

1 ***In vitro* and *in vivo* activity of new strains of *Bacillus subtilis***  
2 **against ESBL-producing *Escherichia coli*: an experimental study.**

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19 **Key-words:** *Bacillus subtilis*, *Escherichia coli*, ESBL, extended-spectrum beta-lactamase,  
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24 **Running title:** *B. subtilis* strains against ESBL-*E. coli*

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35 **ABSTRACT**

36 **Aims:** The gastro-intestinal tract is a major reservoir of extended-spectrum beta-lactamase  
37 (ESBL) producing *E. coli*. *Bacillus* spores may be used as probiotics to decrease digestive  
38 colonization by ESBL-*E. coli*. Our aim was to assess the *in vitro* and *in vivo* activity of new  
39 *Bacillus* strains against ESBL-*E. coli*.

40 **Methods and Results:** We screened the *in vitro* activity of 50 *Bacillus* strains against clinical  
41 isolates of ESBL-*E. coli* and selected *B. subtilis* strains CH311 and S3B. Both strains  
42 decreased ESBL-*E. coli* titers by 4 log<sub>10</sub> CFU L<sup>-1</sup> in an *in vitro* model of gut content, whereas  
43 the *B. subtilis* CU1 strain did not. In a murine model of intestinal colonization by ESBL-*E. coli*,  
44 CH311 and S3B did not decrease fecal titers of ESBL-*E. coli*. Ten sequences of putative  
45 antimicrobial peptides were identified in the genomes of CH311 and S3B, but not in CU1.

46 **Conclusions:** Two new *B. subtilis* strains showed strong *in vitro* activity against ESBL-*E. coli*.

47 **Significance and Impact of Study:** Despite strong *in vitro* activities of new *B. subtilis* strains  
48 against ESBL-*E. coli*, intestinal colonisation was not altered by curative *Bacillus* treatment even  
49 if their spores proved to germinate in the gut. Thus this work underlines the importance of *in*  
50 *vivo* experiments to identify efficient probiotics. The use of potential antimicrobial compounds  
51 identified by genome sequencing remains an attractive alternative to explore.

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56 **Introduction**

57 Third-generation cephalosporins and carbapenem-resistant Enterobacteriaceae are critical  
58 pathogens according to the World Health Organisation priority list of antibiotic resistant  
59 bacteria. The intestinal microbiota is a major reservoir of multidrug-resistant

60 Enterobacteriaceae (MDR-EB), and intestinal colonization by MDR-EB is promoted by  
61 intestinal dysbiosis (Sorbara *et al.* 2019). Intestinal colonization by MDR-EB may evolve from  
62 an asymptomatic carriage to various infections including urinary, gastrointestinal and  
63 bloodstream infections. Furthermore, the intestinal carriage of MDR-EB can lead to  
64 environmental contamination and transmission to healthy or diseased subjects. Hence,  
65 treatments to decrease the intestinal carriage of MDR-EB are crucial to limit the global spread  
66 of antimicrobial resistance. Various strategies have been assessed but none has proved to be  
67 effective so far (Tacconelli & Pezzani, 2019).

68 Probiotics may be considered to restore dysbiotic intestinal microbiota, to treat symptomatic  
69 intestinal infections and/or to decrease asymptomatic intestinal colonization by MDR-EB.  
70 Probiotic preparations, usually consisting of *Bifidobacteria*, *Lactobacilli*, *Enterococci* and/or  
71 *Streptococci* alter intestinal microbiota (Chae *et al.*, 2016) and showed variable efficacy in  
72 preventing experimental intestinal infections due to *E. coli* in animals (Fukuda *et al.* 2011;  
73 Guerra-Ordaz *et al.* 2014). Besides, their efficacy in humans on improving microbiome  
74 reconstitution after an antibacterial treatment has not been demonstrated, and a commercial  
75 probiotic preparation even delayed and decreased post-antibiotic restoration of the gut  
76 microbiota and prevented post-antibiotics reduction of antimicrobial resistance genes (Suez  
77 *et al.*, 2018; Montassier *et al.*, 2021). Furthermore, few randomized controlled trials have been  
78 conducted to assess if probiotics may decrease intestinal colonization by MDR-EB in humans  
79 (Newman & Arshad, 2020). Hua *et al.* found no effect of a commercial probiotic on rectal  
80 colonization by ESBL-producing bacteria in preterm newborns, although subgroup analysis  
81 suggested an effect in non breastfed patients (Hua, Tang & Mu, 2014). *Saccharomyces*  
82 *boulardii* and other commercial probiotic preparations consisting of *Lactobacillus*,  
83 *Bifidobacterium* and *Streptococcus* (Vivomixx®) or *S. boulardii*, *L. acidophilus*, *B. animalis*  
84 *subsp. lactis* and *Lacticaseibacillus paracasei* (Bactiol Duo®) failed to significantly reduce  
85 intestinal colonization by Extended Spectrum Beta-Lactamase producing Enterobacteriaceae  
86 (ESBL-EB) in adult patients (Ljungquist *et al.*, 2020; Wieërs *et al.*, 2020). Hence, there is a  
87 need for probiotics that would reduce intestinal colonization by MDR-EB. *Bacillus* spp consists

88 of different species of spore-forming soil bacteria that are commonly ingested with vegetables,  
89 and reach a concentration of about  $10^{4-5}$  colony-forming units (CFU) per gram of animal and  
90 human faeces (Elshaghabe *et al.* 2017). *Bacillus* spores are resistant to the acidity of gastric  
91 contents and are stable for long periods in various environments (Cutting 2011). After  
92 germination, vegetative cells of *Bacillus* produce antimicrobial compounds (AMC) that confer  
93 an antimicrobial activity against various pathogens (Caulier *et al.* 2019). Most of these  
94 compounds have bactericidal activity against gram positive bacteria but a few, such as  
95 Gramicidin S and Polymyxins, are active against gram negative bacteria (Mogi & Kita, 2009).  
96 Unfortunately, the latter antimicrobial peptides (AMPs) have toxicity and can induce colistin  
97 resistance in other enterobacteria (Halaby *et al.* 2013).

