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► To cite this version:

M. Imran, N. Desmasures, M. Coton, Anne Le Fleche-Mateos, F. Irlinger, et al.. Safety assessment of Gram-negative bacteria associated with traditional French cheeses. *Food Microbiology*, 2019, 79, pp.1-10. 10.1016/j.fm.2018.11.001 . hal-02086922

HAL Id: hal-02086922

<https://normandie-univ.hal.science/hal-02086922v1>

Submitted on 5 Jun 2020

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3 **1 Safety assessment of Gram-negative bacteria associated with traditional French cheeses**

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40 **18 Running title: Safety of Gram-negative dairy isolates**

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61
62 **21 ABSTRACT**
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64 22 Twenty Gram-negative bacterial (GNB) strains were selected based on the biodiversity
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66 23 previously observed in French traditional cheeses and their safety was assessed considering
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68 24 various safety criteria. For the majority of tested GNB strains, only gastric stress at pH 2 (vs pH
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70 25 4) resulted in low survival and no regrowth after an additional simulated gastro-intestinal stress.
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72 26 Presence of milk was shown to be rarely protective. The majority of strains was resistant to
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74 27 human serum and had a low level of adherence to Caco-2 cells. When tested for virulence in
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76 28 *Galleria mellonella* larvae, GNB strains had LD 50 values similar to that of safe controls.
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78 29 However, four strains, *Hafnia paralvei* 920, *Proteus* sp. (close to *P. hauseri*) UCMA 3780,
79
80 30 *Providencia heimbachae* GR4, and *Morganella morganii* 3A2A were highly toxic to the larvae,
81
82 31 which suggests the presence of potential virulent factors in these strains. Noteworthy, to our
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84 32 knowledge, no foodborne intoxication or outbreak has been reported so far for any of the GNB
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86 33 belonging to the genera/species associated with the tested strains. The role of multiple dynamic
87
88 34 interactions between cheese microbiota and GIT barriers could be key factors explaining safe
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90 35 consumption of the corresponding cheeses.
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96 37 **Keywords:** Caco-2, *Galleria mellonella*, gastro-intestinal stress, human serum bactericidal
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98 38 assay, risk factors
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102 **40 1. Introduction**
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104 41 Cheese microbiota consists of diverse microorganisms, including yeasts, moulds, Gram-
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106 42 positive and -negative bacteria (Dugat-Bony et al., 2016; Gori et al., 2013; Imran et al., 2012;
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108 43 Irlinger and Mounier, 2009; Larpin-Laborde et al., 2011; Martín and Coton, 2017; Mounier et
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110 44 al., 2009, 2017; Wolfe et al., 2014). Microbial community diversity is important during cheese
111
112 45 making as it is associated with cheese sensorial quality but can also contribute to ensure
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114 46 microbiological control and safety (Delbes et al., 2007; Delbès-Paus et al., 2012; Irlinger and

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120
121 47 Mounier, 2009). While literature data are abundant concerning yeasts/molds and Gram-positive
122
123 48 bacteria (GPB) in cheese, Gram-negative bacteria (GNB) have been seldomly studied.
124
125 49 However, a wide diversity of GNB, found at relatively high levels in raw milk (around 3 to 4
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127 50 Log CFU/mL), has been reported (Desmaures et al., 1997; Fréтин et al., 2018; Kable et al.,
128
129 51 2016). GNB usually represent from 18 to 60 % of the bacteria isolated from the surface of
130
131 52 European smear cheeses (Larpin-Laborde et al., 2011; Maoz et al., 2003; Montel et al., 2014;
132
133 53 Mounier et al., 2005, 2017; Wolfe et al., 2014). Most of the microorganisms found in cheese
134
135 54 originate from raw milk, (animal, milking machine, environment /air) (Desmaures et al., 1997;
136
137 55 Fréтин et al., 2018), processing steps, plant, transportation equipment, labor and cheese factory
138
139 56 sources (Mounier et al., 2006). GNB present on the surface of the ripened soft cheese mainly
140
141 57 belong to *Enterobacteriaceae*, *Moraxellaceae*, *Pseudoalteromonadaceae*, *Pseudomonadaceae*,
142
143 58 *Sphingobacteriaceae* and *Vibrionaceae* families (Bockelmann et al., 2005; Chaves-Lopez et
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145 59 al., 2006; Maoz et al., 2003; Mounier et al., 2005; Tornadijo et al., 1993). Previous work, done
146
147 60 between 2008 and 2010 aimed at studying GNB associated with French milk and corresponding
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149 61 cheeses. The obtained 173 isolates corresponded to at least 26 genera and 68 species, including
150
151 62 potential new species. *Pseudomonas*, *Chryseobacterium*, *Enterobacter*, and *Stenotrophomonas*
152
153 63 were the most frequent genera found in cheese core and milk samples, while *Proteus*,
154
155 64 *Psychrobacter*, *Halomonas* and *Pseudomonas* were the most frequent genera isolated from
156
157 65 cheese surface (Coton et al., 2012).

161
162 66 Some GNB species found in cheese were reported to include some non-foodborne
163
164 67 strains associated with clinical cases (Delbès-Paus et al., 2012). Criteria to classify bacteria as
165
166 68 human pathogens or non-pathogens depends on the presence or absence of virulence factors.
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168 69 Survival during gastro intestinal transit and then adhesion to enterocyte cell surfaces is often
169
170 70 the first step in establishing potential bacterial disease. For extracellular pathogens, adhesion is
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172 71 a means to withstand mechanical cleaning. For intracellular pathogens, adhesion is often a
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180 72 prerequisite for invasion (dos Santos et al., 2015). The human adenocarcinoma cell line Caco-
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182 73 2, isolated from an adult human colon, which expresses several markers characteristic of normal
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184 74 small villi cells (Fogh et al., 1977; Pinto et al., 1983) has been extensively used to study bacterial
185
186 75 adhesion mechanisms for pathogens and probiotic strains (Greene and Klaenhammer, 1994).
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189 76 Another barrier in the human body against invading pathogens is the action of serum
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191 77 through a series of serum proteins interacting in a regulated sequential manner that eventually
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193 78 leads to bacterial death due to either lysis or opsonisation (Morgan et al., 2000). Furthermore,
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195 79 the safety status evaluation of a given microorganism must also be assessed by its actual
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197 80 pathogenicity on an animal model. Mammals have been used for a long time to evaluate
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199 81 microbial pathogen virulence but it is time consuming, labor intensive, and expensive in terms
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201 82 of purchasing animals, feeding, and housing. An alternative option is the use of an insect model
202
203 83 (Kavanagh and Reeves, 2004) because the innate immune responses are similar. A useful model
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205 84 is *Galleria mellonella* (wax moth) larvae (Ramarao et al., 2012). It has been used to evaluate
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207 85 the pathogenicity of various GNB such as *Proteus mirabilis* (Morton et al., 1987), *Francisella*
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209 86 *tularensis* (Aperis et al., 2007), *Yersinia pseudotuberculosis* (Champion et al., 2009),
210
211 87 *Stenotrophomonas maltophilia* (Nicoletti et al., 2011) and *Escherichia coli* (Walters and
212
213 88 Ratcliffe, 1983). For example, *Pseudomonas aeruginosa* virulence or that of *Bacillus*
214
215 89 *thuringiensis* and *Bacillus cereus* were correlated in *Galleria* larvae and in mice (Jander et al.,
216
217 90 2000; Salamitou et al., 2000). In fact, the virulence of many pathogens is similar in wax moth
218
219 91 larvae and mammals, including Humans (Desbois and Coote, 2012).
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222
223 92 The present study is a continuation of the previous work reported by Coton et al., (2012)
224
225 93 which focused on the diversity and potential risk factors (antibiotic resistance and biogenic
226
227 94 amines production) of milk and cheese GNB isolates. It aimed at further evaluating the safety
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229 95 aspects of selected representative GNB strains from each genus identified in the cited study.
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231 96 Growth in conditions mimicking the gastrointestinal tract (GIT) transit, Caco 2 cell adhesion,
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97 survival in serum conditions and pathogenicity on insect larvae, were assessed to provide a
98 more comprehensive view about the safety these GNB isolates.

