Bacillus cereus Isolation and Identification: From Traditional Microbiology Methods to Molecular Methods

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Introduction

Bacillus cereus is a spore-forming, facultative anaerobic bacterium, well known for its ability to cause **food poisoning** and **spoilage of milk** and **dairy products**. Identification of *B. cereus* in contaminated milk can be laborious and time-consuming. The current widely used method for the isolation and identification of *B. cereus* includes cultivation in mannitol egg yolk polymyxin (MYP) agar, forming pink-purple colonies surrounded by a pink halo of egg-yolk precipitate (lecithinase positive) (ISO 7932: 2004). PCR methods targeting the 16S rRNA gene have also been established for identifying *B. cereus* in milk products, and can be more sensitive and rapid.

Purpose

B. cereus can be detected in dairy products using both microbiological and molecular methods. This study aims to **improve** the ***B. cereus*** **isolation and identification** in pasteurized milk, by comparing the direct plating method in MYP and BRI (Brilliance *B. cereus*) agar and the 16S rRNA PCR and sequencing method.

Significance

Both traditional microbiological methods and 16S rRNA sequencing methods have their own advantages and limitations for the identification of *B. cereus* in milk. This study **compares** available **methods for the identification** of *B. cereus* from milk isolates and highlights the **advantage of molecular techniques**.

Materials & Methods

Isolates from pasteurized milk (n=194)
of Greek dairy industry

Microbiological Method

Mannitol Egg Yolk Polymyxin Agar (MYP) (Oxoid)
Brilliance™ Bacillus Cereus Agar (Oxoid)
30 °C for 24h

Molecular Method

DNA extraction
ZymoBIOMICS DNA Miniprep kit
(Zymo Research Corp.)

16S rRNA gene amplification
27F (5'-AGAGTTTGATCMTGGCTCAG-3')
1518R (5'-AAGGAGGTGATCCANCCRCCA-3')

Amplicon Sequencing
BigDye™ Terminator kit
(Applied Biosystems™, ThermoFisher Scientific Inc.)
3500 Genetic Analyzer
(Applied Biosystems™, ThermoFisher Scientific Inc.)

Bioinformatic Analysis
BLASTn algorithm
NCBI nt Database

Results

The results obtained indicated that the **chromogenic Brilliance agar performed better** than the recommended MYP agar in identifying the *B. cereus group* isolates from pasteurized milk (Figure 1). Among the 194 isolates, 177 and 115 were identified as *B. cereus group* on MYP and Brilliance agar, respectively (Figure 2). Moreover, the 16S rRNA method provided the most accurate identification and revealed that a high number of isolates belonged to the *B. cereus group*. Based on the taxonomic results obtained from the 16S rRNA gene sequencing, **49.2 % of the isolates were correctly identified using selective media** in classic microbiology methods. For **31.2 % of the isolates, selective media were unable to discriminate them** ("Probably *B. cereus group*" in Figure 3). Of these, **78.7 % were identified by Sanger sequencing** as *B. cereus group* isolates while 21.3 % were not. Among the isolates identified as "Other" (not *B. cereus group*), **57.1 % were identified as one of the *B. cereus group* species** using the sequencing method, while 42.9 % were identified as *Lysinibacillus* and *Pseudomonas* species. Additionally, the molecular method allowed for the study of the genetic distance of the isolates, as shown in Figure 4.

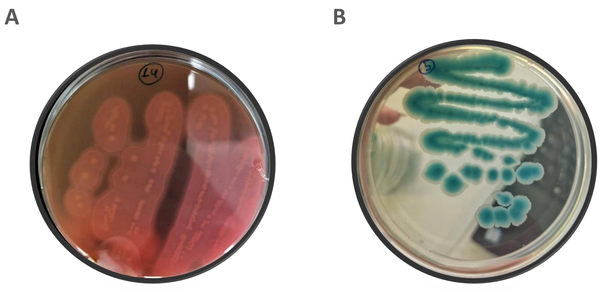

Figure 1. Representative colony morphology of *B. cereus* isolated form pasteurized milk on MYP (A) and Brilliance (B) agar. Colonies of *B. cereus* on MYP are pink and lecitinase positive, however other bacteria are not inhibited (eg *Lysinibaciilus* and *Pseudomonas* spp). On Brilliance, *B. cereus* colonies are blue/green, since the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-glucopyranoside is cleaved by the enzyme β-glucosidase present in *B. cereus.* The growth of *Lysinibaciilus* and *Pseudomonas* spp was inhibited. All agar plates were incubated at 30 °C for 24h.