98 Therefore, alternative *Bacillus* strains that could serve as a source for safer AMCs have been  
99 sought (Latorre *et al.* 2016; Guo *et al.* 2017; Caulier *et al.* 2019). Treatment with *Bacillus*  
100 spores has been shown to prevent various gastrointestinal infections, including infections due  
101 to *E. coli* in piglet, chicken, rabbit and mouse (La Ragione *et al.* 2001; La Ragione &  
102 Woodward, 2003; D'Arienzo *et al.* 2006; Tsukahara *et al.* 2013; Guo *et al.* 2017). *Bacillus* also  
103 abrogated asymptomatic intestinal colonization of methicillin resistant *S. aureus* in mice  
104 (Piewngam *et al.* 2018). However, whether *Bacillus* treatment decrease the intestinal  
105 colonization by MDREc remains unknown.

106 Consequently, we searched for new strains of *Bacillus* that would inhibit ESBL-producing *E.*  
107 *coli* (ESBL-*E. coli*) under in vitro conditions that mimic the gut environment. We also  
108 characterised their genome and tested their efficacy as a curative treatment of intestinal  
109 colonization by an ESBL-*E. coli* in a murine model.

## 110 **Materials and Methods**

### 111 **Bacterial strains and media**

112 Clinical isolates of ESBL-*E. coli* were collected at the University Hospital of Nantes. One of  
113 them was also resistant to carbapenems through an OXA-48 carbapenemase and was used

114 in the *in vitro* culture test and in the murine model of intestinal colonization. The reference  
115 strain for *Bacillus subtilis* was the commercially available CU1 strain (Lefevre *et al.* 2015). For  
116 bacterial numeration, we used CHROMID™ ESBL agar plates (Biomerieux) for ESBL-*E. coli*,  
117 and LB agar NaCl plates (75 g L<sup>-1</sup> NaCl) for *Bacillus* and *Staphylococcus* spp strains.

### 118 **Isolation of *Bacillus* strains from the environment and identification**

119 Samples were collected from different environmental sources such as soil, mice food, lake and  
120 river mud as well as faeces from a variety of organisms (human, mouse, dog and snail). Each  
121 sample was mixed with water. One ml of each mix was heated at 80°C for 20 min to kill  
122 vegetative cells, leaving *Bacillus* spores. Then they were spread on LB NaCl agar plates. For  
123 identification, 16S RNA genes were amplified with universal primers D88 (5'-  
124 AGAGTTTGATCCTGGCTCAG-3') and F17 (5'-CCGTCAATTCCTTTGAGTTT), and *gyrA*  
125 genes were amplified with primers *gyrA*-f (5'-CAGTCAGGAAATGCGTACGTCCTT) and *gyrA*-  
126 r (5'-CAAGGTAATGCTCCAGGCATTGCT). Sequences were blasted in NCBI.

### 127 **Spores production**

128 In order to produce large amounts of spores, an overnight culture of *Bacillus* in 10 ml LB  
129 medium was inoculated into a flask containing 400 ml of Difco Sporulation Medium (Monteiro  
130 *et al.* 2005). After 3 days of shaking at 37°C, most of the *Bacillus* bacteria were in a sporulated  
131 form. The culture was then centrifuged, washed and resuspended in 3 ml of sterile water.  
132 Titration of spores was carried out after heating this suspension for 20 min at 80°C and then  
133 stored (about 10<sup>10</sup>-10<sup>11</sup> spores ml<sup>-1</sup>) at -80° C.

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### 135 **Colony diffusion assay**

136 The *in vitro* inhibitory activity of *Bacillus* against ESBL-*E. coli* was screened as follows. Two  
137 µL of fresh *Bacillus* suspension (10<sup>6</sup> CFU ml<sup>-1</sup>) were inoculated on the surface of a dried LB or  
138 minimal medium with 0,2% inulin agar plates where an ESBL-*E. coli* isolate had been spread.  
139 Fifteen clinical isolates of ESBL-*E. coli* were used. After incubation for 24 hr at 37°C, the areas  
140 of inhibition around *Bacillus* colonies were determined. The *in vitro* activity of strains was

141 classified as strong, medium and weak for inhibition diameter higher than 20mm, between 10  
142 and 20 mm, and lower than 10 mm, respectively.

### 143 **Co-culture of *Bacillus* and ESBL-*E. coli* isolates in murine faeces**

144 In order to mimic the culture conditions in colonic content (including low oxygen pressure), 50  
145 mg of faeces from an antibiotic naive mouse were suspended in 1 ml of sterile water and  
146 shaken at 20 Hz (Mixer Mill MM 400, RETSCH's) for 5 min to make a homogenous suspension.  
147 Then, 100 µl of this suspension was mixed with suspensions of vegetative cells of *Bacillus* and  
148 ESBL-*E. coli* strains (in a 1:1 ratio) to obtain 10<sup>6</sup> CFU ml<sup>-1</sup> of each bacterium (final volume, 1  
149 mL), supplemented or not with 0,2% inulin (Bulk powders). Inulin was tested since it is a  
150 prebiotic which could be used by *Bacillus* as a carbon source. The 2 ml tubes (containing a  
151 1ml suspension) were shaken horizontally for 24 hr at 37°C (orbital shaker-incubator ES-20,  
152 Grant bio). Titers of *E. coli* and *Bacillus* were assessed after incubation at 37°C for 24 hr and  
153 48 hr, respectively. All experiments were done in triplicate.