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100 **2. Materials and methods**

101 *2.1 Strains and culture conditions*

102 Starting from an initial collection of 173 GNB strains (Coton et al., 2012), 20 strains from the
103 raw milk or milk, cheese core or cheese surface representative of the main identified genera and
104 of different safety status (evaluated according to low or high antibiotic resistance and biogenic
105 amine production *in vitro*) were selected (Table 1).

106 All these strains had been previously identified by 16S rRNA or *rpoB* gene sequencing and
107 their antibiotic resistance and biogenic amines production *in vitro* were determined (Coton et
108 al., 2012). For initial propagation, all control and tested strains were precultured in Tryptic Soy
109 Broth (Merck, KGaA, Darmstadt, Germany) supplemented with 2.5 g/L yeast extract (Oxoid,
110 Basingstoke, Hampshire, England) (TSB-YE) and incubated at 37°C or 25°C (for strains unable
111 to grow at 37°C) for 24 h under aerobic conditions.

112 For adhesion and pathogenicity assays, bacterial strains were grown in TSB-YE and
113 incubated at 37°C or 25°C (for strains unable to grow at 37°C) for 24 h with shaking (120 rpm)
114 using a Novotron shaker (VWR, Fontenay sous bois, France).

115

116 *2.2 Growth at 37°C*

117 Each precultured strain (cf. §2.1) was isolated on three plates poured with Brain Heart
118 Infusion agar (BHA, AES, France). For each strain, three plates were inoculated
119 simultaneously: two plates were incubated at 37°C respectively under aerobic and anaerobic
120 (AnaeroGen Pack, Oxoid, France) conditions for 14 days and one plate was incubated at 25°C
121 under aerobic conditions. If growth appeared as expected in aerobiosis on at least one plate and

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298 122 for one temperature, results for growth/no growth at 37°C under anaerobic conditions were
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300 123 considered.

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304 125 *2.3 Gastric and gastro-intestinal media and stress simulation*

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307 126 For gastro-intestinal stress simulation tests, bacterial strains were first precultured in J
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309 127 broth (JB) (5 g/L peptone, 15 g/L yeast extract, 2 g/L glucose, 3 g/L K₂HPO₄, adjusted to pH
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311 128 7.2) (Clavel et al., 2004) and incubated at 25°C for 24 h with shaking to obtain optimal growth.
312
313 129 For all enumerations, bacteria were plated on J agar (JA) (JB supplemented with 15 g/L agar),
314
315 130 incubated at 25°C under aerobic conditions for 24 h.

316
317 131 Simulated gastric medium (GM) was prepared by adding one volume of a sterile
318
319 132 (autoclaved 121°C, 15 min) gastric electrolyte solution (Gänzle et al., 1999) containing 4.8 g/L
320
321 133 NaCl, 1.56 g/L NaHCO₃, 2.2 g/L KCl and 0.22 g/L CaCl₂ to one volume of JB (GM-JB;
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323 134 autoclaved 121°C, 15 min) or milk medium (100g/L of half-skim milk powder; autoclaved
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325 135 100°C, 30 min) (GM-milk (Clavel et al., 2004, 2007). After sterilization, the pH of the GM-JB
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327 136 or GM-milk media were adjusted to 2 or 4 with sterile 1N HCl to simulate the acidic
328
329 137 environment of human gastric fluids. Finally, media were supplemented with 500U/l of a filter
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331 138 sterilized (0.22µm) pepsin (gastric juice enzyme) solution prepared in water (P6887; Sigma-
332
333 139 Aldrich, France) just before use.

334
335 140 For gastric stress experiments, GM-JB and GM-milk media were inoculated with each
336
337 141 strain at initial populations of $N_0 = 1.10^6$ CFU/mL and incubated at 37°C with shaking (160
338
339 142 rpm) for 3 h to simulate human stomach conditions. Numerations were carried out during the
340
341 143 gastric stress simulation at 0, 1.5 and 3 h.

342
343 144 Following the 3 h gastric stress, the inoculated GM-JB or GM-milk medium was
344
345 145 modified by adding one volume of intestinal medium (IM, composed of double strength
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347 146 sterilized JB medium adjusted to pH 6.5 with sterile 1N HCl, (Clavel et al., 2004, 2007)), to
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357 147 obtain the simulated gastrointestinal medium (GIM). These two media were named GIM-JB
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359 148 and GIM-milk. Bovine bile (B3883, Sigma-Aldrich, France) was added or not at 1.5 g/L.
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361 149 Incubation in GIM-JB and GIM-milk was done at 37°C without shaking for 28 h to simulate
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363 150 the gastro-intestinal stress. Numerations were carried out at different time intervals: 0, 1.5, 3,
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365 151 5, 21 and 28 h.

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368 152 Bacterial survival was expressed as the log (N/N₀) where N₀ is the initial population that was
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370 153 adjusted to 10⁶ CFU/ml and N is the number of CFU/mL obtained after 3 h (gastric stress) or
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372 154 31 h (gastro-intestinal stress). The two-fold dilution, due to changing the gastric stress medium
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374 155 to the gastro-intestinal stress medium, was taken into account.

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376 156 All numerations were done by serially diluting bacterial suspensions in Trytone Salt (TS)
377
378 157 diluent and plating on JA medium with a spiral system (Intersciences, France). Cell
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380 158 concentrations were expressed as CFU/mL.

