Discrimination between the Two Closely Related Species of the Operational Group *B. amyloliquefaciens* Based on Whole-Cell Fatty Acid Profiling

Thu Huynh 1,2,3, Mónika Vörös 1, Orsolya Kedves 1, Adiyadolgor Turbat 1, György Sipos 4, Balázs Leitgeb 5, László Kredics 1, Csaba Vágvölgyi 1 and András Szekeres 1, **

**Abstract:** (1) Background: *Bacillus velezensis* and *Bacillus amyloliquefaciens* are closely related members of the "operational group B. amyloliquefaciens", a taxonomical unit above species level within the *Bacillus subtilis* species complex. They have similar morphological, physiological, biochemical, phenotypic, and phylogenetic characteristics. Thus, separating these two taxa from each other has proven to be difficult to implement and could not be pushed easily into the line of routine analyses. (2) Methods: The aim of this study was to determine whether whole FAME profiling could be used to distinguish between these two species, using both type strains and environmental isolates. Initially, the classification was determined by partial sequences of the gyrA and rpoB genes and the classified isolates and type strains were considered as samples to develop the identification method, based on FAME profiles. (3) Results: The dissimilarities in 16:0, 17:0 iso, and 17:0 FA components have drawn a distinction between the two species and minor differences in FA 14:0, 15:0 iso, and 16:0 iso were also visible. The statistical analysis of the FA profiles confirmed that the two taxa can be distinguished into two separate groups, where the isolates are identified without misreading. (4) Conclusions: Our study proposes that the developed easy and fast-automated identification tool based on cellular FA profiles can be routinely applied to distinguish *B. velezensis* and *B. amyloliquefaciens*.

**Keywords:** Bacillus taxonomy; Bacillus velezensis; Bacillus amyloliquefaciens; fatty acid profiling; chemotaxonomy

1. Introduction

*Bacillus velezensis* and *B. amyloliquefaciens*, together with the *B. siamensis* and a black-pigment-producing strain, *B. nakamurai*, are members of the operational group *B. amyloliquefaciens*. This operational group belongs to the *B. subtilis* species complex with an eventful taxonomic history [1]. Both taxa are beneficial species, they have played increasingly important roles in applied microbiology [2-5]. Furthermore, several plant growth-promoting and biocontrol products from *B. amyloliquefaciens* and *B. velezensis* are now commercially available, including RhizoVital® (*B. velezensis* DSM 23117®; ABiTEP, GmbH, Berlin, Germany), Amylo-X® WG (*B. amyloliquefaciens* subsp. *plantarum* D747; Certis Europe BV, Utrecht, The Netherlands).
Netherlands), RhizoPlus® (B. amyloliquefaciens FZB24; ABiTEP), and Taegro® (B. subtilis var. amyloliquefaciens FZB24; Novozymes Biologicals, Inc., Salem, VA, USA) [6].

B. amyloliquefaciens was first described by Fukumoto [7,8] and later revised by Priest et al. [7,8] as an industrial producer of amylase, while B. velezensis, first isolated from the river Vélez in Málaga (southern Spain), was initially described as a distinct ecotype of B. amyloliquefaciens by Ruiz-García et al. [9]. Morphological, physiological, chemotaxonomic, and phylogenetic interrelations have indicated that the two taxa are highly similar [2,7,10,11]. Furthermore, detailed examinations of the members of these two species, including phenotype analysis, phylogenetics, fatty acid methyl ester (FAME) analysis, DNA–DNA hybridization, microarray-based comparative genomic hybridization, genomic analysis, HPLC-electrospray ionization MS, and MALDI-TOF MS have revealed B. velezensis as plant-associated B. amyloliquefaciens subsp. plantarum subsp. nov., and B. amyloliquefaciens as non-plant-associated B. amyloliquefaciens subsp. amyloliquefaciens subsp. nov., respectively [12]. Subsequently, B. methylotrophicus, B. amyloliquefaciens subsp. plantarum, and B. oryzicola were reclassified later as heterotypic synonyms of B. velezensis, while B. amyloliquefaciens subsp. amyloliquefaciens was considered as B. amyloliquefaciens [13].

The earliest description differentiated B. velezensis and B. amyloliquefaciens, as well as other closely related taxa based on phenotypic and genetic differences [9], but in many cases, these taxonomical descriptions were later revised according to the current state of Bacillus taxonomy. A typical example for this is the identification history of strain DSM 23117T, which was first identified as B. amyloliquefaciens in 2008 [14], later revised as B. amyloliquefaciens subsp. plantarum in 2011 [12], and finally reclassified as B. velezensis in 2016 based on DNA–DNA hybridization, as well as phenotypic and phylogenetic analyses [13]. Furthermore, this statement was strongly confirmed for this strain using molecular methods [1]. Therefore, this well-defined strain was used as the type strain of B. velezensis in our study, although it is still named as B. amyloliquefaciens in several recent publications and GenBank sequences [15,16].