### 154 **Murine model of intestinal colonization by ESBL-*E. coli***

155 All experiments were approved by the Animal Experiment Committee of Pays de la Loire  
156 (France, authorization number APAFIS#18120) and ARRIVE Guidelines were followed.  
157 Six weeks old male Swiss mice (Janvier Labs, Saint-Berthevin, France) were kept in individual  
158 cages with free access to food and water. First, intestinal dysbiosis was induced with  
159 amoxicillin (0,5 g L<sup>-1</sup>) in drinking water for 3 days. Pantoprazole (0,1 g L<sup>-1</sup>) and inulin 0.2% (Bulk  
160 powders) were added to drinking water of all mice, from the experiment 1st day and the day of  
161 amoxicillin discontinuation, respectively. Pantoprazole was added because it is known to  
162 suppress the gastric acid production and to promote intestinal colonization by certain bacteria  
163 (Stiefel *et al.* 2006). ESBL-*E. coli* (10<sup>6</sup> CFU) was instilled intragastrically the day of amoxicillin  
164 discontinuation. At one day post *E. coli* inoculation (dpi), 10<sup>9</sup> spores of *Bacillus* CH311 were  
165 daily administered by intragastric instillation, for 4 days. The experimental protocol was slightly  
166 modified to assess activity of *Bacillus* S3B: amoxicillin was administered for 5 days, and ESBL-  
167 *E. coli* was inoculated 2 days after amoxicillin was stopped. Faecal samples were collected

168 daily. Faecal titers of *Bacillus*, *Staphylococci* and ESBL-*E. coli* were assessed by plating  
169 serially diluted faeces. There were 4-7 mice per group. No mouse was excluded from analysis.  
170 Researchers were aware of the group allocation during the experiment.

171 To enumerate the total number of *Bacillus* (spores and vegetative cells) per gram of faeces,  
172 faeces were homogenized in sterile water before plating serial dilutions in LB NaCl agar and  
173 incubating at 37C° for 24 h. To enumerate the total number of spores, suspensions were  
174 heated for 20 min at 80°C and then spread on the same medium. The difference in the number  
175 of CFU between the non heat-treated and heat-treated suspensions was considered as the  
176 number of spores that germinated. The spores of S3B and CH311 in frozen stocks were used  
177 as control to check the efficiency of this procedure.

## 178 **Genome sequencing and assembly**

179 Whole-genome sequencing was performed using the Illumina MiSeq platform, with a paired-  
180 end library (2 x 150 bp paired-end read mode). Illumina reads were trimmed using  
181 Trimmomatic (Bolger, Lohse & Usadel, 2014), quality filtered using the Fastx-toolkit  
182 ([http://hannonlab.cshl.edu/fastx\\_toolkit/](http://hannonlab.cshl.edu/fastx_toolkit/)), and then assembled using the SPAdes software  
183 (Bankevich *et al.* 2012; Nurk *et al.* 2013). SIS and GapFiller version 1.10 (Boetzer & Pirovano,  
184 2012; Nadalin, Vezzi & Policriti, 2012) were used to improve the initial set of contigs, and the  
185 closest complete genome was used as the reference to order and orient the contigs. The draft  
186 genome sequences of CH311 and S3B were deposited in NCBI under accession numbers  
187 JAAUXF000000000 and JAAUXE000000000 respectively.

## 188 **Genome annotation, comparative genomics and phylogeny**

189 The nucleotide sequences of CH311 and S3B strains were also submitted to ResFinder 3.2  
190 servers (<https://cge.cbs.dtu.dk>) for identification of acquired resistance genes. All annotated  
191 proteins with unknown function were submitted to Deep-AmPEP30 (Yan *et al.* 2020) to predict  
192 peptides with antimicrobial activities. The genomes of 423 *B. subtilis* strains were downloaded  
193 from Genbank (<http://www.ncbi.nlm.nih.gov/genome>), redundant sequenced strains and very  
194 poor genome quality was removed resulting in a total number of 405 genomes. The 405 *B.*

195 *subtilis* genomes were submitted to CSIphylogeny  
196 (<https://cge.cbs.dtu.dk/services/CSIPhylogeny/>). The core genome polymorphic sites were  
197 retrieved and phylogenetic tree constructed using Fasttree (Price, Dehal & Arkin, 2010). To  
198 normalize genes predictions, all genomes were annotated using Prokka (Seemann, 2014). The  
199 pangenomic analysis was performed using Roary software (Page *et al.* 2015). The gene  
200 homologies between the strains were assessed with >98% nucleotide identity and >80%  
201 sequence coverage cutoffs.

## 202 **Statistical analyses**

203 Means were reported with standard deviations. Difference in mean titers were tested with t test  
204 or ANOVA according of the number of compared groups. The efficacy of treatments was  
205 assessed using linear mixed effect models, where faecal titer was the dependent variable,  
206 treatment and time being the fixed independent variable, and mouse the random effect.  
207 Statistical analyses were performed with R 3.6.3, R Foundation for Statistical Computing,  
208 Vienna, Austria.