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384 385 160 *2.4 Tissue culture and in vitro adhesion assay*

386
387 161 The strains were tested for their adhesion ability *in vitro* on epithelial intestinal cells (Caco-2:
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389 162 colon adenocarcinoma human, ATCC, USA, Lot# 4129634). The intestinal cells were routinely
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391 163 cultured and used as already described (Tareb et al., 2013). Cultures were used at post-
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393 164 confluence after 15 days of culture (differentiated cells). To determine the number of Caco-2
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395 165 cells in a monolayer, cells were detached for 2 min with Splittix and Splitstop (Bio Media) at
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397 166 ambient temperature and counted using a Thoma cell. Three non-pathogenic control strains
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399 167 were used: *E. coli* Nissle 1917 (obtained from Dr. Ulrich Sonnerborn from Ardeypharm,
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401 168 Germany GmbH), with a long history of safe use as a probiotic and large body of acquired
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403 169 knowledge (EFSA Panel on Biological Hazards (BIOHAZ), 2014; Wassenaar, 2016), *E. coli*
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405 170 K12 (ATCC 10798) and *Lactobacillus rhamnosus* GG strain. In addition, another control was
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407 171 used: *Escherichia coli* O157: H7 C267 (*stx*⁻, *eae*⁺), this strain is a shiga toxin negative mutant
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416 172 (*stx1*- and *stx2*-) which has kept the gamma-intimin (adherence protein) producing gene
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418 173 (*eaeA*+) (Vernozy-Rozand et al., 2000). Bacterial strains were cultured in TSB-YE incubated
419
420 174 at 37°C or 25°C (for strains unable to grow at 37°C) for 24 h under shaking (120 rpm) and then
421
422
423 175 washed twice with PBS. Concentrations were adjusted to 2×10^8 cells/mL and cells were labeled
424
425 176 with 0.2% aqueous solution of 4', 6-diamidino-2-phenylindole (DAPI, Sigma) by incubating
426
427 177 for 15 min. Three washing steps were performed with PBS (0.01 M, pH 7.4) to remove excess
428
429 178 unbound DAPI, then cells were suspended in 0.2mL Dulbecco's Modified Eagle Medium
430
431 179 (DMEM) without antibiotics and put into contact with Caco-2 cells at a final concentration of
432
433 180 10^7 per 7×10^4 Caco-2 cells for 2h under standard Caco-2 growing conditions (incubation at
434
435 181 37°C, 5% CO₂, 95% humidity). Not adherent bacterial cells were removed by three washing
436
437 182 steps with PBS solution and cell fixation was performed using a 3.7% (w/v) solution of
438
439 183 formaldehyde in PBS.

441
442 184 Enumeration of adherent bacteria was performed using an epifluorescence microscope.
443
444 185 Results were determined as an average of ten observations per assay. Adhesion was calculated
445
446 186 by enumerating the adhered bacterial cells in 10 microscopic fields for each strain and was
447
448 187 expressed as the average number of adhered bacterial cells per 100 Caco-2 cells. For selected
449
450 188 strains, validation of bacterial adhesion was performed by using electron microscopy as
451
452 189 routinely done (Tareb et al., 2013).

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455 190

456 191 *2.5 Human serum bactericidal assay*

458
459 192 To determine the sensitivity of GNB strains to human serum, human serum type male,
460
461 193 blood group: AB, HIV negative (Biowest, Nauville-France) was used. The bacterial strains were
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463 194 cultivated overnight in TSB under shaking at 30 or 37°C, depending on the strain. GNB strains
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465 195 and negative/positive control strains (see below) were added to 50% human serum solution to
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467 196 obtain the initial cell density of 10^5 cells per mL (Jankowski et al., 1996). Each mixture was
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475 197 separated in two aliquots, one was plated onto Tryptic Soy Agar (TSA) in Petri plate and was
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477 198 incubated at 30 or 37°C according to the tested strain. The other one was placed in a water bath
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479 199 for 3 hours at 37°C then plated onto TSA and incubated at 30 or 37°C. The results were
480
481 200 validated by using *Hafnia alvei* 56.85 (resistant strain) as a positive control and *Hafnia alvei*
482
483 201 31.86 (sensitive strain) as a negative control (Jankowski et al., 1996).
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488 203 *2.6 Pathogenicity evaluation:*
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490 204 *Galleria mellonella* larvae were grown in medium containing 50.6% wheat flour, 19%
491
492 205 honey, 19% glycerol, 7.7% brewer's yeast and 3.7% bee hive wax. All bacterial strains were
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494 206 grown as mentioned above, washed three times and cell concentrations were adjusted to $\sim 10^{10}$
495
496 207 cells/mL of PBS by optical density (OD 600 nm) determination using pre-enumeration data for
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498 208 each strain. The two non-pathogenic *Escherichia coli* K12 and *E. coli* Nissle 1917 strains were
499
500 209 used as safe controls. Additionally, as above, *E. coli* C267, O157: H7 (*stx1*-, *stx2*-, *eaeA*+) was
501
502 210 also used because of the presence of an adherence protein (intimin coding gene *eaeA*). *Galleria*
503
504 211 *mellonella* larvae, in groups of ten, were inoculated by injection into the haemocoel through the
505
506 212 last pro-leg by using a sterile syringe (Needle: 0.33x 12mm; 0.3mL U-100 insulin- TERUMO,
507
508 213 Belgium) with a needle diameter of 0.33 mm. Ten μ l of sterile PBS (negative control) and 10
509
510 214 μ l of a dose series corresponding to successive 10-fold dilutions of bacterial cell suspensions
511
512 215 (ranging from 2 to 10 logs) were injected. After inoculation, larvae were placed in sterile Petri
513
514 216 dishes and incubated at 30°C for 72 h and up to the moth stage (10-15 days). Mortality rate was
515
516 217 assessed by the lack of movement of larvae in response to stimulation and observation of
517
518 218 concomitant melanization of the cuticle. LD 50 (cfu/g of larvae) was estimated at 72 h in two
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520 219 independent assays as the geometric mean of the doses for which the first 100% mortality and
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522 220 0% mortality were found (Lorke, 1983).
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222 2.7 Statistical analysis

223 Principal Component Analysis (PCA) using Pearson correlation (n) method to determine the
224 correlation between different variables was carried out by using XLSTAT 2014.5.03 program.

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226 **3. Results and Discussion**

227 3.1 Growth Assessment of Gram negative bacterial species in different conditions related to the
228 human gastro intestinal tract (GIT) environment

229 3.1.1 Growth at 37°C

230 The ability to grow at 37°C under anaerobic conditions was first tested on Petri dishes
231 as it is an easy way to screen bacteria in order to evaluate their potential to survive in human
232 body temperature conditions. All strains grew at 25°C in aerobic conditions. At 37°C under
233 aerobic conditions, only one strain *Alcaligenes faecalis* 904 did not grow. Twelve out of the 20
234 selected isolates (60%) were able to grow at 37°C under anaerobic conditions (Table 1). As
235 expected most of the tested *Enterobacteriaceae* (e.g. *Citrobacter*, *Klebsiella*, *Morganella*,
236 *Proteus* or *Hafnia alvei*) were able to grow in these conditions, which is in accordance with
237 their general physiological characteristics. Surprisingly, some *Enterobacteriaceae* (namely,
238 *Hafnia paralvei* 920, *Proteus sp* UCMA 3779 (close to *P. hauseri*)) did not grow in these
239 conditions. Growth of *Pseudomonas sp.* depended on the considered strain, and it was positive
240 for *Pseudomonas putida* CV30.6 and negative for *Pseudomonas putida* VRBG37.3.
241 Interestingly, other GNB (*i.e.* strains belonging to the *Acinetobacter*, *Alcaligenes*,
242 *Chryseobacterium*, *Halomonas*, *Pseudomonas*, *Psychrobacter*, *Sphingobacterium* and
243 *Stenotrophomonas* genera) showed variable growth in these conditions and were not all able to
244 withstand anaerobic conditions. However, a limitation of this test was that it was performed in
245 laboratory conditions with pure cultures at a relatively neutral pH without bile salts, these
246 conditions being different from *in vivo* conditions encountered in the GIT.

