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***Virgibacillus ihumii* sp. nov., a new bacterium isolated from the stool of healthy African children**

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Keywords: *Virgibacillus ihumii* sp. nov., Culturomics, Taxonogenomics, Severe Acute Malnutrition, Mali.

1 **Abstract:**

2 During a case-control study on severe acute malnutrition, strain Marseille-Q1233 was
3 isolated. It is a Gram positive, rod-shaped and halophilic bacillus isolated from a stool sample
4 of Malian child under the age of five. The fatty acid profile of the strain consisted of C_{15:0}-
5 anteiso and C_{14:0}-iso as major components. dDDH and ANI calculation showed 23.10% and
6 80.81% of similarity respectively, between strain Marseille-Q1233 and *Virgibacillus*
7 *siamensis* strain Marseille-P2607, the phylogenetically closely related species. Based on these
8 results, we report here the description of *Virgibacillus ihumii* sp. nov., strain Marseille-
9 Q1233, as new bacterial species.

10

Introduction

Severe acute malnutrition (SAM) is a real public health problem that particularly affects children under the age of five [1]. Several factors are implicated, such as the increase in *Proteobacteria* [2], the involvement of intestinal microbes by anaerobic depletion [3] and the decrease in antioxidants [4], all of which are involved in the progressive establishment of SAM [5]. In this study, we used a new approach, called culturomics, which consists in isolating bacteria under various culture conditions and identifying them using matrix-assisted laser desorption ionization - time-of-flight mass spectrometry [6, 7]. Heyndrickx *et al.* (1998) described the genus *Virgibacillus* by analyzing the diversified genus *Bacillus* [8]. Since, some species have been discovered and many further species have been reclassified in the genus [9–11]. The genus *Virgibacillus* is composed of 40 validly published species with correct names [12]. Most of them are isolated from different biotopes, such as seawater, animal, sediment or soil [13–17]. The members of the genus *Virgibacillus* are characterized by its motility and staining Gram-positive. In particular, *Virgibacillus* species possesses C_{15:0}-anteiso as a major fatty acid and MK-7 as the main menaquinone [18]. *Virgibacillus ihumii* sp. nov., a new bacterial species, was characterized using a combination of genotypic and phenotypic criteria, following a taxono-genomics concept previously reported [19–20]. To this end, we performed a description of this new species belonging to the genus *Virgibacillus* that was isolated from the stool of a healthy Malian child as part of the exploration of the intestinal microbiota in malnourished children.

Isolation and growth conditions

In 2019, a bacterial strain was isolated from the stool sample of Malian child as part of a case-control study on severe acute malnutrition. The growth of this strain was obtained following 48 hours of incubation at 37°C on halophilic medium at pH 7.5. It was able to grow after 24 hours of incubation at temperatures ranging from 28°C to 42°C with an optimal pH of 7.5

under aerobic conditions. However, no cell growth was observed in an anaerobic or microaerophilic atmosphere. Following the identification protocol by MALDI-TOF as previously reported [20], no accurate identification was obtained for strain Marseille-Q1233 despite three tests performed. MALDI spectra generated with strain Marseille-Q1233 were imported using the Biotyper 3.0 software and compared to those of the local URMS database, which was continuously incremented (<https://www.mediterranee-infection.com/urms-database>).

Phenotypics characteristics

Virgibacillus ihumii sp. nov., strain Marseille-Q1233 is a Gram positive bacilli. It is motile and exhibited catalase positive and oxidase negative activities. Bacterial cells measured 1.5 µm in length and 0.3 µm in diameter. Using the TM4000 instrument (Hitachi Group, Krefeld, Germany), a scanning electron microscope (SEM) examination was performed on this strain (Figure 1). Using API ZYM strip (bioMérieux), positive reactions were observed for alkaline phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase. Using API 50 CH strip, only esculin ferric citrate and D-turanose were positive. Negative reactions with these API strips are noted in Table 1. The strain Marseille-Q1233 is sensible to tobramycin, rifampicin, oxacilline, vancomycin, tetracycline, erythromycin, amoxicillin, benzylpenicillin, ciprofloxacin, clindamycin, gentamicin, linezolid, ceftazidime, ampicilline, daptomycin, but resistant to amikacin, doxycycline, imipenem and mezlocillin. Some discriminatory criteria for distinguishing Marseille strain-Q1233 from its closely related species are presented in Table 2. Gaz Chromatography/Mass Spectrometry test was carried out to reveal the existing cellular fatty acid in this strain, as previously reported [21]. The only abundant fatty acid from strain Marseille-Q1233 was 12-methyl-tetradecanoic acid (52.2 %). Minor amounts of unsaturated, branched and other saturated fatty acids were also detected (Table 3).