## 209 **Results**

### 210 ***Identification of Bacillus strains from the environment and screening for inhibitory*** 211 ***activity against ESBL-E. coli***

212 Fifty different *Bacillus* strains were isolated from environmental samples. Based on their 16S  
213 RNA and *gyrA* genes sequences, they were identified as *B. subtilis*, *B. licheniformis* or *B.*  
214 *pumilus*. We screened their inhibitory activity against 15 clinical isolates of ESBL-*E. coli*  
215 isolates using the colony diffusion assay. Nine *Bacillus* isolates inhibited growth of ESBL-*E.*  
216 *coli* isolates (Table 1). Among these, two *Bacillus subtilis* strains CH311 and S3B, isolated  
217 from dog and snail faeces respectively, presented the highest inhibition against 12 or 13  
218 different strains of clinical isolates of ESBL-*E. coli*. For several strains, inhibition diameters  
219 were higher in minimal medium with inulin than in LB (data not shown).

### 220 ***In vitro activity against ESBL-E. coli***



221 In order to assess the activity of these strains in conditions mimicking the gut environment, co-  
222 cultures of an ESBL-*E. coli* and *Bacillus* strains were performed in faeces suspension with or  
223 without inulin, and ESBL-*E. coli* titers were determined after 24 hr. *Bacillus* titers increased  
224 after 24-h culture in the medium without inulin and remained steady in the medium with inulin  
225 (Fig. 1A). However, after a 24-h culture, titers of *Bacillus* isolates in the medium without inulin  
226 showed a high inter-isolate variability, whereas they did not when cultivated with inulin.

227 After a 24-h culture without *Bacillus*, mean ESBL-*E. coli* titers increased in the medium with or  
228 without inulin. ESBL-*E. coli* titers after a 24-h co-culture with *Bacillus* without inulin were  
229 significantly different from the control for one *Bacillus* isolate (isolate Bac2,  $7.4 \pm 0.3$  vs  $8.1 \pm$   
230  $0.3 \log_{10}$  CFU ml<sup>-1</sup>, t test P-value, 0.04, Fig.1B). The combination of *Bacillus* and inulin was  
231 more effective, as 5 among 11 *Bacillus* strains significantly decreased ESBL-*E. coli* titers, the  
232 latter ranging from  $3.7 \pm 0.5$  to  $4.8 \pm 1.0 \log_{10}$  CFU ml<sup>-1</sup>. Regardless of the culture conditions,  
233 the commercial *B. subtilis* strain (CU1) did not show inhibition (Fig 1AB). For subsequent *in*  
234 *vivo* experiments, we selected the 2 most active strains on ESBL-*E. coli* (*B. subtilis* CH311  
235 and S3B). Of note, these strains also had strong *in vitro* activity against *S. aureus* (data not  
236 shown).

### 237 ***In vivo* activity in a murine model of intestinal colonization by ESBL-*E. coli***

238 Then, we assessed the activity of curative treatment with *Bacillus* spores on ESBL-*E. coli* fecal  
239 titers. First, we tested the *B. subtilis* S3B strain. Between the 2nd and the 5th day after starting  
240 treatment with *Bacillus* S3B (i.e. between 3 and 6 days post *E. coli* inoculation), fecal titers of  
241 *Bacillus* in treated mice exceeded those of control mice by  $3.0 \pm 0.2 \log_{10}$  CFU g<sup>-1</sup> (P-value <  
242 0.00001, Fig. 2A). Although *Bacillus* titers decreased when *Bacillus* administration was  
243 discontinued, they were still higher than controls at 6 dpi ( $4.5 \pm 0.2 \log_{10}$  CFU g<sup>-1</sup>). Germination  
244 rates of a *Bacillus* spores in faeces are reported in Table 2. During the same period, neither  
245 ESBL-*E. coli* or commensal *Staphylococcus* titers were significantly altered (differences with  
246 control,  $-0.2 \pm 0.7 \log_{10}$  CFU g<sup>-1</sup>, P-value, 0.82; and  $-0.3 \pm 0.7 \log_{10}$  CFU g<sup>-1</sup>, P-value 0.64,  
247 respectively, Fig. 2B and 2C).

248 Second, we tested the activity of *B. subtilis* CH311. Fecal titers obtained during and after  
249 treatment with *Bacillus* CH311 were slightly higher than those with *Bacillus* S3B. Indeed,  
250 between the 1st and the 5th day after starting treatment with *Bacillus* CH311, *Bacillus* fecal  
251 titers were higher by  $3.7 \pm 0.4 \log_{10}$  CFU g<sup>-1</sup> in comparison with control mice (P-value <  
252 0.00001, Fig. 2D). Meanwhile, *Staphylococcus* faecal titers were lower by  $1.3 \pm 0.3 \log_{10}$  CFU  
253 g<sup>-1</sup> (P-value < 0.00001; Fig. 2E). However, ESBL-*E. coli* titers were not significantly altered by  
254 *Bacillus* treatment (difference with control,  $-0.5 \pm 0.6 \log_{10}$  CFU g<sup>-1</sup>, P-value, 0.42, Fig. 2F).