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592
593 247 3.1.2 Gastric and gastrointestinal stress simulation
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595 248 To get closer to *in vivo* conditions, survival of 20 GNB dairy strains was evaluated
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597 249 successively in simulated gastric fluids and gastrointestinal stress environments over time, in
598
599 250 the presence or absence of milk (to mimic ingestion of a dairy product), *in vitro*. For all strains,
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601 251 growth data after 3 h gastric stress and 28 h gastrointestinal stress at 37°C were plotted together
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603 252 and are presented in Table 2. Additionally, changes in counts for four strains representative of
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605 253 the observed behaviours during the 31h gastric and gastrointestinal stress simulation are
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607 254 presented in Figures 1 A to D.
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611 255 The effects of *in vitro* simulated gastric stress were assessed over a 3 h period, for the
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613 256 20 selected GNB dairy isolates, in the presence of gastric juices (containing 500 U/l pepsin),
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615 257 and at pH 2 and pH 4, supplemented or not with milk. A good survival, corresponding to stable
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617 258 or increased population counts, was observed for the majority of the 20 GNB isolates in gastric
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619 259 juices initially adjusted to pH 4, both in the presence or absence of milk, except for *Alcaligenes*
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621 260 *faecalis* 904 and to a lesser extent for *Pseudomonas putida* VRBG37.3, and in GM-milk for
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623 261 *Halomonas venusta/alkaliphila/hydrothermas* 4C1A and *Sphingobacterium* sp. PCAi F2.5
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625 262 (Table 2). At pH 2.0 in the absence of milk, a decrease in survival for the majority (14 strains)
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627 263 of strains was reported. Six strains actually grew or presented relatively stable populations in
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629 264 these conditions: *Acinetobacter* sp. PCAi E6.10, *Hafnia paralvei* 920, *Halomonas*
630
631 265 *venusta/alkaliphila/hydrothermas* 4C1A (Figure 1A), *Pantoea agglomerans* Q6.3, *Providencia*
632
633 266 *heimbachae* GR4, and *Sphingobacterium faecium* F2.5. In the presence of milk, a decrease in
634
635 267 survival was also mainly observed for 13 strains. In these conditions, populations remained
636
637 268 relatively stable for only 7 strains ($\log(N/N_0) > -0.5$): *Chryseobacterium bovis* Pi18 (Figure
638
639 269 1B), *Hafnia paralvei* 920, *Halomonas venusta/alkaliphila/hydrothermalis* 4C1A (Figure 1A),
640
641 270 *Halomonas* sp. B39, *Morganella morganii* 3A2A, *Providencia heimbachae* GR4 and
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643 271 *Pseudomonas putida* CV30.6 (Table 2).
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652 272 These results showed that under simulated gastric stress conditions (no bile salts added),
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654 273 pH 2 had a different effect on growth than pH 4; presence of milk had only a slight impact on
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656 274 growth for both tested pH values.

658 275 The effects of an additional 28 h simulated gastrointestinal stress (with or without 1.5 g/L
659
660 276 bile salts) after 3 h gastric stress treatment, at pH 2 or pH 4, on the viability of the 20 GNB
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662 277 dairy isolates was then evaluated at neutral pH (pH 6.5). In all the tested conditions, no or low
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664 278 survival was observed for *Alcaligenes faecalis* 904 while two other strains exhibited good
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666 279 survival in all conditions as observed by an increase in population counts or re-growth during
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668 280 the simulated stress (i.e. *Halomonas* sp B39, *Panteoa agglomerans* PCA Q6.3). Surprisingly,
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670 281 bile salts (studied concentration was 1.5 g/L), that are normally encountered during
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672 282 gastrointestinal stress, did not modify strain survival or had only slight effects when compared
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674 283 to the same conditions without bile salts (for example among others, *Chryseobacterium bovis*
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676 284 Pi18 (Figure 1B) or *Pseudomonas putida* VRBG 37.3).

679 285 No or low survival of a large number of strains was observed after a pH 2.0 gastric fluids
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681 286 treatment regardless of the conditions encountered in the simulated gastrointestinal tract. No or
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683 287 low survival was observed for 7 strains at pH 2 , contrary to a high growth observed at pH 4 for
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685 288 these strains. This was the case for *Hafnia alvei* (Biogroup 1) B16, *Proteus* sp. (close to *P.*
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687 289 *hauseri*) UCMA 3779 (Figure 1C), *Pseudomonas putida* CV 30.6, *Pseudomonas stutzeri*
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689 290 UCMA 3883, and *Psychrobacter celer* 91. Survival in the simulated gastrointestinal tract
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691 291 containing milk was better for some strains: *Hafnia paralvei* 920, *Halomonas* sp. B39 and
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693 292 *Proteus* sp. (close to *P. hauseri*) UCMA 3780, suggesting a protective effect on the overall
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695 293 survival in GIM-media of these dairy isolates.

698 294 These results showed that the determinant role of initial pH persisted while bile salts
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700 295 concentration had a negligible effect on growth; the addition of milk rarely showed a protective
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702 296 survival effect for the tested GNB strains at both pHs after the total of 31 h of incubation.
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711 297 Noteworthy, the simulated TGI stress conditions could also potentially lead to a viable but non-
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713 298 culturable state for some cells and under some conditions, thus potentially underestimating
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715 299 viable cell counts. This state has been previously observed for some foodborne pathogens in
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718 300 environmentally limiting conditions during food processing and conservation such as drastic
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720 301 temperatures or the use of preservatives (Zhao et al, 2017).

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722 302 The protective survival effect of food in gastrointestinal simulated media has already
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724 303 been observed for *Bacillus cereus* (milk media) (Clavel et al., 2007), *Bifidobacterium* (soymilk)
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726 304 (Shimakawa et al., 2003) and *Lactobacillus curvatus* (meat based medium) (Gänzle et al., 1999)
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728 305 and is often linked to food components such as proteins and fats. Additionally, in the intestinal
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730 306 tract, bile reacts with cell membrane phospholipids and proteins and disrupts cellular
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732 307 homeostasis (Begley et al., 2005). However, in this study, the presence of bile salts in the
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734 308 intestinal media only had an effect on a limited number of strains and low survival was rather
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736 309 due to the acidic environment encountered in the gastric fluids simulation before the
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738 310 gastrointestinal stress. High variability in bile salt tolerance has also been previously observed
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740 311 for GPB such as lactic acid bacteria and *Listeria monocytogenes* (Begley et al., 2005; Chateau
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742 312 et al., 1994; Hyronimus et al., 2000). Finally, some species including those belonging to
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744 313 *Chryseobacterium* sp., *Proteus* sp., *Halomonas* sp. and *Psychrobacter* sp., recently identified
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746 314 as corresponding to the most frequent surface and/or core genera of French dairy products
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748 315 (cheeses and milk, (Dugat-Bony et al., 2016)) did not survive well in the simulated gastric and
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750 316 gastrointestinal media used in this study.