Strain identification

To identify the strain Marseille-Q1233, the 16S rRNA gene was amplified using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequenced using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xL Genetic Analyzer capillary sequencer (ThermoFisher, Saint-Aubin, France), as previously described [22]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (<http://www.codoncode.com>). Strain Marseille-Q1233 had a 99.28% 16S rRNA similarity with *Virgibacillus siamensis* strain MS3-4 (Genbank accession number: NR_112738.1), the phylogenetically closest species with standing in nomenclature (Figure 2). Thus, this result, which is in disagreement with the obtained phenotypic and biochemical characteristics, led us to compare genomics data with *V. siamensis*, the closest phylogenetic neighbor with standing in nomenclature.

Genome sequencing

Genomic DNA was extracted using the EZ1 biorobot with the EZ1 DNA tissue kit (Qiagen, Hilden, Germany) and then sequenced on a MiSeq sequencer (Illumina Inc, San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired End (Illumina), as previously described [23]. The assembly was performed using a pipeline containing several softwares (Velvet [24], Spades [25] and Soap Denovo [26]), and trimmed (MiSeq and Trimmomatic [27] softwares) or untrimmed data (only MiSeq software). GapCloser software [28] was used to decrease assembly gaps. Scaffolds that had a nucleotide number <800 (bp) and scaffolds that had a depth value lower than 25% of the mean depths were removed. The best assembly was selected using different criteria (number of scaffolds, N50, number of N). The genome of strain Marseille-Q1233 was 3,813,121 bp long with a 41.1 % of G+C content. Whole genome sequences analysis by Blast in Genbank database revealed that strain Marseille-Q1233 was 90.30% similar to *Virgibacillus siamensis*. In addition, dDDH values

were below the recommended threshold value of >70% (Table 4). Indeed, the highest value from Genome-to-Genome Distance Calculator 2.1 (<http://ggdc.dsmz.de>) was 23.10% between strain Marseille-Q1233 and *V. siamensis* strain Marseille-P2607. This strongly suggests that this strain is a new member of genus *Virgibacillus* [29-30]. The degree of genomic similarity of the strain with closely related species was calculated using the OAT software [31]. OrthoANI values among *Virgibacillus* species (Figure 3) ranged from 66.14% between *V. soli* and *V. ihumii* to 79.67 % between *V. ihumii* and *V. siamensis*. In addition, when the Average Nucleotide Identity was calculated using ANI calculator (<http://enve-omics.ce.gatech.edu/ani/index>), we found 80.81% similarity with *Virgibacillus siamensis* strain Marseille-P2607. This result is below the cut-off value 92% [32] recommended to delineate new bacterial species.

Despite high similarity in the 16S sequence with the closest neighbor (92%), the careful characterization confirmed the identification of a new species based on phenotypic (endospore formation, oxidase, urease, metabolism of D-glucose and D-maltose) and genomics (dDDH 23%, orthoANI 80% and ANI 81%). Indeed, nowadays, 16S similarity is not the only criterion to delineate a new species. Phenotypic and genomic characteristics should be taken into account with different thresholds. For digital DNA-DNA Hybridization (dDDH), the threshold is 70%. Here, the dDDH is much lower than this threshold (23%). For orthoANI (average nucleotide identity based on orthologous genes), the threshold was set at 95%. Here, the orthoANI is as low as 80%. Finally, for the average nucleotide identity, the usual threshold is 92%. Here, the ANI is 81%, well below this threshold. All these results confirm the description of a new species.

Conclusion

109 On the basis of unique phenotypic, phylogenetic and genomic features, we formally propose
110 strain Marseille-Q1233 as type strain of *Virgibacillus ihumii* sp. nov., a new species within
111 the genus *Virgibacillus*.