### 255 **Genome sequencing of S3B and CH311 strains**

256 Genomes of S3B and CH311 strains were sequenced in order to (i) determine whether they  
257 are novel strains and (ii) try to identify the genes responsible for the strong *in vitro* inhibitory  
258 activity against *E. coli*. Comparative genomics statistics are summarized in table 3. To specify  
259 the relationship between the isolates, we performed a pangenomic analysis of the 405 *B.*  
260 *subtilis* genomes. We predicted a total of 894 orthologous genes representing the core genome  
261 (genes shared by all strains) and 46,942 orthologous genes and singletons representing the  
262 accessory genome (genes encoded in one or more isolates, but not in all). Strain CH311  
263 shared 86% of gene content with strain GXA-28 and 85% with strain H19. The strain S3B  
264 shared 97% of gene content with strains MSP1 and MSP5. The strains CH311 and S3B shared  
265 83% of their gene content. We further analysed SNPs in the core genome and detected 38,016  
266 polymorphic sites between all *B. subtilis* genomes. For strain CH311 we detected 1,521 SNPs  
267 with the closest strain H19. For strain S3B we detected 140 SNPs with the closest strains  
268 MSP1 and MSP5. We also detected 2,198 SNPs between strains CH311 and S3B. Altogether,  
269 phylogenetic and comparative genomic analyses demonstrated a polyclonal population,  
270 suggesting that CH311 and S3B are phylogenetically distinct from other strains (Fig. S1  
271 available as supplementary data).

272 We then searched 50 antimicrobial compounds (AMCs) encoding genes (Sumi *et al.*, 2015;  
273 Caulier *et al.*, 2019) within the annotated genomes of the two newly identified CH311 and S3B  
274 *B. subtilis* strains. Nine different antimicrobial compounds were detected in both strains,

275 including 4 genes reported to have a mild inhibitory activity against Gram-negative bacteria  
276 (Table 4). Of note, these 4 genes have also been reported in CU1 genome. Then, we pursued  
277 this analysis and all peptides with unknown function were submitted to the software Deep-  
278 AmPEP30 (Yan *et al.*, 2020) to predict potential new peptides with anti-microbial activities. As  
279 presented by the table 3, we found respectively 5 and 9 peptides in the genomes of CH311  
280 and S3B with a probability above 0.7. All of them are different but 4 predicted peptides from  
281 CH311 genome are also present in the CU1 genome. Thus sequences of putative antimicrobial  
282 peptides only found in CH311 and S3B are presented in the figure S2 (available as  
283 supplementary data).

## 284 Discussion

285 From our environmental sample screening, we isolated new *Bacillus* strains. About 10% of  
286 them displayed a strong *in vitro* activity against ESBL-*E. coli* not only in the colony diffusion  
287 assays but also in conditions that mimic faecal microbiota. Thus most of the collected strains  
288 of *Bacillus* did not show such bactericidal activity, as did the reference CU1 *Bacillus* strain and  
289 in agreement with previous reports (Caulier *et al.*, 2019).

290 The *in vitro* activities of the newly isolated *Bacillus* in gut mimicking conditions were strongly  
291 related to the presence of inulin, albeit *Bacillus* grew well in faeces without this oligosaccharide.  
292 However, without addition of these *Bacillus*, inulin 0.2% alone was not able to decrease ESBL-  
293 *E. coli* titers in a suspension of antibiotic naïve mouse faeces. The *in vitro* efficacy of *Bacillus*  
294 with inulin on ESBL-*E. coli* may be mediated by the fermentation of inulin by *Bacillus*,  
295 production of short chain fatty acids (SCFAs) and colonic acidification. Indeed, *Bacillus*  
296 produces SCFAs, mainly acetate, and the combination of pH under 5,75 and SCFAs proved  
297 to be deleterious for *E. coli* survival (Nakano *et al.* 1997; Sorbara *et al.* 2019). However, this  
298 mode of action was not predominant in our experiments, as pH after a 24-h co-culture ranged  
299 between 6 and 7 (data not shown). Alternatively, inulin may induce the synthesis of  
300 antimicrobial compounds by *Bacillus* as it was shown for the antifungal compound Bacillomycin

301 (Qian *et al.* 2015). It is thus likely that the *in vitro* bactericidal activities of *Bacillus* were due to  
302 the production of AMCs.

303 Despite using inulin and the strains S3B and CH311 that exhibited the most potent *in vitro*  
304 activity, we could not decrease ESBL-*E. coli* faecal titers in a mouse model of intestinal  
305 colonization. Several causes could explain this result. First, inulin concentration in the gut was  
306 not assessed and we cannot affirm that it was high enough to induce the activity of *Bacillus*.  
307 Second, the intestinal transit time of the mouse is as short as a few hours, which quickly  
308 eliminates this genus of the intestinal flora, as shown by the rapid decrease of *Bacillus* titers in  
309 our mice faeces. Third, although the *Bacillus* strains proved to germinate at quite high level,  
310 they are subjected to harsh environmental conditions including low oxygen tension and high  
311 faeces concentration. Inhibitors may reduce growth and/or gene expression of *B. subtilis* in  
312 comparison to what we observed in our *in vitro* experiments. As the bactericidal activity of  
313 *Bacillus* on *Staphylococci* was previously described in mice gut (Piewngam *et al.*, 2018), we  
314 tested it as a control of the metabolic activity of our strains in these conditions. Activity against  
315 endogenous *Staphylococci* was found for the CH311 strain, but not for S3B, suggesting that  
316 AMCs are differentially expressed in the gut. This result suggests that the lack of activity of  
317 *Bacillus* in our murine model is likely to be caused by insufficient expression/activity of the  
318 AMCs.