Over the last 15 years, interest in understanding the genetic relationship of the two taxa has led to many studies being published [1,3,10–14,17–20]. The two taxa share similar morphological, physiological, and phenotypic traits (Table 1) as well as 16S rRNA gene sequences, tetranucleotide frequency distribution, and DNA G+C contents [1,13]. Their average nucleotide identity and average amino acid identity is approximately 93.6–94.5% and 97.8% similarity, respectively [1,17], and their high DNA–DNA relatedness values showed 20% [9], 55% [1,13,17], or 80% similarity [14] in various studies. Strains B. velezensis DSM 23117T and B. amyloliquefaciens DSM 7T share 3345 genes in their core genomes, which have 97.89% similarity at the amino acid level [12]. Furthermore, the phylogenomic tree based on the core genome (799 genes) also indicates their close genetic relationship [13]. Both taxa are characterized by substantial production of secondary metabolites via non-ribosomal synthesis. However, only B. velezensis contains gene clusters synthesizing macrolactin and difficidin, which are lacking in B. amyloliquefaciens [1]. On the other hand, only B. amyloliquefaciens contains the amyA gene for industrial starch-liquefying α-amylase [12].

Previously, the whole-cell FAME profiles of B. velezensis and B. amyloliquefaciens have been studied only to a limited extent, therefore FAs were not considered as biomarkers distinguishing between them [2,10,11]. FAs are part of the bacterial cell membrane structure, and specific FAs and their ratios in cellular membranes have usually been revealed as biomarkers to distinguish closely related species of bacteria [2,4]. This study considered the possibility of using the cellular FAs with the application of a Sherlock chromatographic analysis system (CAS) as a taxonomic and diagnostic tool. The method, using FAs of 9–20 carbons in length and automated GC analysis, qualitatively and quantitatively analyzes bacterial whole-cell FAME [21]. Since the Sherlock CAS has developed, it has become capable of performing cost-effective, sensitive, reliable, and rapid analyses with a small amount of cell mass.
Table 1. Characteristics of *B. velezensis* and *B. amyloliquefaciens* determined by different techniques.

| Characteristics                  | B. velezensis <sup>b</sup> | B. amyloliquefaciens <sup>b</sup> | References |
|----------------------------------|----------------------------|---------------------------------|------------|
| Pigmentation                     | Creamy white              | Creamy white                    |            |
| Oxidase                          | +                          | +                               |            |
| Acid in API system from:         |                            |                                 |            |
| - Glycogen                       | +                          | nd                              | [9]        |
| - Lactose                        | +                          | +                               |            |
| - Melibiose                      | -                          | +                               |            |
| - Methyl α-D-glycoside           | +                          | +                               |            |
| - D-Raffinose                    | +                          | +                               |            |
| - D-Turanose                     | -                          | +                               |            |
| Hydrolysis of                    |                            |                                 |            |
| - Tween 20                       | –                          | +                               |            |
| - Tween 80                       | –                          | nd                              |            |
| - DNA                            | –                          | –                               |            |
| Arginine dihydrolase             | –                          | –                               |            |
| ONPG                             | +                          | –                               |            |
| Non-ribosomally synthesized      |                            |                                 |            |
| secondary metabolites            |                            |                                 |            |
| - Surfactin                      | +                          | +                               |            |
| - Macrolactin                    | +                          | –                               |            |
| - Bacillaene                     | +                          | +                               |            |
| - Fengycin                       | +                          | –                               |            |
| - Difficidin                     | +                          | –                               |            |
| - Bacillibactin                  | +                          | +                               | [1,10,12] |
| - Bacilysin                      | +                          | +                               |            |
| Ribosomally synthesized antimicrobial compounds | | | |
| - Sublancin                      | –                          | –                               |            |
| - Subtilosin                     | –                          | –                               |            |
| - Amylocyclicin                  | +                          | +                               |            |
| - Plantazolicin                  | +                          | –                               |            |
| Plant colonization               | +                          | –                               |            |
| AZCL-HE cellulose liquefaction   | +                          | –                               |            |
| Growth in lactose minimal medium | +                          | +                               | [12]       |
| Amylase AmyA                     | –                          | +                               |            |
| Amylase AmyE                     | +                          | –                               |            |
| Cellulase BglC                   | +                          | –                               |            |
| Xylanase XynA                    | +                          | –                               |            |
| 16S rRNA gene sequence similarity (%) | 99.7%                     |                                  | [14]       |
| rpoB gene sequence similarity (%) | >98%                      |                                  | [1]        |
| gyrB gene sequence similarity (%) | >95.5%                    |                                  | [14]       |
| DNA relatedness value (%)        | 74–84%                     |                                  | [14]       |

<sup>a</sup> ONPG: O-nitrophenyl β-D-galactopyranoside, AZCL-HE: endocellulase activity determined by using insoluble azurine cross-linked (AZCL)-HE-cellulose. <sup>b</sup>: detected, –: not detected, nd: not determined.

