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

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Running title: *Virgibacillus ihumii* sp. nov.

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

11 **Introduction**

12 Severe acute malnutrition (SAM) is a real public health problem that particularly affects
13 children under the age of five [1]. Several factors are implicated, such as the increase in
14 *Proteobacteria* [2], the involvement of intestinal microbes by anaerobic depletion [3] and the
15 decrease in antioxidants [4], all of which are involved in the progressive establishment of
16 SAM [5]. In this study, we used a new approach, called culturomics, which consists in
17 isolating bacteria under various culture conditions and identifying them using matrix-assisted
18 laser desorption ionization - time-of-flight mass spectrometry [6, 7].

19 Heyndrickx *et al.* (1998) described the genus *Virgibacillus* by analyzing the diversified genus
20 *Bacillus* [8]. Since, some species have been discovered and many further species have been
21 reclassified in the genus [9–11]. The genus *Virgibacillus* is composed of 40 validly published
22 species with correct names [12]. Most of them are isolated from different biotopes, such as
23 seawater, animal, sediment or soil [13-17]. The members of the genus *Virgibacillus* are
24 characterized by its motility and staining Gram-positive. In particular, *Virgibacillus* species
25 possesses C_{15:0-anteiso} as a major fatty acid and MK-7 as the main menaquinone [18].

26 *Virgibacillus ihumii* sp. nov., a new bacterial species, was characterized using a combination
27 of genotypic and phenotypic criteria, following a taxono-genomics concept previously
28 reported [19-20]. To this end, we performed a description of this new species belonging to
29 the genus *Virgibacillus* that was isolated from the stool of a healthy Malian child as part of the
30 exploration of the intestinal microbiota in malnourished children.

31 **Isolation and growth conditions**

32 In 2019, a bacterial strain was isolated from the stool sample of Malian child as part of a case-
33 control study on severe acute malnutrition. The growth of this strain was obtained following
34 48 hours of incubation at 37°C on halophilic medium at pH 7.5. It was able to grow after 24
35 hours of incubation at temperatures ranging from 28°C to 42°C with an optimal pH of 7.5

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

43 **Phenotypics characteristics**

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

61 **Strain identification**

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

73 **Genome sequencing**

74 Genomic DNA was extracted using the EZ1 biorobot with the EZ1 DNA tissue kit (Qiagen,
75 Hilden, Germany) and then sequenced on a MiSeq sequencer (Illumina Inc, San Diego, CA,
76 USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired End (Illumina), as
77 previously described [23]. The assembly was performed using a pipeline containing several
78 softwares (Velvet [24], Spades [25] and Soap Denovo [26]), and trimmed (MiSeq and
79 Trimmomatic [27] softwares) or untrimmed data (only MiSeq software). GapCloser software
80 [28] was used to decrease assembly gaps. Scaffolds that had a nucleotide number <800 (bp)
81 and scaffolds that had a depth value lower than 25% of the mean depths were removed. The
82 best assembly was selected using different criteria (number of scaffolds, N50, number of N).
83 The genome of strain Marseille-Q1233 was 3,813,121 bp long with a 41.1 % of G+C content.

84 **Whole genome sequences analysis by Blast in Genbank database revealed that strain**
85 **Marseille-Q1233 was 90.30% similar to *Virgibacillus siamensis*. In addition, dDDH values**

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

97 Despite high similarity in the 16S sequence with the closest neighbor (92%), the careful
98 characterization confirmed the identification of a new species based on phenotypic (endospore
99 formation, oxidase, urease, metabolism of D-glucose and D-maltose) and genomics (dDDH
100 23%, orthoANI 80% and ANI 81%). Indeed, nowadays, 16S similarity is not the only
101 criterion to delineate a new species. Phenotypic and genomic characteristics should be taken
102 into account with different thresholds. For digital DNA-DNA Hybridization (dDDH), the
103 threshold is 70%. Here, the dDDH is much lower than this threshold (23%). For orthoANI
104 (average nucleotide identity based on orthologous genes), the threshold was set at 95%. Here,
105 the orthoANI is as low as 80%. Finally, for the average nucleotide identity, the usual
106 threshold is 92%. Here, the ANI is 81%, well below this threshold. All these results confirm
107 the description of a new species.