319 Genome sequencing of CH311 and S3B showed that these strains potentially expressed at  
320 least nine antimicrobial compounds. Four of these AMCs are reported to have inhibitory activity  
321 against Gram-negative bacteria, but these effects are often described as mild and all of them  
322 are also potentially produced by the CU1 strain that showed no *in vitro* activity against *E. coli*.  
323 Only two compounds, Subtilin and Kanosamine, were found in CH311 and/or S3B and not in  
324 the CU1 strain but they have no activity against Gram-negative bacteria (Sumi *et al.* 2015;  
325 Caulier *et al.* 2019). Therefore, it was not possible to attribute the inhibitory activity against  
326 ESBL-*E. coli* to any of these compounds, although it cannot be excluded that it could be due  
327 to different levels of gene expression of the gram-negative-active AMCs. In addition, some  
328 *Bacillus* AMCs likely remain unidentified and those that have been detected using Deep-

329 AmPEP30 must be investigated. Thus, further biochemical analyses are required to identify  
330 the origin of the bactericidal activity of these new strains of *Bacillus* against *E. coli*.

331 In conclusion, we described two new strains of *Bacillus* exhibiting an *in vitro* activity against  
332 ESBL-*E. coli*. Further studies are needed to elucidate their mechanisms of action and to assess  
333 if they could be used to treat infections or decrease colonization due to MDR *E. coli*.

334

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339

## 340 **Conflict of interest**

341 No conflict of interest declared.

342

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474 **Authors contribution statement:**

475 1) Substantial contribution to conception and design or the acquisition and analysis of data,

476 Murad Ishnaiwer, Yihienew Bezabih, François Javaudin, Mohamed Sassi, Eric Batard and

477 Michel Dion

478 2) Drafting or critically revising the manuscript: Murad Ishnaiwer, Yihienew Bezabih, Eric

479 Batard, Pascale Bemer, François Javaudin, Mohamed Sassi, and Michel Dion.





481 **List of table legends:**

482 **Table1:** *Bacillus* strains: identification and screening for activity against ESBL-*E. coli*

483 **Table2:** Percentage of spores and vegetative cells of *Bacillus* in faeces

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485 **Table 4:** Genes associated with antimicrobial compounds in 3 strains of *Bacillus subtilis*

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502 **List of figure legends:**

503 **Figure 1:** *In vitro* activity of *Bacillus* against ESBL-*E. coli*. *Bacillus* (A) and ESBL-*E. coli* (B)  
504 mean titers after 24 hr cultivation with or without 0.2% inulin in a liquid medium containing  
505 murine native faeces (5 mg ml<sup>-1</sup>). Dark, with inulin; light, without inulin. Error bars, standard  
506 deviation. Stars show statistically significant differences with control.

507 **Figure 2:** Effect of treatment with *Bacillus* on fecal titers of *Bacillus*, commensal *Staphylococci*  
508 and ESBL-*E. coli*. Fecal titers of *Bacillus* (A and D), *Staphylococcus* (B and E) and ESBL-*E.*  
509 *coli* (C and F). Mice were treated with spores of *B. subtilis* S3B (left) or *B. subtilis* CH311 (right).  
510 The experimental design is shown at the top of the figure: mice were treated with amoxicillin  
511 before being inoculated with ESBL-*E. coli*. Spores of *B. subtilis* S3B and CH311 were  
512 inoculated from 1 to 3 dpi, and from 1 to 4 dpi, respectively. Grey lines and circles, control;  
513 black lines and circles, *Bacillus* treatment.

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**Table 1** *Bacillus* strains: identification and screening for activity against ESBL-*E. coli*

| Strain       | Origin       | Identification method | Identification          | Level of activity against ESBL <i>E. coli</i><br>(number of <i>E. coli</i> strains)* |        |      |              |       |
|--------------|--------------|-----------------------|-------------------------|--|--------|------|--------------|-------|
|              |              |                       |                         | strong   | medium | weak | any activity | total |
| <b>CU1</b>   | commercial   | -                     | <i>B. subtilis</i>      | 0  | 0      | 0    | 0            | 15    |
| <b>A532</b>  | Bogoria Lake | 16S RNA               | <i>B. licheniformis</i> | 0  | 0      | 0    | 0            | 15    |
| <b>S2</b>    | Snail faeces | gyrA                  | <i>B. subtilis</i>      | 2  | 4      | 6    | 12           | 15    |
| <b>S3B</b>   | Snail faeces | gyrA                  | <i>B. subtilis</i>      | 6  | 3      | 3    | 12           | 15    |
| <b>S28</b>   | Snail faeces | 16S RNA               | <i>B. pumilus</i>       | 4  | 4      | 3    | 11           | 15    |
| <b>CH311</b> | Dog faeces   | gyrA                  | <i>B. subtilis</i>      | 10   | 1      | 2    | 13           | 15    |
| <b>Bac2</b>  | Mice Aliment | gyrA                  | <i>B. subtilis</i>      | 4  | 0      | 5    | 9            | 15    |
| <b>Bac4</b>  | Mice Aliment | gyrA                  | <i>B. subtilis</i>      | 2  | 2      | 6    | 10           | 14    |
| <b>MD1</b>   | Human faeces | gyrA                  | <i>B. subtilis</i>      | 2  | 0      | 4    | 6            | 15    |
| <b>Mi1</b>   | Mice faeces  | gyrA                  | <i>B. subtilis</i>      | 3  | 0      | 5    | 8            | 15    |
| <b>Mi2</b>   | Mice faeces  | gyrA                  | <i>B. subtilis</i>      | 2  | 5      | 4    | 11           | 15    |

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\*Note. Activity was assessed using a colony diffusion assay on LB agar against 15 clinical isolates of ESBL-*E. coli*. A532 strain was kept to provide a negative control.