112 **Description *Virgibacillus ihumii* sp nov**

113 *Virgibacillus ihumii*, (i.hu.mii, L. fem. adj. *ihumii*, the French acronym for Institut Hospitalo-
114 Universitaire (IHU) Méditerranée Infection (MI) of Marseille, where the type strain was first
115 isolated) is a motile rod-shaped bacterium with 1.5µm of diameter, which grows on a
116 halophilic medium at 100g/L-1 after 24 hours incubation under aerobic conditions. Colonies
117 are translucent and whitish. Positive reactions were observed with alkaline phosphatase,
118 esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, esculin ferric citrate
119 and D-turanose. However, glycerol, arabinose, ribose, xylose, D-adonitol, galactose, glucose,
120 fructose and mannose are not produced. The 16S rRNA gene sequence and whole-genome
121 shotgun sequence of Marseille-Q1233 were deposited in GenBank under accession numbers
122 LR745654 and CACVAN0000000000 respectively. The type strain is Marseille-Q1233 (=
123 CSURQ1233) isolated from the stool sample of a Malian child under five years of age.

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225 **Table 1:** Biochemical tests performed on strain Marseille-Q1233 using API strips ZYM and 50 CH

API ZYM		API 50 CH			
Characteristics	Results	Characteristics	Results	Characteristics	Results
Alkaline phosphatase	+	Control	-	Esculin ferric citrate	+
Esterase (C4)	+	Glycerol	-	Salicin	-
Esterase Lipase (C8)	+	Erythritol	-	D-cellobiose	-
Lipase (C14)	-	D-arabinose	-	D-maltose	-
Leucine arylamidase	-	L-arabinose	-	D-lactose	-
Valine arylamidase	-	D-ribose	-	D-melibiose	-
Cystine arylamidase	-	D-xylose	-	Sucrose	-
Trypsin	-	L-xylose	-	D-trehalose	-
α -chymotrypsin	-	D-Adonitol	-	Inulin	-
Acid phosphatase	-	Methyl β D-xylopyranoside	-	D-melezitose	-
Naphthol-AS-BI-phosphohydrolase	+	D-galactose	-	D-raffinose	-
α -galactosidase	-	D-glucose	-	Starch	-
β -galactosidase	-	D-fructose	-	Glycogen	-
β -glucuronidase	-	D-mannose	-	Xylitol	-

α -glucosidase	-	L-sorbose	-	Gentiobiose	-
β -glucosidase	-	L-rhamnose	-	D-turanose	+
N-acetyl- β -glucosaminidase	-	Dulcitol	-	D-lyxose	-
α -mannosidase	-	Inositol	-	D-tagalose	-
α -fucosidase	-	D-mannitol	-	D-fucose	-
		D-sorbitol	-	L-fucose	-
		Methyl α D-mannopyranoside	-	D-arabitol	-
		Methyl α D-glucopyranoside	-	L-arabitol	-
		N-acetyl-glucosamine	-	Potassium gluconate	-
		Amygdalin	-	Potassium 2-ketogluconate	-
		Arbutin	-	Potassium 5-ketogluconate	-

226 **Table 2:** Biochemical characteristics of **1**, *Virgibacillus ihumii* sp. nov., strain Marseille-
227 Q1233 compared to **2**, *Virgibacillus siamensis* strain JCM 15395 [16]; **3**, *Virgibacillus*
228 *salinus* strain DSM 21756 [10]; **4**, *Virgibacillus phasianinus* strain LM2416 [17]; 5,
229 *Virgibacillus necropolis* strain DSM 14866 [18].

Properties	1	2	3	4	5
Cell diameter (µm)	0.3-1.5	0.5–0.7	0.9	0.3–0.4	0.5–0.7
Oxygen requirement	+	+	+	+	+
Gram stain	+	+	+	+	+
Salt tolerance	+	+	+	+	+
Motility	+	+	+	+	+
Endospore formation	-	+	+		+
Alkaline phosphatase	+	NA	NA	+	+
Catalase	+	+	+	+	+
Oxidase	-	+	-	-	NA
Nitrate reductase	-	-	+	+	+
Urease	+	-	NA	-	-
β-Galactosidase	-	NA	NA	+	NA
N-acetyl-glucosamine	-	NA	NA	+	w
Arabinose	-	-	+	+	-
Esterase lipase (C8)	-	NA	NA	+	NA
Mannose	-	-	-	+	-
Mannitol	-	-	-	+	-
Sucrose	-	-	+	+	-
D-Glucose	-	+	+	+	+
D-Fructose	-	-	+	+	w

D-Maltose	-	+	+	+	-
Source	Human stool sample	Fermented fish	Saline lake	Faecal sample	Deteriorated mural paintings