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755 318 *3.2 Adhesion analyses*

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758 319 The observed adhesion capacities are presented in Table 3 as a mean of two biological
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760 320 replicates. Results for all tested strains varied from 5 to 363 bacterial cells per 100 Caco-2 cells.
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762 321 Value for attachment of *Lactobacillus rhamnosus* GG to Caco-2 cells were similar to that
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770 322 previously published (Gopal et al., 2001), thus confirming the validity of the present results.
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772 323 The results for adherence of the two Gram negative species used as safe control strains were
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774 324 low (<100 cells) as well for the control *E. coli* O157: H7 C267 (*stx1*-, *stx2*-, *eae*+) and twelve
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776 325 other strains. The other remaining eight strains exhibited attachment > 100 cells and the most
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778 326 adherent strain was *Halomonas venusta/alkaliphila/hydrothermalis* 4C1A with 363 cells per
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780 327 100 Caco-2 cells, followed by 200, 152, 145, 141, 138, 114, 100 microbial units for *Alcaligenes*
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782 328 *faecalis* 904, *Proteus sp* (close to *P. hauseri*) UCMA 3779, *Psychrobacter celer* 91,
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784 329 *Pseudomonas group putida* VRBG 37.3, *Klebsiella oxytoca* 927, *Pseudomonas stutzeri* UCMA
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786 330 3883 and *Citrobacter freundii* UCMA 4217, respectively. Adherence is a clue for probiotic
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788 331 potential but is also the first step leading to potential cytotoxicity (dos Santos et al., 2015;
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790 332 Pavlov et al., 2004; Pogačar et al., 2015). In the present study, adhesion capacity was overall
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792 333 low for most of the tested strains and it was lower than previously tested strains of the same
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794 334 species present in drinking water (Pavlov et al., 2004). The low level of adherence was also
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796 335 confirmed by scanning electron microscopy (data not shown).
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801 336 3.3 Bactericidal effect of human serum

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803 337 The strains were described as sensitive or resistant to human serum after incubation in
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805 338 the presence of 50% human serum for three hours in comparison to known resistant and
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807 339 sensitive control strains of *Hafnia alvei* (e.g. in Table 4). Only 5 out of the 20 strain subsets
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809 340 were sensitive, namely *Halomonas* B39, *Halomonas venusta/alkaliphila/hydrothermalis* 4C1A,
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811 341 *Pseudomonas group putida* VRBG 37.3, *Psychrobacter celer* 91 and *Sphingobacterium sp.*
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813 342 (close *S. faecium*) PCAi F2.5 (Table 3). Most of the studied strains showed resistance against
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815 343 human serum as previously reported for GNB like *Acinetobacter sp.* (King et al., 2009). Serum
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817 344 resistance is often related to capsules or lipopolysaccharides which can protect the bacteria from
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819 345 entrance of the bactericidal compounds, but lipopolysaccharides are not solely responsible for
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821 346 resistance (Wand et al., 2013). The complement system is a series of serum proteins interacting
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829 347 in a regulated sequence that could lead to bacterial cell death (Morgan et al., 2000). The
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831 348 resistance mechanism could be due to a bacterial surface protein which binds the human factor
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833 349 H (FH), and thereby inhibits complement deposition on the bacterial surface (Quin et al., 2006).
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836 350 *3.4 Virulence of bacteria to Galleria mellonella*

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839 351 The 20 selected GNB and four control strains were tested for virulence in *G. mellonella*
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841 352 (Table 3). All PBS-injected control larvae grew until the moth stage. Control strains, *E. coli*
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843 353 Nissle 1917, *E. coli* K12 ATCC 10798, and *Lactobacillus rhamnosus* GG had LD 50 from
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845 354 around 10^7 to $>10^8$, respectively. This range was considered as the reference non-toxic range,
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847 355 since it was obtained for the three safe control strains. Thirteen GNB strains showed no
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849 356 virulence score within this range. Among them *Hafnia alvei* (biogroup 1) B16 was found safe
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851 357 which is reassuring as strains from this species are used as commercial ripening cultures for
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853 358 many cheeses (Irlinger et al., 2012). Three strains, including *K. oxytoca* 927, *P. stutzeri* UCMA
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855 359 3883, *Sphingobacterium* sp (close to *S. faecium*) PCAi F2.5 had intermediate LD 50 around 6.5
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857 360 10^6 as observed for *E. coli* O157:H7 (*stx1-*, *stx2-*, *eae+*) C267 which was used as a control. Two
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859 361 strains, *H. paralvei* 920 and *Proteus* sp (close to *P. hauseri*) UCMA 3780, were toxic to larvae
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861 362 with a lower LD 50 around 10^5 . Two other strains were even more virulent, with LD50 value
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863 363 lower than 10^4 , and corresponded to *Providencia heimbachae* GR4 and *Morganella morganii*
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865 364 3A2A.
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868 365 Assuming that *G. mellonella* larvae injection experiments are commonly used to detect
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870 366 bacterial strains presenting virulence factors and that the virulence of many pathogens is similar
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872 367 in wax moth larvae and mammals, including Humans (Desbois and Coote, 2012), it was
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874 368 deduced that only few strains contained efficient virulent factors and could act as direct or
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876 369 indirect pathogens. In the literature, a recent study showed that *Galleria mellonella* can also
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878 370 provide significant insights into virulence mechanisms and that this can be applied to the study
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880 371 of opportunistic human pathogens (Wand et al., 2013). Virulence clearly depended here on the
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888 372 considered strain but not on its origin as two *Proteus* sp. (close to *P. hauseri*) strains, 3779 and
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890 373 3780, having the same origin, were very different in their virulence towards the wax moth
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892 374 larvae. Only *Proteus* sp. (close to *P. hauseri*) UCMA 3780, *H. paralvei* 920, and even more *M.*
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894 375 *morganii* 3A2A and *P. heimbachae* GR4 strains required the lowest concentrations to kill larvae
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896 376 and can be considered as harbouring efficient virulence factors. Overall, the majority of the
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898 377 strains tested for virulence in *Galleria mellonella* larvae were safe for this organism when
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900 378 compared with the three control strains known to be safe for use in Humans through oral
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902 379 absorption.
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907 381 3.5 Data analysis for safety assessment of GNB associated with traditional French cheeses

909 382 A compilation of selected results obtained in the present work (Table S1) provides a global
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911 383 view about main safety characteristics of the tested GNB during simulated GIT transit after oral
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913 384 ingestion of milk products and their potential virulence. In this table, the individual strains were
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915 385 classified from safe to virulent based on the results obtained using the insect model *Galleria*
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917 386 *mellonella* larvae. Then, *in vitro* results are presented by following the GIT transit progression
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919 387 described in Figure 2 for cheese ingestion.
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922 388 From the *in vivo* data, it was concluded that *Proteus* sp (close to *P. hauseri*) UCMA 3780, *M.*
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924 389 *morganii* 3A2A, *H. paralvei* 920 and *P. heimbachae* GR4 strains presented some efficient
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926 390 virulence factors. Among these four strains, *M. morganii* 3A2A and *H. paralvei* 920 survived
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928 391 well in aerobic simulated gastric conditions (with milk and bile salts) while the two others
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930 392 survived to a lesser extent. Only one strain, *H. paralvei* 920, did not grow at 37°C under
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932 393 anaerobic conditions. PCA analysis (Figure S2) of the quantitative results given in Table S1,
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934 394 for pH 2 as primary gastric stress, showed some negative correlation ($r = -0.42$) between LD50
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936 395 in wax moth larvae and survival in GIM-milk at pH 6.5 with bile salt (1.5g/L). So, the more
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938 396 sensitive the strain to pH 2 (Figure S2A), the higher the value of LD50 (=the less toxic it was);
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946 397 at pH4 (Figure S2B), this negative correlation value was weaker ($r = -0.29$) and it could be
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949 398 explained by a lower number of strains sensitive to this less aggressive pH. Furthermore, at
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951 399 pH4, although adherence to Caco-2 cells and survival were opposed on the F1 axis, no negative
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954 400 correlation appeared ($r = -0.21$) between them. Qualitative factors were not specifically
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956 401 associated with any of the quantitative factors studied in Table S1.
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959 403 *3.6 Safety assessment of GNB strains belonging to genera/species of strains assessed in this*
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962 404 *study.*