538 **Table 2** Percentage of spores and vegetative cells of *Bacillus* in faeces

| isolate                            | CH311  | S3B   |
|------------------------------------|--|---|
| <b>total cells, CFU/g</b>          | $2.4 \cdot 10^6 \pm 1.8 \cdot 10^6$                    | $1.2 \cdot 10^5 \pm 8.2 \cdot 10^4$                   |
| <b>Spores, CFU/g (%)</b>           | $1.4 \cdot 10^6 \pm 1.4 \cdot 10^6$<br>(51% $\pm$ 12%) | $5.6 \cdot 10^3 \pm 3 \cdot 10^3$<br>(5% $\pm$ 2%)    |
| <b>Vegetative cells, CFU/g (%)</b> | $1 \cdot 10^6 \pm 5 \cdot 10^5$<br>(49% $\pm$ 12%)     | $1.2 \cdot 10^5 \pm 7.9 \cdot 10^4$<br>(95% $\pm$ 2%) |

539 \*Note. Faeces were sampled in mice treated with *B. subtilis* S3B and CH311 3 and 2 days  
 540 after inoculation of ESBL-*E. coli*. *Bacillus* cells were enumerated in faeces before (total cells)  
 541 and after 20 min heating at 80°C (spores). Vegetative cells were estimated as the difference  
 542 between total cells and spores.

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**Table 3** Genomic and comparative genomic statistics

|  | <b>CH311</b>                                   | <b>S3B</b>                                     |
|--|--|--|
| <b>Genome properties</b>   |  |  |
| Genome size (bp)   | 4,190,428                                      | 4,038,907                                      |
| Number of contigs  | 5  | 6  |
| Total genes  | 4,377  | 4,138  |
| Protein-coding genes   | 4,209  | 3,995  |
| <b>Comparative genomics</b>  |  |  |
| Number of unique genes   | 48   | 8  |
| Closest genomes (Number of SNPs ; % of shared orthologous genes)                         | H19 strain (1,521 SNPs ; 85.8%)                | MSP1 and MSP5 strains (140 SNPs ; 97,4%)       |
| <b>Genome annotation</b>   |  |  |
| Number of genes with unknown function  | 712  | 552  |
| Number of predicted antimicrobial peptides with Deep-AmPEP30 , product probability > 0.7 | 5  | 9  |
| Predicted antibiotic resistance genes (Phenotype)  | <i>mph(K)</i> gene (spiramycin, telithromycin) | <i>mph(K)</i> gene (spiramycin, telithromycin) |
|  | <i>aadK</i> gene (streptomycin)                | <i>aadK</i> gene (streptomycin)                |
|  | <i>tet(L)</i> gene (doxycycline, tetracycline) |  |

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**Table 4** Genes associated with antimicrobial compounds in 3 strains of *Bacillus subtilis*

| antimicrobial compound | inhibition spectrum    |                        |       | gene detected in strain |       |     |
|------------------------|------------------------|------------------------|-------|-------------------------|-------|-----|
|                        | Gram positive bacteria | Gram-negative bacteria | Fungi | CU1                     | CH311 | S3B |
| <b>Subtilin</b>        | +                      |                        | +     |                         |       | +   |
| Subtilosin-A           | +                      | +                      |       | +                       | +     | +   |
| Surfactin              | +                      |                        | +     | +                       | +     | +   |
| Bacilysin              | +                      | +                      | +     | +                       | +     | +   |
| Bacillaene             | +                      | +                      |       | +                       | +     | +   |
| Plipastatin            |                        |                        | +     | +                       | +     | +   |
| Bacillibactin          |                        |                        | +     | +                       | +     | +   |
| Kanosamine             |                        |                        | +     |                         | +     | +   |
| Bacilysozin            |                        | +                      | +     | +                       | +     | +   |

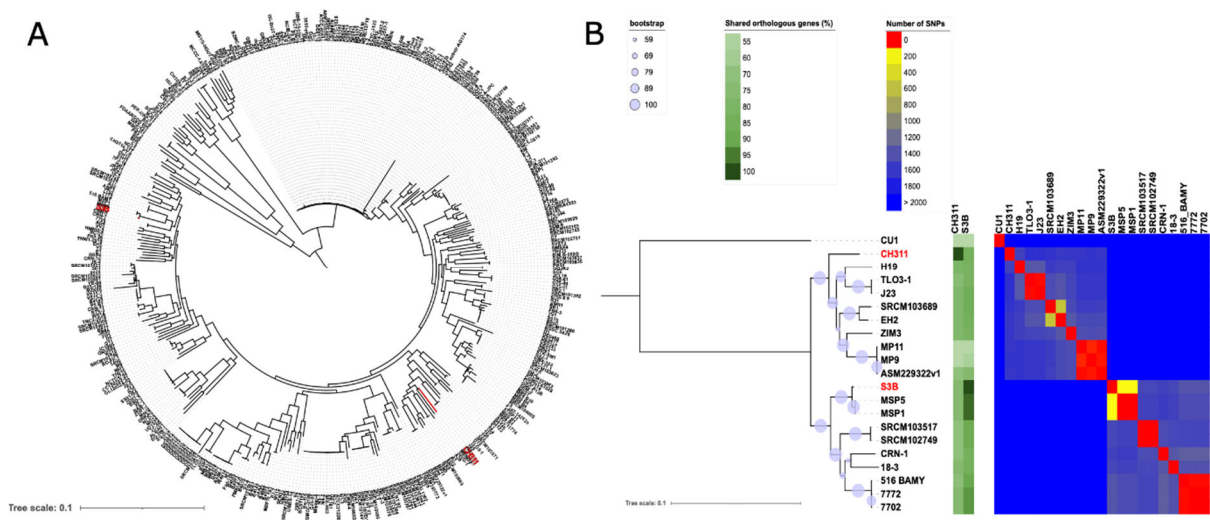
548 \*Note. Ribosomal peptide is indicated in bold character. +, target of the AMC or gene coding  
549 for peptide or AMC synthesising enzyme.