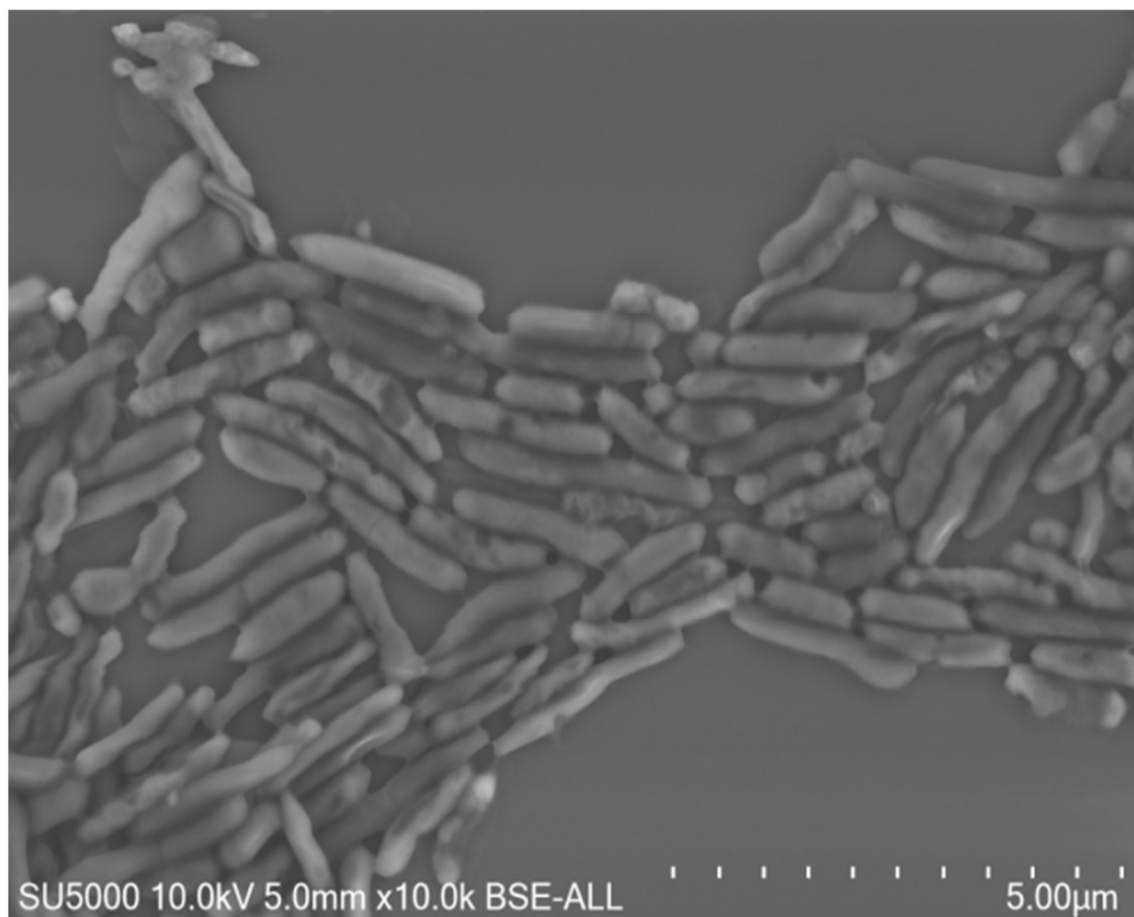
230 **Table 3:** Cellular fatty acid profiles (%) of **1**, *Virgibacillus ihumii* sp. nov., strain Marseille-
231 Q1233^T compared with **2**, *Virgibacillus salinus* strain DSM 21756; **3**, *Virgibacillus*
232 *phasianinus* strain LM2416 and **4**, *Virgibacillus necropolis* strain DSM 14866.

Fatty acids	Names	1	2	3	4
15:0 anteiso	12-methyl-tetradecanoic acid	52.2	32.0	73.0	64.7
14:0 iso	12-methyl-tridecanoic acid	15.2	17.1	3.1	2.5
5:0 iso	3-methyl-butanoic acid	5.3	ND	ND	ND
17:0 iso	15-methyl-hexadecanoic acid	5.3	ND	0.7	0.9
15:0 iso	13-methyl-tetradecanoic acid	4.9	9.4	5.3	3.1
16:00	Hexadecanoic acid	-	20.5	0.9	2.2
13:0 anteiso	10-methyl-dodecanoic acid	TR	TR	TR	-
15:00	Pentadecanoic acid	TR	TR	TR	-

233 TR, trace amounts <1%. -, not detected; ND, not detected.

234 **Table 4:** Genome comparison between strain Marseille-Q1233 and closely related species using GGDC and formula 2 (dDDH estimates based
 235 on identities over HSP length), upper right.

