive *B. cereus* colonies were isolated and kept for further analysis.

Next, different analytical techniques for identification and differentiation in the *B. cereus* group were tested on 21 strains (isolates from a culture collection), whereof 18 *B. cereus* group strains, 2 *B. subtilis* and 1 *B. amyloliquefaciens*, and on 17 presumptive *B. cereus* isolates from the sampled butterhead lettuce (Table 1). The different analytical techniques were selected based on their ease of performance and possibility to incorporate in routine diagnostics, and include:

- Cultural detection on MYP agar and chromogenic media (BACARA, RAPID'*B. cereus* and CHROMagar *B. cereus*).
- Psychrotrophic character: ability to grow at 4, 7 and 10°C in Brain Heart Infusion (BHI) and on Trypton Soy Agar (TSA).
- PCR for presence of toxin genes (*nheA*, *hblA*, *hblD*, *cytK1*, *ces*).
- Immunological assay Duopath® for enterotoxin production (Nhe and Hbl) after overnight incubation in BHI.
- Phase-contrast light microscopy for the observation of parasporal crystals after incubation on nutrient agar supplemented with $CaCl_2$ and $MgCl_2$ for 24 to 48 hours to allow sporulation to occur. Crystals were stained using a Coomassie Brilliant Blue solution.
- Identification with MALDI-TOF MS.

Results and discussion

Cultural detection

It was shown that all *B. cereus* group strains show the same colony morphology on the different tested agar media (Figure 1). Less background microbiota can be seen on BACARA and RAPID'*B. cereus* compared to MYP and CHROMagar *B. cereus*.

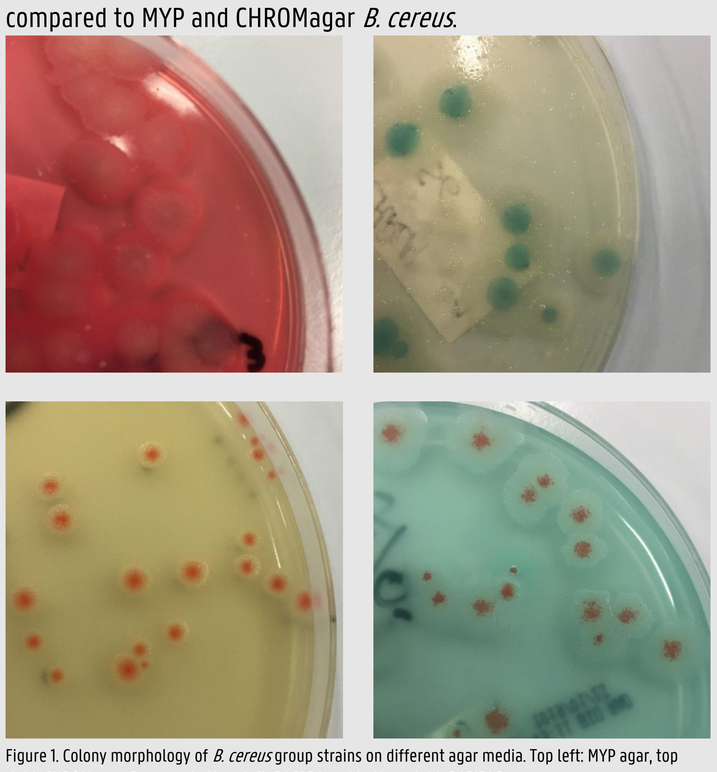

Figure 1. Colony morphology of *B. cereus* group strains on different agar media. Top left: MYP agar, top right: CHROMagar *B. cereus*, bottom left: BACARA and bottom right: RAPID'*B. cereus*.

Phase-contrast light microscopy

B. thuringiensis produces parasporal crystals during sporulation, that are observable using phase-contrast light microscopy and can be seen as less phase-bright inclusions next to the phase-bright spores (Figure 2). These crystals can therefore be used to differentiate *B. thuringiensis* from other *B. cereus* group species. However, there are still some difficulties to incorporate this technique in routine diagnostics:

- the possibility to confuse crystals with other internal bodies
- the time-consuming protocol
- the strain-dependency of the incubation time.

The staining experiment showed that it is not possible to stain the crystals before the lysis of the mother cell. Longer incubation times are needed so that free spores and crystals are obtained.