108 **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

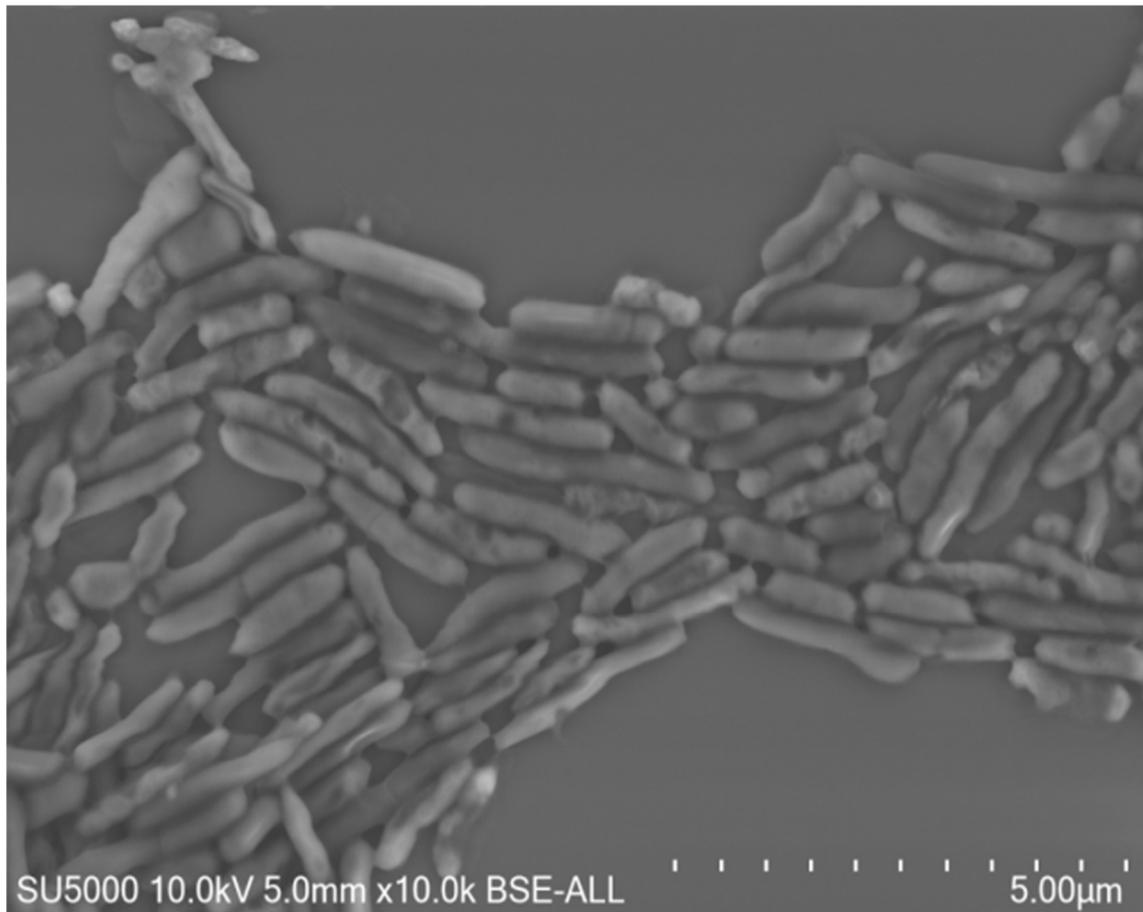
230 **Table 3:** Cellular fatty acid profiles (%) of **1**, *Virgibacillus ihumii* sp. nov., strain Marseille-
 231 Q1233^T compared with **2**, *Virgibacillus salinus* strain DSM 21756; **3**, *Virgibacillus*
 232 *phasianinus* strain LM2416 and **4**, *Virgibacillus necropolis* strain DSM 14866.

