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SÃO PAULO - BRAZIL

Are you having trouble to characterize Bacillus spp.? You should read this

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After uncovering misidentifications of four among six strains of *Heyndrickxia coagulans* received from different microbial banks, a review of existing methods led us to develop and propose a microbiological characterization protocol aimed at minimizing errors and improving accuracy in *Bacillus* species-based products, such as probiotics, thereby enhancing the quality, safety and reproducibility of those products and techniques.

Six strains (A1,A2,B1,C1,D1,D2) presumed to be *Heyndrickxia coagulans* were obtained from four different banks (Banks: A,B,C,D). Cultures of all strains were activated according to the recommendations for *Baciilus* spp. (Poormontaseri et al.,2017). Identification by MALDI-TOF and partial genome sequencing on 16S rRNA for all strains (A1,A2,B1,C1,D1) and Whole Genome Sequencing (WGS) for strains A1 and A2 were performed.

Both identification approaches (MALDI-TOF and 16S-rRNA) revealed that strains A2 and B1 belong to species *Heyndrickxia coagulans*, A1 and D2 to *Bacillus subtilis*, C1 to *Bacillus cereus*, and D1 to *Cytobacillus oceanisediminis*.

MALDI TOF identification is considered as are highly reliable, simple, fast and provided results with scores between 2.0 and 2.3, that indicate highly probable genus identification and probable species identification (Topi? Popovi? et al., 2023). Despite the limitations and challenges existent for the taxonomic identification through 16S-rRNA sequencing method due to the intragenomic variations in the conserved regions of *Bacillus*-related species and high interspecies similarity of their 16S-rRNA gene sequences that can lead to inaccurate identification of closely related strains or species, our results were consistent with those obtained from MALDI-TOF, reinforcing precision of our strain identification. To address the limitations of 16S-rRNA sequencing for characterizing *Bacillus*-related species, further analysis through WGS or even 16S-rRNA next-generation sequencing is considered as relevant step in the appropriate identification, becoming even standard for strains with applications in fermentation processes or putative probiotics (Rizal et al., 2020). In relation to strain A1, based on all preliminary biochemical and physiological tests, MALDI-TOF, 16S-rRNA and WGS was confirmed that strain belong to *Bacillus subtilis* and not *Heyndrickxia coagulans* (Uono et al., 2019). Following the consequences of the experience with strain A1, facts of time- and budget-consuming experience, the team decided to prioritize MALDI-TOF analysis and 16S-rRNA sequencing before WGS. By employing a combination of morphological, biochemical, MALDI-TOF, and 16S-rRNA analyses, we established a more reliable and comprehensive protocol to ensure precise identification of *Bacillus*-related species. The findings demonstrate that while MALDI-TOF and 16S-rRNA sequencing are valuable tools for initial steps for accurate and cost-effective pre-identification of *Bacillus*-related genera, they must be complemented by more definitive methods such as WGS for conclusive results. Morphological, biochemical, such as the simple catalase test and physicochemical analysis should be conducted after the preliminary confirmation through MALDI-TOF and 16S-rRNA sequencing for the better species characterization (Vos et al., 2011). This layered approach not only minimizes errors but also enhances the safety, quality, and reproducibility of *Bacillus*-related species-based products and techniques. Implementing this refined protocol can significantly reduce the incidence of misidentification, ensuring that industrial and probiotic applications of bacterial strains are both effective and safe.

References

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