This study included DNA sequencing, as well as phylogenetic and FAME analyses with the aim of providing a complementary tool to distinguish *B. velezensis* and *B. amyloliquefaciens* based on their cellular FAs.
2. Materials and Methods

2.1. Bacillus Strains and Growth Conditions

_Bacillus_ type strains including DSM 7^T^, DSM 1061^T^, and DSM 23117^T^ were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), while the _Bacillus_ field strains were isolated according to Vágvölgyi et al. [22]. Briefly, soil samples (5 g) were collected from agricultural fields and suspended in 50 mL of 1% NaCl solution with intensive mixing with a glass rod, then the suspensions were allowed to pellet for a minute. The supernatants were used to make a dilution series. Fifty µL of each diluted sample was spread onto the surface of yeast extract–glucose (YEG) medium (glucose 0.2%, yeast extract 0.2%, bacto agar 2%) supplemented with 50 µg mL^−1_ nystatin to suppress fungi. After 7 days of incubation, the dominant bacterial colony morphotypes were picked and cleaned until homogeneity on YEG medium. The isolated strains were deposited in the Szeged Microbiology Collection (SZMC, http://szmc.hu, accessed on 29 December 2021) of the Department of Microbiology, University of Szeged, Hungary.

For molecular taxonomical investigations, _Bacillus_ strains were cultured in YEG medium and incubated at 37 °C overnight. Before FA profiling, bacteria were inoculated on trypticase soy broth agar (TSBA, Becton, Dickinson and Company, Sparks, NV, USA) with the quadrant streaking method and incubated at 28 °C for 24 ± 2 h.

2.2. PCR Amplification of the gyrA and rpoB Genes

Total cellular DNA was extracted by the E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer’s instructions. Amplification of the gyrA gene [22] was performed in 50 µL reaction mixtures containing 10 pmol of each primer (gyrAF: CAGTCAGGAAATGCGTACGTCCTT; gyrAR: CAAGGTAATGCTACCTGCTTT), 10 nmol dNTP mix, 2 µL template DNA, 5 µL 10 × PCR buffer, 6 µL of 25 mM MgCl₂, and 1 U of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The PCR thermocycler (Doppio, VWR International GmbH, Darmstadt, Germany) was set to an initial denaturation step at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. The amplification of the rpoB gene [23] was conducted in 50 µL reaction mixtures containing 20 pmol of each primer (rpoBF: AGGTCAACTAGTTCAGTATGGACG; rpoBRO: GTCCTACATTGGCAAGATCGTATC), 10 nmol dNTP mix, 2 µL template DNA, 5 µL 10 × PCR buffer, 6 µL of 25 mM MgCl₂, and 0.4 U of Taq DNA polymerase. The PCR cycling parameters included an initial denaturation step at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 50 s, and a final extension at 72 °C for 5 min. Sequencing of the amplified DNA fragments was performed on an ABI 3730XL sequencer (Thermo Fisher Scientific, Waltham, MA, USA) using Sanger sequencing.

2.3. Phylogenetic Analysis

Sequences were analyzed using the Mega X software [24]. The NCBI Nucleotide BLAST similarity search was carried out at https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 29 December 2021. Alignments were performed using the MUSCLE algorithm. The phylogenetic tree was inferred using the Neighbor-Joining method [24] with 1000 bootstrap replicates. The evolutionary distances were computed using the Tamura-Nei method and the units of the number of base substitutions per site [25]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.5).