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.

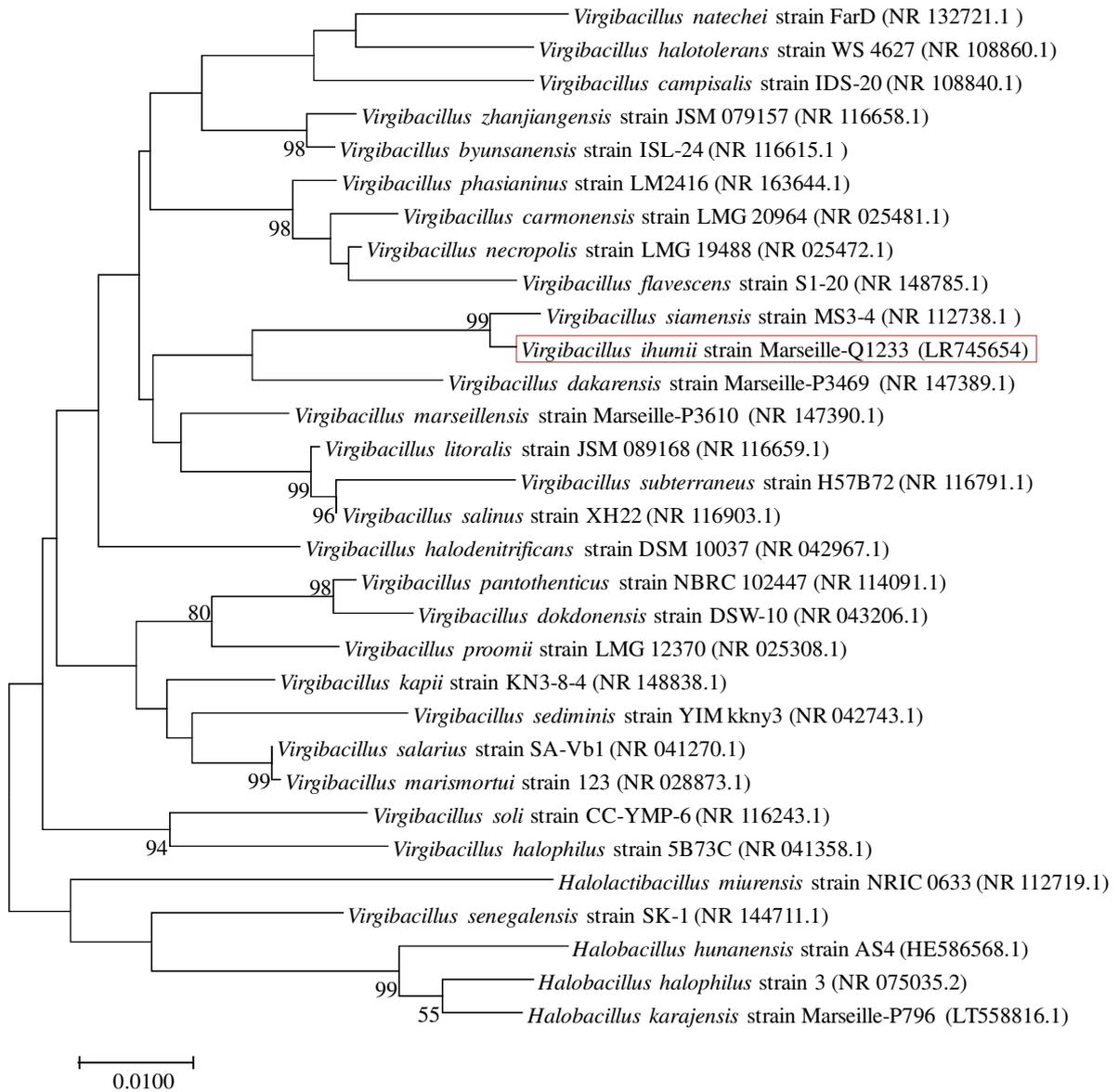
	<i>V. ihumii</i>	<i>V. dokdonensis</i>	<i>V. halodenitrificans</i>	<i>V. necropolis</i>	<i>V. phasianinus</i>	<i>V. salinus</i>	<i>V. siamensis</i>	<i>V. soli</i>
<i>V. ihumii</i>	100%	20.90%	21.50%	21.10%	21.10%	19.60%	23.10%	21.00%
<i>V. dokdonensis</i>		100%	23.20%	24.40%	28.50%	21.90%	22.50%	32.10%
<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.