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964 405 To assess the safety status of GNB strains of dairy origin studied here, a bibliographic
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966 406 review on the potential human pathogenicity of strains belonging to the corresponding species,
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968 407 was conducted and is listed in Table S3. Four bacteria, namely *Chryseobacterium* sp.,
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970 408 *Psychrobacter celer*, *Sphingobacterium* sp., and *Stenotrophomonas maltophilia*, were so far
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972 409 never reported in any clinical cases. Only individual clinical cases were reported for the GNB
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974 410 species studied in Table S3 but not associated with food vectors. The only one exception was
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976 411 for *Proteus* sp. that caused biogenic amine (BA) related intoxications (after eating BA
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978 412 contaminated fish). BA could pose a safety risk for human health. Fortunately, the
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980 413 corresponding clinical effect appeared to be very moderate except in rare cases and it could
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982 414 explain that there is no regulation for BA in food, except for histamine levels in fish products.
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984 415 Furthermore, a study (Delbès-Paus et al., 2012) involving eleven strains used *in vitro* in the
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986 416 present study, reported that only negligible biogenic amine amounts were produced *in situ* (in
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988 417 model cheeses), contrary to BA production observed *in vitro* by Coton et al., (2012).
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990 418 Nevertheless, as BA production is a strain-dependant trait, strain effect and cheese type must
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992 419 also be considered.
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998 421 **4. General discussion and conclusion**

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1006 422 The safety status as well as contribution of most GNB to the cheese process is poorly
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1008 423 documented. In previous years, GNB were classically considered as indicators of hygienic
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1010 424 problems (Bockelmann et al., 2005; Tornadijo et al., 1993) and responsible for defects in cheese
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1012 425 texture and flavor due to the production of extracellular proteolytic and lipolytic enzymes
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1014 426 (Amato et al., 2012; Martins et al., 2006). For example, dairy related *Pseudomonas* spp. strains
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1016 427 have been shown to produce volatile compounds such as ethyl esters and alcohols that may
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1018 428 negatively affect cheese sensory characteristics (Arslan et al., 2011; Morales et al., 2005). The
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1020 429 idea that GNB species can be normal and interesting elements of cheese microbiota is more
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1022 430 recent (Larpin-Laborde et al., 2011). In this sense, for example, *Proteus* sp. (close to *P. hauseri*)
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1024 431 and *Psychrobacter* sp. have been shown to significantly contribute to flavor production in smear
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1026 432 cheeses (Deetae et al., 2007; Irlinger et al., 2012) raising questions about the potential beneficial
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1028 433 effects of GNB in general. Recently, it was confirmed that some GNB strains have interesting
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1030 434 specific technological properties (Schmitz-Esser et al., 2018).
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1034 435 In this context, to delve deeper into the knowledge about GNB, it was necessary to
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1036 436 assess the safety status of these species. The present study was designed to evaluate the potential
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1038 437 health risks, if any, associated with the consumption of GNB present in cheeses (and coming
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1040 438 from raw milk, milk, or the milking environment), in simulated body conditions. However, risk
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1042 439 analysis is complex. You need to know, successively, the danger and its characteristics, the
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1044 440 exposure level and to set a safe threshold value, then, and only in that case, risk assessment can
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1046 441 be performed. In this study, from the present data and by using a general exposure context, only
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1048 442 part of risk analysis (i.e. the qualification of the danger) was done through an *in vivo* study of
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1050 443 pathogenicity in *Galleria mellonella* larvae and by using *in vitro* determinations of bacterial
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1052 444 growth in the simulated GIT, on Caco-2 cells and in the presence of human serum. The presence
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1054 445 of virulent factors was suggested here for some specific GNB strains but their virulence in human
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446 gut also depends on their active dose which is modulated by the effect of different gut barriers,
447 as reported in Figure 2.

448 Data from human consumption can however be considered. In fact, the safety status of
449 cheese in healthy consumers has been recognized for a long time, despite or thanks to microbial
450 strain diversity and microbial interactions in the cheese microbiota that evolves during
451 fermentation and ripening (Imran et al., 2012). Although cheeses have been implicated in 7.8%
452 of strongly evidenced foodborne outbreaks due to zoonotic agents in the EU (European Food
453 Safety Authority and European Centre for Disease Prevention and Control, 2016; Imran et al.,
454 2012), none of the GNB strains studied here (nor other strains belonging to the same species
455 than those assessed in this study) have been determined to be responsible for any known
456 foodborne outbreaks associated to cheeses. This was also confirmed by our literature analysis
457 described in Table S3. However, two key food risk factors (potential antibiotic resistances and
458 biogenic amine (BA) production, see Table S1) can modulate GNB safety. Assessment of these
459 traits has already been discussed for the 20 selected strains as well as some others (Coton et al.,
460 2012). Particularly of interest is the variable antibiotic resistance profiles observed among
461 certain GNB strains since their genetic material may harbour antibiotic resistance associated
462 genes that may be potentially transferable. Additionally, the presence of potentially
463 transferable virulence factors in cheese GNB strains must also be underlined.

464 Up until now, the absence of cheese foodborne outbreaks, might be explained by the positive
465 bioactive impact of the cheese microbial community, as previously demonstrated in a model
466 cheese ecosystem against *Listeria monocytogenes* (Imran et al., 2013). Additionally, it was
467 also recently shown that *Hafnia alvei* B16 had an inhibitory effect in a model cheese
468 ecosystem against *Escherichia coli* O26:H11 (Callon et al., 2016; Delbès-Paus et al., 2013).
469 Multiple dynamic interactions between GIT microbiota, cheese matrix and its initial
470 associated microbiota (detailed in Figure 2) must be taken into account. Among these barriers,

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1123
1124 471 and of special importance is the gut microbiota which was shown to play a crucial role for
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1126 472 protection against some pathogenic bacteria (Ubeda et al., 2017). This dense resident
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1128 473 microbial community, referred as the commensal microbiota, has a major function of
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1130 474 protection against colonization of pathogens and this ability to restrain pathogen growth
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1132
1133 475 include competitive metabolic interaction, localization to intestinal niches, and induction of
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1135 476 host immune responses (Kumada et al., 2013).

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1137 477 The impact of the dairy matrix on microbial interactions can also lead to changes in
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1139 478 functionality such as a means to combat against pathogens as already described (Imran et al.,
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1141 479 2012; Imran and Desmasures, 2015). In very recent scientific reports, it has been demonstrated
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1143 480 that the dairy matrix also has a significant impact on the survival and immunostimulant ability
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1145 481 (treatment of Inflammatory Bowel Diseases) of microbiota in a model gastrointestinal tract and
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1147 482 mouse model (Adouard et al., 2016; Foligné et al., 2016; Plé et al., 2015). The role of biliary
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1149 483 salts to prevent microbial adherence (Begley et al., 2005) was also recently confirmed (Sanchez
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1151 484 et al., 2016). The role of metabolites appearing during digestion must also be detailed.