2.4. The Fatty Acid Methyl Ester (FAME) Analysis

The MIDI Sherlock® Microbial Identification System (MIS, MIDI Inc., Newark, NJ, USA) was applied for the data acquisition [21]. The composition of whole-cell FAs was determined by the Sherlock CAS Software ver. 6.4 (Microbial ID Inc., Newark, DE, USA) operating through the LabSolution ver. 5.97 software on a Nexera GC-2030 gas chromatograph.
equipped with an AOC-20i Plus autoinjector (Shimadzu, Kyoto, Japan). For the separation of the FAs, the RTSBA6 method provided by the manufacturer was applied on a HP-Ultra 2, 25 m × 0.2 mm × 0.33 µm film thickness, fused, silica capillary column (Agilent, Santa Clara, CA, USA). Injector and detector temperatures were 250 °C and 300 °C, respectively. Carrier gas was hydrogen at a flow rate of 1.48 mL min⁻¹, while the detector gases were nitrogen (make up), oxygen and hydrogen with the follow flows of 30, 30, and 350 mL min⁻¹, respectively. Samples were introduced in an injection volume of 2 µL in split mode with a 40:1 split ratio. The oven program started at 168.1 °C, which ramped up to 291 °C with 28 °C min⁻¹, and then up to 300 °C with 60 °C min⁻¹, holding at this temperature for 1.50 min. The total column oven program time was 6.04 min. The 1300-C rapid calibration standard mix (Microbial ID Inc., Newark, DE, USA) was used for retention time calibration and system suitability purposes. The B. subtilis strain ATCC 6633T and pure hexane were considered as the quality control and the negative control, respectively. Whole-cell FAME profiles were analyzed by the library RTSBA6.21 (Microbial ID Inc., Newark, DE, USA).

2.5. Sample Pretreatment
Sample processing was carried out according to the Sherlock™ Operating CAS Manual [21]. Briefly, 20–40 mg of cells was harvested and placed in a clean glass tube. Then, 1 mL of reagent 1 (45 g NaOH, 150 mL of methanol and 150 mL of distilled water) was added to the sample and heated at 95–100 °C in a water bath (Precision water bath NB-301, HandyLAB® System, N-BIOTEK, Bucheon-si, Korea). After 5 min, the sample was removed from the water bath, vortexed and heated for an additional 25 min. The sample was mixed with 2 mL of reagent 2 (325 mL of 6.0 N HCl, 275 mL of methanol) and incubated at 80 °C in a water bath for 10 min. Subsequently, 1.25 mL of reagent 3 (200 mL of hexane, 200 mL of methyl tert-butyl ether) was added and the derivatized FAs were extracted for 10 min in a laboratory rotator (Rotator drive STR4 Stuart, Cole-Parmer™, Vernon Hills, IL, USA). The organic (upper) phase was recovered and washed with 3 mL of reagent 4 (10.8 g NaOH, 900 mL distilled water) for 5 min in a laboratory rotator. The resulting organic (upper) phase from the tube was transferred to a clean vial for GC analysis.

2.6. Statistical Analysis
The library generation function of MIS Sherlock Command Centre ver. 6.4 was applied to install a new library of Bacillus named RTSBA7. New entries of Bacillus species were added by a statistical summary of a set of related samples. The MIS Sherlock Command Centre had been applied also for data analysis. The Dendrogram cluster analysis technique, using Euclidian distance (ED) metric, was applied for determining the distance between individual FAs, producing unweighted pair matchings based on FA compositions. The results were displayed graphically to depict the relatedness between organisms. The 2D plot cluster analysis technique using a principal component (PC) analysis was used to separate groups of samples in an n-dimensional space.

3. Results
3.1. The Classification of B. velezensis and B. amyloliquefaciens Based on Molecular Markers
The application of the type strains is necessary for the development of a reliable FA profiling method capable of differentiating between the closely related B. velezensis and B. amyloliquefaciens species, and the use of a confirmatory method is also essential for the classification of unknown Bacillus isolates. Therefore, as a confirmatory analysis, the partial sequences of the genes encoding the subunit A protein of DNA gyrase (gyrA) and the RNA polymerase beta-subunit (rpoB) were determined to identify the isolated strains. The BLASTN comparison showed high similarities between the examined strains (Table 2) and corresponding records of B. velezensis and B. amyloliquefaciens strains in the GenBank database. Accordingly, the gyrA and rpoB sequences of B. velezensis DSM 23117T and the isolated strains displayed approximately 100% similarity with both B. velezensis and B. amyloliquefaciens records in GenBank. Furthermore, the gyrA and rpoB genes of B. amyloliq-
uefaciens DSM 7T and DSM 1061T strains shared also approximately 100% similarities with numerous B. amyloliquefaciens and B. velezensis strains in GenBank, respectively. Thus, the classification using both gyrA and rpoB genes revealed high relatedness values between B. velezensis and B. amyloliquefaciens, and the BLAST results also showed the presence of sequences from possibly misidentified strains deposited in the GenBank.