239

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

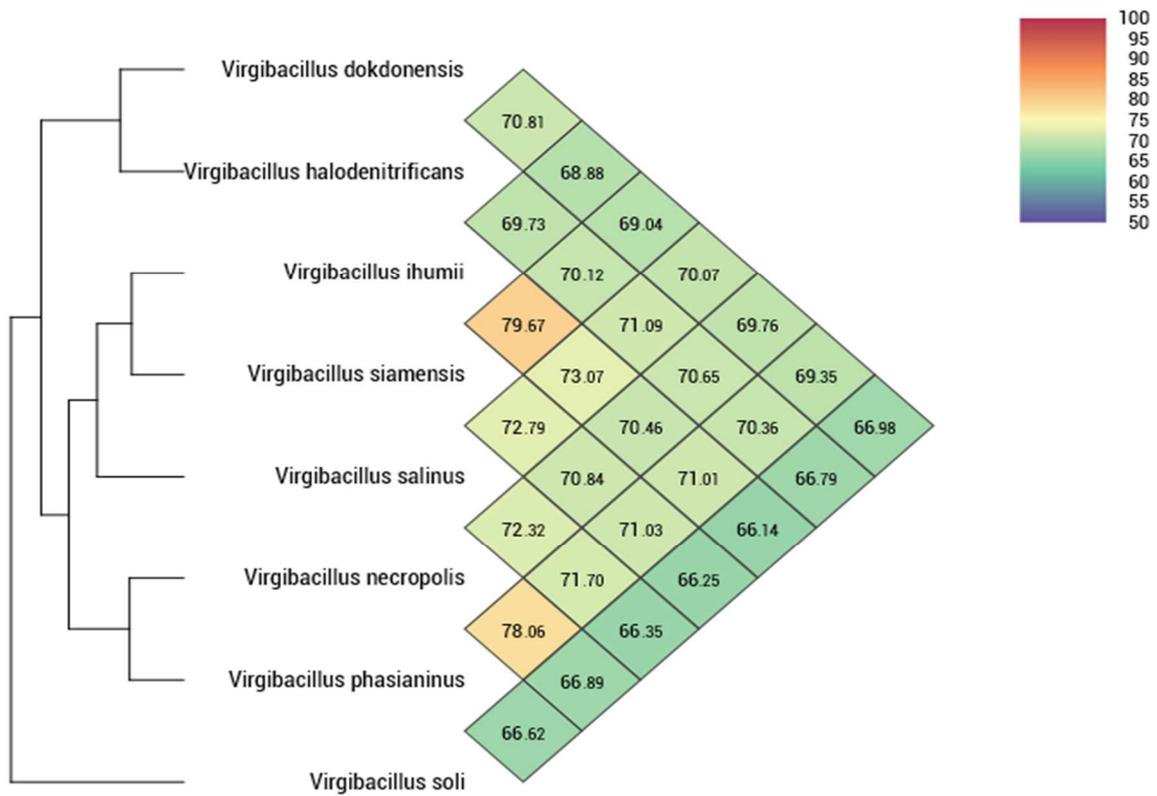


Figure 3: Heatmap generated with OrthoANI values calculated using the OAT software between *Virgibacillus ihumii* sp. nov., strain Marseille-Q1233 and other closely related species with standing in nomenclature.