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1153
1154 485 *In silico* studies could of help to complete our understanding of these interactions, but
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1156 486 it is missing for the GNB studied here. Till now only the genome sequence of several other
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1158 487 cheese-related GNB, which include the strain *P. heimbachae* GR4, was determined in a
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1160 488 subsequent study (Almeida et al., 2014). It indicated that this strain, studied in this work, is not
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1162 489 closely related to the reference strain; an adaptation to the dairy environment could be supposed.
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1164 490 For the future, *in vivo* approaches using wax worm could be further combined with *in silico*
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1166 491 approaches to seek for pathogenicity factors and their potential mobility, in the genome
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1168 492 sequence of these strains.

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1171 493 To conclude, presumption of safety of most GNB strains studied here was successfully
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1173 494 established, while some could harbour virulence factors. These factors (including some
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1175 495 antibiotic resistances) could be considered as a direct or indirect risk when dissemination to

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496 other bacteria of the human gut occurred. The role of multiple dynamic interactions between
497 cheese microbiota and GIT barriers including gut microbiota could be key factors explaining
498 safe consumption of the corresponding cheeses. However, the present study, which was partly
499 limited to *in vitro* studies of selected individual strains, cannot replace *in vivo* studies involving
500 actual cheese and gut microbiota ecosystem. There is always a gap between microbial behavior
501 in *in vitro* and *in vivo* conditions, especially taking into account the complexity of the
502 considered systems (cheese, alimentary bolus, human body). The present approach is a first step
503 for future studies aiming at elucidating the role of each microbial component (GNB or others)
504 within such complex microbial communities.

505 However, the present study, which was partly limited to *in vitro* studies of selected
506 individual strains, cannot replace *in vivo* studies involving actual cheese and gut microbiota
507 ecosystem.

508
509 **Acknowledgements**

510 This work was funded by the French “Agence Nationale de la Recherche” in the framework of
511 the ANR Gramme project n° ANR-07-PNRA-010. The authors would like to thank Valérie Stahl
512 for supplying GNB strains from the Aerial collection, Dr Ulrich Sonnerborn from Ardeypharm
513 (Germany GmbH) for supplying *E. coli* Nissle 1917, Pr Vernozy-Rozand (Biomérieux, Lyon)
514 for supplying *E. coli*.C267 strain. Authors also acknowledged the technical support from Dr.
515 Marion Bernardeau (Unite MILA), Dr. Didier Goux (CMABio) and Marie Joel Jacob at the
516 University of Caen (France). M. Imran Ph D scholarship at University of Caen Normandy was
517 funded by the Higher Education Commission of Pakistan (HEC).

518
519 **Declarations of interest conflict:** None

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Table 1. Identification of representative Gram-negative bacterial strains of dairy origin used in this study. Growth in anaerobic or aerobic conditions at 37°C was assessed in the present study.

Species	Code	Dairy source	Growth in anaerobic/aerobic condition at 37°C*
<i>Acinetobacter</i> close to <i>A. genospecies 3</i>	PCAi E6.10	Milk from Salers cow	-/+
<i>Alcaligenes faecalis</i>	904	Smear-ripened cheese	-/-
<i>Chryseobacterium</i> close to <i>C. bovis</i>	Pi18	Uncooked pressed cheese	+/+
<i>Citrobacter freundii</i>	UCMA 4217	Smear-ripened cheese	+/+
<i>Hafnia alvei</i> (biogroup 1)	B16	Smear-ripened cheese	+/+
<i>Hafnia paralvei</i>	920	Smear-ripened cheese	-/+
<i>Halomonas venusta/ alkaliphila/ hydrothermalis</i>	4C1A	Smear-ripened cheese	+/+
<i>Halomonas</i> sp. nov.	B39	Smear-ripened cheese	-/+
<i>Klebsiella oxytoca</i>	927	Smear-ripened cheese	+/+
<i>Morganella morganii</i>	3A2A	Smear-ripened cheese	+/+
<i>Pantoea agglomerans</i>	PCA Q6.3	Milk	+/+
<i>Proteus</i> sp. close to <i>P. hauseri</i>	UCMA 3779	Smear-ripened cheese	-/+
<i>Proteus</i> sp. close to <i>P. hauseri</i>	UCMA 3780	Smear-ripened cheese	+/+
<i>Providencia heimbachae</i>	GR4	Smear-ripened cheese	+/+
<i>Pseudomonas</i> group <i>putida</i>	VRBG37.3	Milk	+/+
<i>Pseudomonas</i> group <i>putida</i>	CV30.6	Milk	-/+
<i>Pseudomonas stutzeri</i>	UCMA 3883	Smear-ripened cheese	-/+
<i>Psychrobacter celer</i>	91	Mould-ripened soft cheese	-/+
<i>Sphingobacterium</i> sp. close to <i>S. faecium</i>	PCAi F2.5	Milk	+/+
<i>Stenotrophomonas maltophilia/rhizophila</i>	PCAi D6.5	Milk	+/+

*all strains grew at 25°C in aerobic conditions

Table 2. Survival of Gram-negative bacteria of dairy origin (inoculated at $N_0=10^6$ cfu/ml) in *in vitro* simulated conditions related to gastric media (GM) and gastrointestinal (GIM) stress. Results are presented as the log (N/N₀) where N is the number of CFU/ml after 3h gastric stress (GM-3) followed by 28h gastrointestinal stress simulation, with or without bile salts (GIM-28), corresponding to a total of 31 h of (GM+GIM) stress.