Table 2. Bacillus strains used in the FA profiling study.

| Strains a | Genbank Accession Number | Origin |
|-----------|--------------------------|--------|
|           | gyrA                     | rpoB   |
| B. velezensis |                          |        |
| SZMC 24980 | OK256097                 | OK256115 | soil sample from pepper field, Totovo selo, Serbia |
| SZMC 24981 | OK256098                 | OK256116 | soil sample from pepper field, Totovo selo, Serbia |
| SZMC 24982 | OK256099                 | OK256117 | soil sample from pepper field, Totovo selo, Serbia |
| SZMC 24983 | OK256100                 | OK256118 | soil sample from pepper field, Totovo selo, Serbia |
| SZMC 24984 | OK256101                 | OK256119 | soil sample from pepper field, Cantavi, Serbia |
| SZMC 24985 | OK256102                 | OK256120 | soil sample from pepper field, Cantavi, Serbia |
| SZMC 24986 | OK256103                 | OK256121 | soil sample from tomato field, Cantavi, Serbia |
| SZMC 24987 | OK256104                 | OK256122 | soil sample from tomato field, Cantavi, Serbia |
| SZMC 25020 | OK256105                 | OK256123 | soil sample from tomato field, Cenej, Serbia |
| SZMC 25646 | OK256106                 | OK256124 | pea rhizosphere, Madaras, Hungary |
| SZMC 25647 | OK256107                 | OK256125 | pea rhizosphere, Madaras, Hungary |
| SZMC 25610 | OK256108                 | OK256126 | maize rhizosphere, Vaszar, Hungary |
| SZMC 6046  | OK256109                 | OK256127 | tomato rhizosphere, Hungary |
| SZMC 16093B | OK256110                | OK256128 | tomato rhizosphere, Hungary |
| SZMC 6387 | OK256111                 | OK256129 | tomato rhizosphere, Hungary |
| DSM 23117T (=BGSC 10A6 = FZB42 = LMG 26770 = SZMC 27497) | OK256112                  | OK256130 | plant pathogen-infested soil of a sugar beet field, Brandenburg, Germany |
| DSM 7T (=ATCC 23350 = SZMC 6027) | OK256113                  | OK256131 | soil and industrial amylase fermentations, Japan |
| DSM 1061T (=IAM 1523 = SZMC 6222) | OK256114                  | OK256132 | unknown origin |
| B. subtilis |                          |        |
| ATCC 6633T | CP039755.1               | CP039755.1 | Japan |

* ATCC—American Type Culture Collection, BGSC—Bacillus Genetic Stock Center, DSM—German Collection of Microorganisms and Cell Cultures, FZB—Research Center Borstel, IAM—Institute of Applied Microbiology, University of Tokyo, LMG—Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection, SZMC—Szeged Microbiology Collection.

To investigate the relatedness of strains, a phylogenetic tree was also built using the Neighbor-Joining method with 1000 bootstrap replicates. The B. subtilis ATCC 6633 strain was considered as the outgroup. Analyses with the gyrA sequences (Figure S1), rpoB sequences (Figure S2), and their concatenation (Figure 1) generated similar phylogenetic trees without notable differences. The analysis separated B. velezensis and B. amyloliquefaciens strains into two corresponding clades of the phylogenetic tree in the case of the type strains; other databases collected strains and the field isolates (Table S1) with the bootstrap values of 83 and 98%, respectively (Figure 1). The phylogenetic tree, based on gyrA and rpoB sequences, shows the existence of two tightly related monophyletic groups: (1) B. velezensis, containing our field Bacillus isolates, type strain DSM 23117T together with the other reference strains At1, AS43.3, BIM B-439D, AP183, KKLW, SQR9, S141, QST713, BvL03, LF01, WRNf014, and SGAir0473; (2) B. amyloliquefaciens, containing type strain DSM 7T and DSM 1061T together with the other reference strains LL3, TA208, and XH7.
3.2. FAME Profiles of *B. velezensis* and *B. amyloliquefaciens* Strains