	<i>V. ihumii</i>	<i>V. dokdonensis</i>	<i>V. halodenitrificans</i>	<i>V. necropolis</i>	<i>V. phasianinus</i>	<i>V. salinus</i>	<i>V. siamensis</i>	<i>V. soli</i>
<i>V. ihumii</i>	100%	20.90%	21.50%	21.10%	21.10%	19.60%	23.10%	21.00%
<i>V. dokdonensis</i>		100%	23.20%	24.40%	28.50%	21.90%	22.50%	32.10%
<i>V. halodenitrificans</i>			100%	23.40%	23.00%	20.70%	21.70%	27.20%
<i>V. necropolis</i>				100%	22.90%	21.00%	22.60%	29.00%
<i>V. phasianinus</i>					100%	20.70%	21.30%	34.40%
<i>V. salinus</i>						100%	18.80%	22.70%
<i>V. siamensis</i>							100%	41.00%
<i>V. soli</i>								100%



236

237 **Figure 1:** Scanning electron micrograph of *Virgibacillus ihumii* sp. nov., strain Marseille-

238 Q1233 obtained from TM4000 microscope.

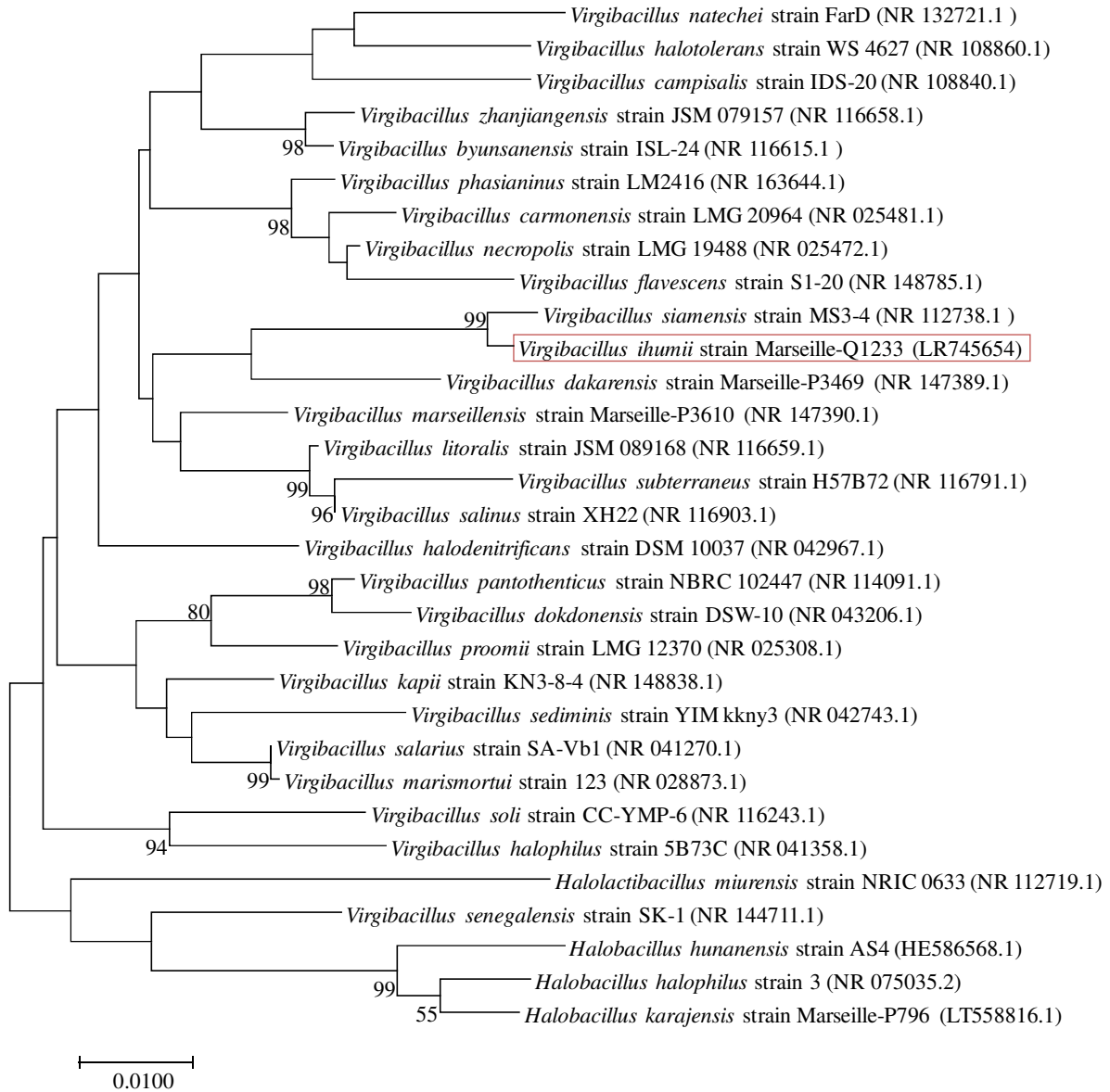