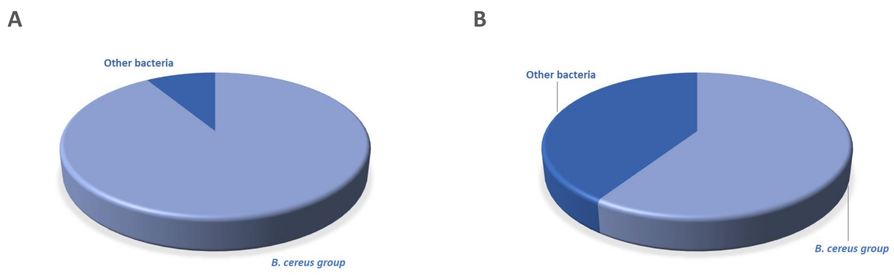

Figure 2. Percentage of pasteurized milk isolates identified as *B. cereus group* on MYP (A) and Brilliance (B) agar. 91.2 and 59.2 % of the tested isolates were found to belong to *B. cereus group* when MYP and Brilliance agar were used, respectively.

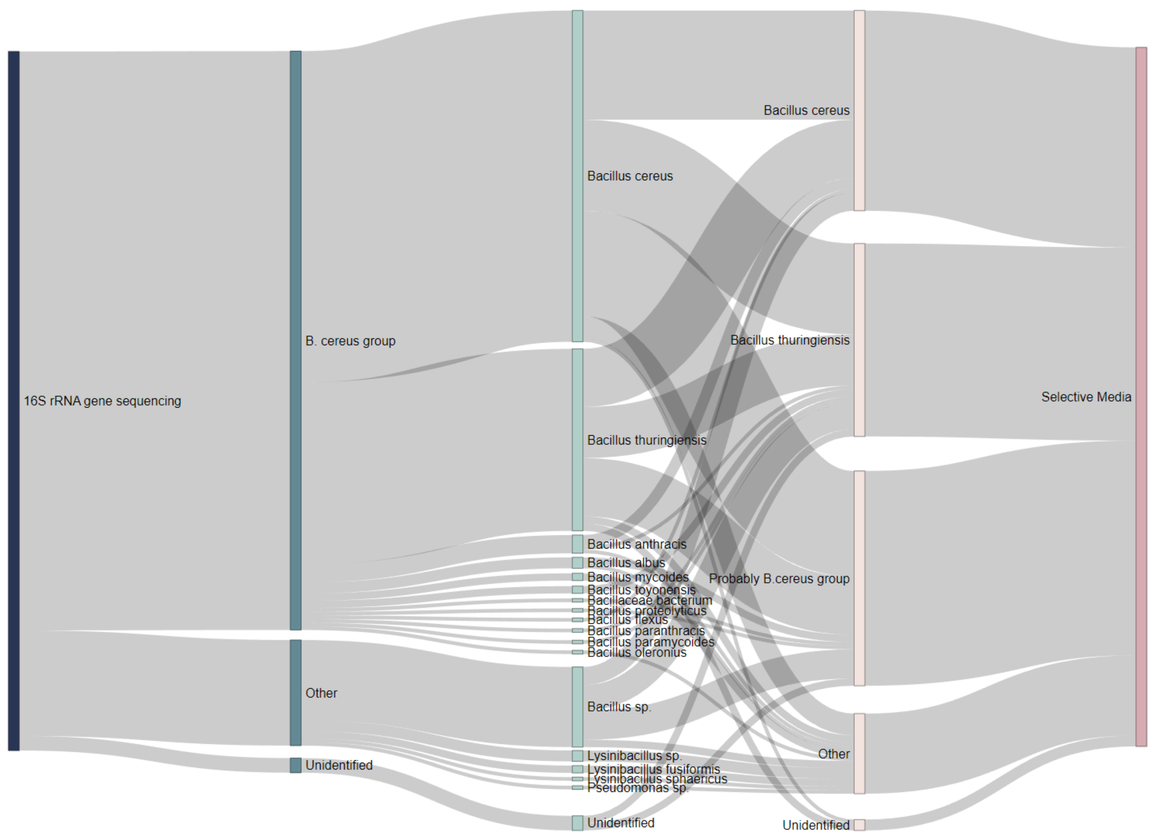

Figure 3. Schematic depiction of the isolates identification results between the two methods. Dark blue colour represents all the isolates that were sequenced, coral blue the results at group level, light blue the results in detail, ivory the results of identification by phenotype in selective media and pink the isolates grown in selective media.

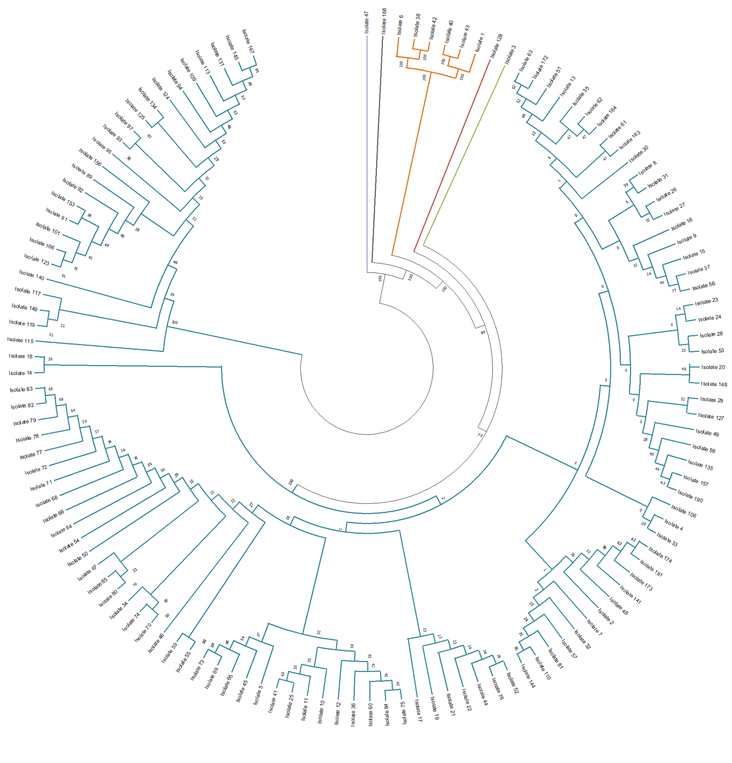

Figure 4. UPGMA phylogenetic tree of the isolates based on the 16S rRNA gene sequences. Blue indicates all the isolates identified as *B. cereus group*, green the isolate identified as *B. oleronius*, red the isolate identified as *B. flexus*, orange the isolates identified as *Lysinibacillus* species, dark grey the isolates identified as *Bacillus sp.*, and purple the isolate identified as *Pseudomonas sp*.

Conclusions

Comparison of microbiological and molecular methods for the identification of isolates from pasteurized milk, resulted in great differences, with the molecular method being more accurate. However, the molecular method's accuracy depends on the quality of the publicly available databases. Additionally, closely related organisms have well-preserved 16s rRNA genes, making it difficult to identify them at the species level. Overall, the two methods could be used synergistically to take advantage of both the phenotype and genotype for their identification.

References

ISO 7932:2004 Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of presumptive Bacillus cereus — Colony-count technique at 30 degrees C, ISO Standard

Acknowledgments

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