The content of FAs was revealed (Table 3, Figure S3) and the features were constructed from the MIS analysis of sixteen *B. velezensis* strains (n = 3) and two *B. amyloliquefaciens* strains (n = 25). The 15:0 iso (13-methyltetradecanoic), 15:0 anteiso (12-methyltetradecanoic), 16:0 (n-hexadecanoic), 17:0 iso (15-methylhexadecanoic), and 17:0 anteiso (14-methylhexadecanoic) have been primary FA components in both taxa. The predominant content of 15:0 iso and 15:0 anteiso are 30.39 ± 2.53 and 32.13 ± 2.33 (%) in *B. velezensis* and 27.85 ± 1.67 and 31.88 ± 1.98 (%) in *B. amyloliquefaciens*, respectively. Besides, the minor content of FA 14:0 iso (12-methyltridecanoic), 14:0 (n-tetradecanoic), 16:0 iso (14-methylpentadecanoic), 16:1 ω11c (cis-5-hexadecenoic), and 17:1 iso ω10c ((6Z)-15-methyl-6-hexadecenoic) are approximately from 1.0 to 3.5% in both taxa (Table 3). Especially, the FA 16:0, 17:0 iso, and 17:0 have drawn a distinction between *B. velezensis* and *B. amyloliquefaciens*. In the case of *B. velezensis*, the proportions of 16:0, 17:0 iso, and 17:0 anteiso were 12.53 ± 1.82, 8.52 ± 0.96, and 5.50 ± 0.85 (%), respectively, which were compared with those for the *B. amyloliquefaciens* strains, which were 4.55 ± 0.54, 15.98 ± 1.95, and 8.97 ± 0.73 (%), respectively.
Table 3. Cellular fatty acid compositions (mean (%) ± SD).

| Feature/FA | B. velezensis | B. amyloliquefaciens |
|------------|---------------|----------------------|
| 12:0       | 0.48 ± 0.23   | 0.54 ± 0.17          |
| 13:0 iso   | 0.89 ± 0.22   | 0.50 ± 0.19          |
| 14:0 iso   | 1.18 ± 0.58   | 1.44 ± 0.11          |
| 14:0       | 2.87 ± 0.70   | 0.61 ± 0.14          |
| 15:0 iso   | 30.39 ± 2.53  | 27.84 ± 1.65         |
| 15:0 anteiso | 32.13 ± 2.33 | 31.92 ± 1.98         |
| 16:0 iso   | 1.70 ± 0.77   | 3.51 ± 0.19          |
| 16:1 ω11c  | 1.65 ± 0.42   | 1.09 ± 0.28          |
| 16:0       | 12.53 ± 1.82  | 4.57 ± 0.55          |
| 17:1 iso ω10c | 0.85 ± 0.47 | 1.07 ± 0.37          |
| 17:0 iso   | 8.52 ± 0.96   | 15.92 ± 1.96         |
| 17:0 anteiso | 5.50 ± 0.85  | 8.99 ± 0.73          |
| 18:0       | 0.60 ± 0.14   | 0.59 ± 0.23          |

3.3. Differentiation of FAME Profiles between B. velezensis and B. amyloliquefaciens

The FA profiles were consistently typical and distinguishable between B. velezensis and B. amyloliquefaciens. Principal component analysis enabled us to look at data with high dimensionality and observe the most critical aspects of the data in two or three dimensions. The 2D plot built from PC 1 and PC 2 (Figure 2) showed a separation of the two taxa in n-dimensional space. The group A represented FA components of B. amyloliquefaciens with ED² (Euclidian distance squared) ~ 36, and the group B represented FAs of B. velezensis with ED² ~ 100. A group with calculated ED² ≤ 100 was considered as the same species. The FA profiles of the two taxa could be distinguished into two separate groups of strains.

![Figure 2](image-url)
The dendrogram analysis drawn from diverse FA profiles of Bacillus species showed a relationship between them (Figure 3) via the ED index. These profiles were obtained from the Sherlock library that had been built from diverse strains (more than 20 strains) within each species. The samples were collected from across the world to avoid potential geographic bias and carefully analyzed with many replications to make the entries of the library [21]. The FA profiles of B. velezensis and B. amyloliquefaciens—together with B. agaradhaerens, B. pumilus, B. licheniformis, and B. subtilis—formed one cluster, which could be distinguished from other clusters. Furthermore, FAs of B. velezensis, B. agaradhaerens, and B. amyloliquefaciens formed a tight phylogenetic branch, which showed the highest phenotypic similarity. The FA profiles of group-related species were highly similar and were also determined previously [26–28].

![Dendrogram](image.png)

**Figure 3.** The relationship of FA profiles among the Bacillus species in the MIS library.

4. Discussion

The classification of B. velezensis and B. amyloliquefaciens has usually been a particularly confounding taxonomic problem. Moreover, it was also concluded in previous reports that the whole-cell FAME profiles had not yielded satisfying results for discriminating these two species [2,10,11]. However, our research efforts, aimed at developing a whole-cell FAME profile-based method for distinguishing both taxa, led to other conclusions.

The gyrA and rpoB sequences proved to be effective for resolving these closely related species of the B. subtilis group [1,19,26]. The previous use of gyrA [12,19] and rpoB [1,11] as phylogenetic markers had drawn clear distinction between the two taxa. Accordingly, their highly conserved rpoB sequences shared approximately 98% similarity [1], but the NJ tree obtained from their gyrA sequences distinguished them with bootstrap values of 100 and 52% [12].