Figure 2: Phylogenetic tree showing the position of *Virgibacillus ihmii* sp. nov., strain Marseille-Q1233. The GenBank accession numbers for 16S rRNA genes are indicated in parenthesis. Sequences were aligned using MUSCLE v7.0.26 with default parameters and phylogenetic inferences were obtained using the maximum likelihood method with MEGA 7 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. Only bootstrap values > 70% were retained. The scale bar indicates a 1% nucleotide sequence divergence.

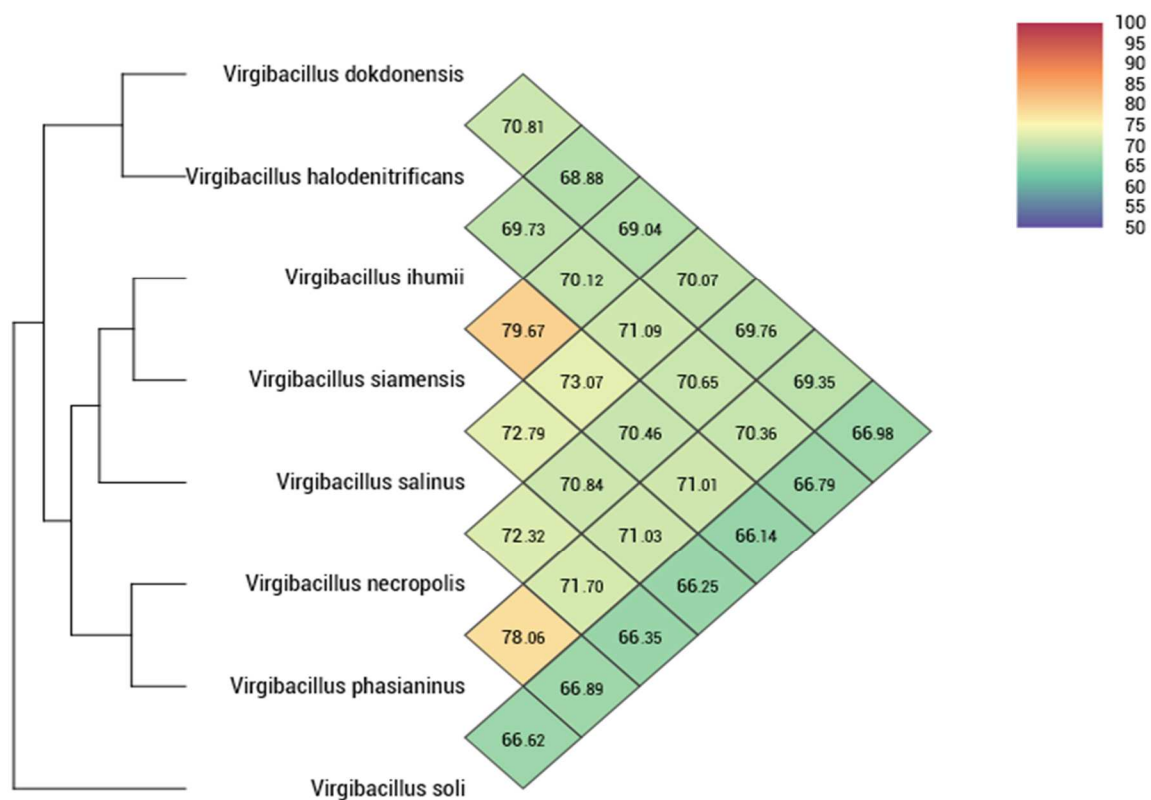


Figure 3: Heatmap generated with OrthoANI values calculated using the OAT software between *Virgibacillus ihumii* sp. nov., strain Marseille-Q1233 and other closely related species with standing in nomenclature.