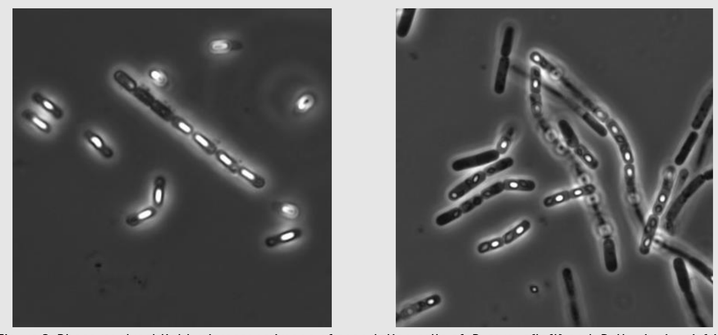

Figure 2. Phase-contrast light microscope image of sporulating cells of *B. cereus* (left) and *B. thuringiensis* (right)

Summary of the different tested analytical techniques

The results of the different techniques are summarized in Table 1.

Table 1. Results for the different tested analytical techniques for the strains from the culture collection and the isolates from the sampled butterhead lettuce

	Strains from culture collection				Isolates from butterhead lettuce	
	B. cereus	*B. thuringiensis*	Other *B. cereus* group	Other *Bacillus* species	Producing parasporal crystal	Not producing parasporal crystal
Total number	10	3	5	3	11	6
Typical *B. cereus* colony	10	3	5	0	11	6
Growth at 7°C	4	0	3	0	2	4
Presence of *nheA*	10	3	3	0	11	6
Presence of *hblA*	5	3	3	0	11	5
Presence of *hblD*	5	3	2	0	11	5
Presence of *cytK1*	0	0	1	0	0	0
Presence of *ces*	3	0	0	0	0	0
Production of Nhe	10	3	3	0	11	6
Production of Hbl	2	1	0	0	1	0
Parasporal crystal	0	3	0	0	11	6

The results show that, although some psychrotrophic *B. cereus* strains exist, the tested *B. thuringiensis* are not able to grow at 7°C, with the biocontrol strains having a minimum growth temperature even higher than 10°C. This is an interesting finding, as the *B. thuringiensis* strains present on butterhead lettuce after treatment, will per definition not be able to grow post-harvest in cold storage if temperature is ≤ 10°C.

Enterotoxin genes are present in all species of the *B. cereus* group, and therefore also in *B. thuringiensis*. This is not unexpectedly given the high genetic similarity and the fact that the enterotoxins are chromosomally encoded. The enterotoxins are also actively produced by *B. thuringiensis*, as shown by the Duopath® immunological assay.

Natural occurrence

Table 2 shows the presumptive *B. cereus* counts for the butterhead lettuce samples.

Table 2. Presumptive *B. cereus* counts for thirteen butterhead lettuce samples.

Conventional butterhead lettuce		Organic butterhead lettuce	
Sample	Presumptive *B. cereus* (log CFU/g)	Sample	Presumptive *B. cereus* (log CFU/g)
1	< 2.00	8	3.60
2	2.00	9	< 2.00
3	< 2.00	10	3.00
4	2.00	11	2.85
5	2.48	12	2.48
6	2.00	13	2.60
7	4.04		

From these samples, 17 presumptive *B. cereus* isolates were further characterized (Table 1). Eleven isolates were identified as *B. thuringiensis* according to the presence of parasporal crystals. Some of these as *B. thuringiensis* identified isolates can be excluded as possibly being a biocontrol strain based on differences between these isolates and the biocontrol strains: 4 isolates are excluded based on their ability to grow at 7 or 10°C, while 1 isolate is excluded based on its ability to produce Hbl in vitro. These are both characteristics that are not present in the biocontrol strains that are allowed on lettuce in Belgium. These 5 isolates are therefore assumed to be naturally occurring *B. thuringiensis* strains, rather than biocontrol strains. The remaining isolates have the same characteristics compared to the biocontrol strains an might therefore be biocontrol strains, although this has to be confirmed using other techniques.

Identification with MALDI-TOF MS

Nine out of 14 tested isolates from the sampled butterhead lettuce were identified as *B. anthracis* using MALDI-TOF MS, while all being haemolyse positive (*B. anthracis* is generally known to be haemolyse negative). Furthermore, isolates identified as *B. thuringiensis* using phase-contrast light microscopy were not identified as *B. thuringiensis* using MALDI-TOF MS. It is therefore concluded that MALDI-TOF MS is not able to identify *B. cereus* group species to the species level.

Conclusion

B. thuringiensis could not be differentiated from *B. cereus* using colony morphology, psychrotrophic character, presence of toxin genes, production of toxins or MALDI-TOF MS. **Phase-contrast light microscopy for the detection of the parasporal crystal seems to be a possible differentiation technique**, as *B. thuringiensis* is the only member of the *B. cereus* group which produces these crystals. **However, using this technique, the origin of the *B. thuringiensis* isolate cannot be determined.** To identify an isolate as a biocontrol strain, and therefore differentiate the isolate from a naturally occurring *B. thuringiensis*, identification to the strain level with e.g. Whole Genome Sequencing or biocontrol-strain-specific PCR is needed.

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