In the present study, gyrA and rpoB sequences were amplified and aligned to determine Bacillus strains to the species level. The result of BLAST alignments showed high relatedness between the sequences of the studied strains and records of both B. velezensis and B. amyloliquefaciens from the GenBank database. Thus, the discrimination of the two taxa were confused, because, unfortunately, several B. velezensis strains are still named as B. amyloliquefaciens in the GenBank and vice versa, which makes the identifications difficult. Therefore, for the detailed analysis, the partial sequences of gyrA and rpoB genes were concatenated and included in the phylogenetic analysis for comparative purposes. The selection of reference sequences was carefully considered according to previous classifications to avoid misinterpretations [2,7,12,16,29]. The studied strains clustered into two separate
clades on the phylogenetic tree (Figure 1) differentiating B. velezensis strains from the cluster of strains related to B. amyloliquefaciens DSM 7T and DSM 1061T. Then the result of this classification was used for developing the identification method based on FA profiles.

In the current work, FAs were identified based on their estimated carbon lengths determined relative to the calibration standard and by comparing with the peak table. It could be concluded that certain deviations can be found between our results and previously published FA profiles [2,10,11]. In general, both taxa possess a higher content of branched-odd FAs, including 15:0 iso, 15:0 anteiso, 17:0 iso, and 17:0 anteiso than other FAs. The presence of branched-chain FAs is expected to increase the membrane’s fluidity because of their low melting point temperatures, and are already remarkable biomarkers used in Bacillus taxonomy [5]. The 15:0 iso and 15:0 anteiso FAs have shared a prominent proportion, similar to other species within the “B. subtilis species complex” [9] and their high ratio has been indicated as a common feature in Bacillus species [5]. These FAs had been considered as being distinguishable features between many other Bacillus species reported previously [25]. Discriminating biomarkers useful for distinguishing between the two taxa were 14:0, 16:0, 16:0 iso, 17:0 iso, and 17:0 anteiso. The FA profiles of B. velezensis could be characterized by higher 14:0 and 16:0 contents and lower 16:0 iso, 17:0 iso and 17:0 anteiso contents in comparison to B. amyloliquefaciens (Figure 4).

![Figure 4](image.png)

*Figure 4.* Comparison charts of (A) B. velezensis and (B) B. amyloliquefaciens based on FA profiles created in the MIS library.

Our comprehensive study proved that these features are valuable taxonomical biomarkers with high discriminatory power, even though previous studies reported on the insufficiency of FA components in the discrimination of the two taxa. In the previous studies, certain involved isolates were misidentified and the novel classification of these strains, proven by many recent scientific contributions, helped us in giving a better conclusion for this issue. As shown in Table 4, the present investigation was similar to the report of Wang et al. [14]. However, it was a misapprehension that the publication considered Bacillus strain BCRC 14193 as B. amyloliquefaciens, which was later reclassified as B. velezensis by Dunlap et al. [13]. Currently, considering strain BCRC 14193 as B. velezensis, a distinguishable FA comparison was obviously drawn between the two taxa (Table 4). In 2011, a comparison among FAs from six strains of B. velezensis and five strains of B. amyloliquefaciens had been reported, with varied cellular FA compositions showing differences between the two taxa in the case of FA 14:0, 16:0, and 16:0 iso [12]. However, B. velezensis DSM 23117T and B. amyloliquefaciens DSM 7T contained a high content of FA 17:0, but lacked 17:0 anteiso [12], which made a difference with other Bacillus strains, and it is difficult to find a comparison, as well as the relatedness with our present study, due to insufficient data. The FA profiles in our study also shared high similarity to those of B. velezensis sp. nov. CR-502T and B. amyloliquefaciens DSM 7T with some minor differences [9]. It is interesting that only B.velezensis
Table 4. Comparison of FA components of B. velezensis and B. amyloliquefaciens reported in the literature.