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552 **Supporting information**

553 **Figure S1:** (A) Phylogenetic tree of 405 strains of *B. subtilis*. The 405 *B. subtilis* genomes were  
 554 submitted to CSIphylogeny (<https://cge.cbs.dtu.dk/services/CSIPhylogeny/>). The core genome  
 555 polymorphic sites were retrieved and phylogenetic tree constructed using Fasttree. (B)  
 556 Phylogenetic tree of 10 closest *B. subtilis* genomes to S3B and CH311. According to core  
 557 genome SNPs analysis, 10 closest genomes were selected for phylogenetic analysis  
 558 Maximum likelihood (ML) phylogeny was constructed using iqtree2 with 100 non-parametric  
 559 bootstrap iterations (Nguyen *et al.* 2015).



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562 **Figure S2:** Sequences of putative antimicrobial peptides of strains CH311 and S3B that were  
563 not present in the strain CU1, as determined by the software Deep-AmPEP30

564 **CH311**

565 MTIYEQIKDALKNKINELVSPQEVKKTLLQEKYGTNPDSIILSDYCYNRYNKGISFNKHLFEYMN  
566 RSSYKYLGENSLYTGILFRKSKGEDKEVIVGEWVNGVKSRLREASVTNNQINDQAEIISKEQLV  
567 NLYNEYNQILR

568 **S3B**

- 569 1. MIQYASESINLPGEIAFKDVREIFFYQIAKISCFYFLLFCAIFAAVNFINGWPRIVYGSDA  
570 LNLFMMSLIIVMSVLFLLLLLLLLLYVKFSRAYKKNERMKSkrTYTLNQEgIRICSKKYDLIFNW  
571 NEITAVFEYKNIFRINTSSSQYIAIPKHFFHSEVEMNRFKEIILKNTETKklKFKKQDQH  
572 2. MKILGVTGVILICLLAISVLMMLQGFSLTKAVYNNMSSFKMTTFAEWVWILFFVLVLV  
573 REMYVIYKSKKKNP
- 574 3. MTEKKQQNKPENPEHNDLTDPIPNEELKENMNDEKHKRQQRDNSQSERYDTK  
575 4. MFRIFKMSFAVIIIILALIAFNYTEHTSVIQSVMLVFLGAVMFMQGLEERKKENDGSGA  
576 FNIYTAVFVWSVSLIGFTLHII  
577 5. MVREAAMLHIKEGLEQEFEDAFRQAAPIISGMKGYITHSLSKCMEETHKYLLLVEWE  
578 TLEDHTEGFRGSSEYQEWKALLHRFYTPFPTVEHFQDV  
579 6. MLQTPIGRLRTMGFIEGMSLLILLFIAMPLKYWAGLPLAVTIVGSVHGGLFILYLLVLAY  
580 ATFSVKWPLKWSAAGFVAAFVFPFGNFLYDRGLRKYK  
581 7. MNYKGITLLCVMMLLFSAIASFPVSAQAKDQDAGILIIYSTLDGKESSQVKMLDLAG  
582 HFTSHVTVKKDLDEASDFKGDHVIYYGQTKRKLskKLLSLISGVKNPVVAIGYNAGQINQF  
583 SGLSLARKENVFQVHSRSEKADVSLESGLNVLSISGLKGTALYTFKADEGTTHSFIWKTkkG  
584 NVYIGLTNLLNDNLIVAKQLREAFGEKAGTLLYLRLDISPMSDEKLLLQAGTYLHKRHIPFIL  
585 AVIPVYLNPETGDKVYLSDKPKMVKVLKklQSMGGSIIVHGYTHAYRYSETGEGFEFWDak  
586 ADQPITSGNAEDPPSILEKEQDFPNEQAYHSYLEPFREKEETYTKQKLTRAIEDLTSSGLYPL  
587 AFEAPHYTMSEYGYQIASQYFTSIFGQVQLSSTTWKTSGAPPFVTAPSMLHGMTLYPETIGF  
588 VDTSKQNPLGEMEERISQMIDFEGGVAGGFYHPYLGmKYLPelVDQMERIPDSEWLDLkkt  
589 KQTVKTDKVEIHTSGDGTIQVKNGVSPIDEFFDHRQTPLEKALWILSAVVLLFVIMFVSyTFY  
590 LRATLKKRIFKERRSLG  
591 8. MLSFLVSLVVAIVIGLIGSAIVGNRLPGGIFGSMIAGLIGAWIGHGllGTWGPslAGFAI  
592 FPAIIGAAIFVFLGLIFRGLRKEAMPWSMKDYPASLKNLEKpVRKKAIIDIANAMIDEGYEEGR  
593 APIATSKAKewaENASTDEIDDFLTHDDETERDADPSSGSGPELMNKAehVIKHKKGWAVK  
594 AEGAKRVSEIKDTKKEAIERAKEIAAHKgieVIVHLADGSVQRKIktGS

595