| Feature          | This Study | Ruiz-García et al. [9] | Wang et al. [14] | Borriss et al. [12] |
|------------------|------------|-------------------------|------------------|---------------------|
|                  | B. v. a    | B. a. b                 | B. v.            | B. a.              | B. a. |
| 12:0             | 0.48       | 0.54                    | -                | -                   | -     |
| 13:0 iso         | 0.89       | 0.50                    | 0.87             | -                   | -     |
| 14:0             | 1.18       | 1.44                    | 1.08             | 2.46               | 1.3   |
| 14:0 iso         | 2.87       | 0.61                    | 2.96             | 3.8                | 3.1   |
| 15:0             | 30.39      | 27.84                   | 29.86            | 30.50              | 30.4  |
| 15:0 anteiso     | 32.13      | 31.92                   | 32.70            | 36.48              | 27.6  |
| 16:0             | 1.70       | 3.51                    | 1.31             | 4.52               | 1.0   |
| 16:0 iso         | -          | -                       | -                | 2.14               | -     |
| 16:0 iso         | -          | -                       | -                | 1.67               | -     |
| 16:1 ω5c         | 1.65       | 1.09                    | 4.42             | 0.62               | -     |
| 16:1 ω7c         | -          | -                       | -                | 3.5                | 2.7   |
| 16:1 ω9c         | -          | -                       | -                | 1.7                | -     |
| 17:0             | 12.53      | 4.57                    | 13.41            | 4.52               | 18.3  |
| 17:0 iso         | -          | -                       | -                | 1.67               | -     |
| 17:0 iso ω7c     | -          | -                       | -                | -                  | -     |
| 17:1 iso ω7c     | -          | -                       | -                | -                  | -     |
| 17:1 iso ω10c    | 0.85       | 1.07                    | 1.44             | -                  | 1.3   |
| 17:0 iso ω10c    | 8.52       | 15.92                   | 7.67             | 9.01               | 7.8   |
| 17:0 anteiso     | 5.50       | 8.99                    | 4.27             | 7.06               | 3.4   |
| 17:0              | 0.17       | 0.22                    | -                | -                  | -     |
| 18:0             | 0.60       | 0.59                    | -                | -                  | 1.1   |

* a. B. v.: B. velezensis; b. B. a.: B. amyloliquefaciens.

A high-quality library plays an important role in the classification. This study carefully constructed the library RTSBA7 from 16 strains of B. velezensis (n = 3) and 2 strains of B. amyloliquefaciens (n = 25) and from the data available in the RTSBA6, containing B. amyloliquefaciens using various strains constructed by MIDI (Table 2).

Altogether a total of 31 FAs were detected in B. velezensis (Table S2) and 38 FAs were determined in B. amyloliquefaciens (Table S3) during the analyses. The calculations were interfered with by occasionally detected peaks irregularly observed on the chromatograms with small peak areas; however the MIS analysis, with many replicates of samples, can detect and remove these variations, creating precise whole-cell FA features (Figure 4). For example, in the case of B. velezensis, the MIS analysis did not consider FA 9:0, because there were only 3 out of 48 samples containing it, with a low mean (=0.06) and high SD/mean (=4.15). Otherwise, FA 14:0 (2.87 ± 0.70 (%)) detected from 1.69 to 4.89% in all 48 samples was considered as a valuable parameter. Accordingly, characterizations of both taxa have contained 13 FAs as features of the analyses (Table 2), which are reported in the Supplementary Tables (Tables S2 and S3).

Once a sample has been analyzed by Sherlock, its FA composition can be matched with those of known organisms that are stored in the library. The Sherlock Library search lists the most likely matches to the query composition, and provides an SI for each match, which is a numerical value expressing how closely the FA composition of the query compares with the mean FA composition of the strains used to create the library entry listed as its match. The database search presents the best matches and associated SI. This value is a software-generated calculation of the distance in multi-dimensional space between the profile of the query and the mean profile of the closest library entry. Our results showed good matches between the experimental samples and the FA composition in the library, with SI > 0.5 and well-separated SI (>0.1). In addition, the FA compositions could be separated between the two taxa and among other Bacillus species.
5. Conclusions

In our study, a method using FAs of 9–20 carbons in length and automated GC analysis were developed to qualitatively and quantitatively analyze the bacterial whole-cell FAs as taxonomical markers. To the best of our knowledge, this is the first time that the method based on whole-cell FA profiles operated by MIS has been applied to distinguish between *B. velezensis* and *B. amyloliquefaciens* with comprehensive evidence. By taking advantage of the current knowledge regarding biomarkers, the FA-based identification proved to be applicable for the differentiation between these closely related species. Our experiments provided a cost-effective, reliable, and fast-automated solution for discrimination between these taxa.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10020418/s1, Table S1: Reference Bacillus strains and their sequence data used in phylogenetic construction; Table S2: Parameters of the whole-cell FA features of *B. velezensis*; Table S3: Parameters of the whole-cell FA features of *B. amyloliquefaciens*; Figure S1: The Neighbor-Joining phylogenetic tree based on gyrA gene sequences; Figure S2: The Neighbor-Joining phylogenetic tree based on rpoB gene sequences; Figure S3: Chromatograms of the FAs of *B. velezensis* (A) and *B. amyloliquefaciens* (B).

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