Gram negative bacterial strains	Bile g/L	pH 4.0 at T ₀				pH 2.0 at T ₀			
		GM-JB		GM-milk		GM-JB		GM-milk	
		Time (h) of exposure with pH change to 6.5 at T+3h							
		3	31	3	31	3	31	3	31
<i>Acinetobacter</i> (close to <i>A. genospecies 3</i>) PCAi E6.10	0	0.49	2.72	0.01	3.23	0.44	2.66	-5.74	-5.74
	1.5		3.01		2.62		0.73		-2.84
<i>Alcaligenes faecalis</i> 904	0	-5.20	-5.20	-4.81	-4.81	-3.30	-3.30	-5.20	-5.20
	1.5		-5.20		-4.81		-3.30		-5.20
<i>Chryseobacterium</i> (close to <i>C. bovis</i>) Pi18	0	0.22	1.15	0.06	-1.48	-2.24	-5.84	-0.17	-3.55
	1.5		2.57		-1.47		-5.84		-5.85
<i>Citrobacter freundii</i> UCMA 4217	0	-0.11	4.04	0.85	3.25	-5.55	-2.94	-5.85	-2.85
	1.5		3.91		2.95		-2.77		-2.64
<i>Hafnia alvei</i> (biogroup 1) B16	0	0.40	3.43	0.53	3.16	-5.90	-5.90	-6.06	-2.86
	1.5		3.53		3.08		-5.90		-2.99
<i>Hafnia paralvei</i> 920	0	-0.48	3.26	0.15	3.12	-0.32	-5.99	-0.11	2.56
	1.5		3.14		3.46		-5.99		2.95
<i>Halomonas venusta/alkaliphila/hydrothermas</i> 4C1A	0	0.65	3.10	-1.28	2.03	0.92	3.55	-0.39	-5.82
	1.5		3.23		1.73		3.62		-5.82
<i>Halomonas</i> sp. nov. B39	0	-0.10	3.91	0.22	3.64	-5.66	-1.03	-0.17	3.87
	1.5		3.89		3.40		-0.88		3.84
<i>Klebsiella oxytoca</i> 927	0	-0.62	3.86	-0.26	3.59	-3.60	-3.60	-5.55	0.95
	1.5		3.64		3.93		-3.60		1.24
<i>Morganella morganii</i> 3A2A	0	-0.29	2.75	-0.34	-0.13	-0.93	-6.24	-0.06	-6.12
	1.5		2.85		-0.07		-3.64		0.20
<i>Pantoea agglomerans</i> PCA Q6.3	0	0.40	3.37	0.23	3.17	0.51	3.50	-5.41	-0.94
	1.5		3.42		3.19		3.42		-0.93
<i>Proteus</i> sp. (close <i>P. hauseri</i>) UCMA 3779	0	-0.24	2.91	0.21	2.86	-6.00	-6.00	-6.70	-6.70
	1.5		3.47		2.15		-6.00		-6.70
<i>Proteus</i> sp. (close <i>P. hauseri</i>) UCMA 3780	0	-0.05	3.41	-0.54	-0.74	-5.83	-5.83	-1.39	2.00
	1.5		3.90		2.90		-5.83		2.17
<i>Providencia heimbachae</i> GR4	0	0.19	2.87	-0.17	0.86	0.58	3.09	-0.46	-2.97
	1.5		2.76		0.98		2.19		-2.85
<i>Pseudomonas</i> group <i>putida</i> VRBG37.3	0	-1.04	-5.45	-1.99	-5.59	-5.61	-3.31	-4.20	-4.20
	1.5		2.56		-3.29		-2.83		-4.20
<i>Pseudomonas</i> group <i>putida</i> CV30.6	0	0.38	3.44	-0.13	2.61	-6.38	-6.38	-0.32	-6.31
	1.5		3.37		3.01		-6.38		-6.31
<i>Pseudomonas stutzeri</i> UCMA 3883	0	0.68	3.97	0.16	3.30	-5.92	-5.92	-5.75	-5.75
	1.5		3.34		4.45		-5.92		-5.75
<i>Psychrobacter celer</i> 91	0	0.47	3.26	-0.16	3.05	-6.05	-6.05	-5.68	-5.68
	1.5		3.45		3.58		-6.05		-5.68
<i>Sphingobacterium</i> sp. (close to <i>S. faecium</i>) PCAi F2.5	0	0.20	3.56	-1.32	2.71	-0.36	3.90	-5.30	-2.15
	1.5		3.63		2.73		3.28		-1.74
<i>Stenotrophomonas maltophilia/rhizophila</i> PCAi D6.5	0	-0.23	4.29	-0.52	0.26	-4.15	-4.15	-5.14	-2.84
	1.5		3.93		-2.79		-4.15		-5.14

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Table 3. Adhesion to Caco-2 cells, sensitivity to human serum and increasing *in vivo* pathogenicity on wax moth worm of the tested Gram-negative bacteria of dairy origin.

Identification	No. bacterial cell adhering to 100 Caco-2*	Sensitivity to human serum	50% lethal dose at 72 h (CFU/g of larva*)
Control strains for safety			
<i>Lactobacillus rhamnosus</i> GG (Gram +)	200	nd	7.00 10 ⁸
<i>Escherichia coli</i> Nissle 1917	23	nd	1.70 10 ⁷
<i>Escherichia coli</i> K12 ATCC 10798	65	nd	3.16 10 ⁷
Strain with intimin			
<i>Escherichia coli</i> O157:H7 C267 (<i>eaec</i> ⁺ , <i>stx</i> ⁻)	35	nd	1.46 10 ⁶
Dairy strains			
<i>Chryseobacterium</i> sp (close to <i>C. bovis</i>) Pi18	73	R	3.71 10 ⁸
<i>Psychrobacter celer</i> 91	145	S	1.41 10 ⁸
<i>Alcaligenes faecalis</i> 904	200	R	9.83 10 ⁷
<i>Proteus</i> sp. (close to <i>P. hauseri</i>) UCMA 3779	58	R	9.78 10 ⁷
<i>Acinetobacter</i> sp. (close to <i>A. genospecies</i> 3) PCAi E6.10	33	R	8.82 10 ⁷
<i>Halomonas venusta/alkaliphila/hydrothermalis</i> 4C1A	363	S	6.06 10 ⁷
<i>Pantoea agglomerans</i> PCA Q6.3	63	R	5.91 10 ⁷
<i>Halomonas</i> sp. nov. B39	30	S	2.54 10 ⁷
<i>Citrobacter freundii</i> UCMA 4217	100	R	2.18 10 ⁷
<i>Pseudomonas</i> group <i>putida</i> VRBG 37.3	141	S	1.91 10 ⁷
<i>Stenotrophomonas maltophilia/rhizophila</i> PCAi D6.5	70	R	1.33 10 ⁷
<i>Pseudomonas</i> group <i>putida</i> CV30.6	5	R	1.25 10 ⁷
<i>Hafnia alvei</i> biogroup 1 B16	43	R	9.48 10 ⁶
<i>Pseudomonas stutzeri</i> UCMA 3883	114	R	6.57 10 ⁶
<i>Sphingobacterium</i> sp. (close to <i>S. faecium</i>) PCAi F2.5	45	S	6.57 10 ⁶
<i>Klebsiella oxytoca</i> 927	138	R	6.13 10 ⁶
<i>Hafnia paralvei</i> 920	36	R	2.80 10 ⁵
<i>Proteus</i> sp. (close to <i>P. hauseri</i>) UCMA 3780	152	R	9.63 10 ⁴
<i>Providencia heimbachae</i> GR4	49	R	< 1.6 10 ⁴
<i>Morganella morganii</i> 3A2A	54	R	< 1.16 10 ⁴

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nd: not determined, R: resistant, S: sensitive
*results are mean of two separate experiments

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Table 4. Impact of human serum on Gram-negative bacteria (GNB) strains after none and 3 hours contact time: examples of results for a sensitive strain (*Pseudomonas* group *putida* VRBG 37.3) and a resistant strain (*Chryseobacterium* sp. (close to *C. bovis*) Pi18).

Dilution	<i>Chryseobacterium</i> sp. (close to <i>C. bovis</i>) Pi18 (number of colonies)	<i>Pseudomonas putida</i> VRBG 37.3 (number of colonies)	Control sensitive (number of colonies)	Control Resistant (number of colonies)
At 0 hour				
10 ⁻¹	>1000	>1000	>1000	>1000
10 ⁻²	>1000	141	>1000	>1000
10 ⁻³	354	6	42	152
10 ⁻⁴	42	0	9	16
At 3rd hour				
10 ⁻¹	>1000	0	0	>1000
10 ⁻²	>1000	0	0	>1000
10 ⁻³	>1000	0	0	>1000
10 ⁻⁴	135	0	0	426

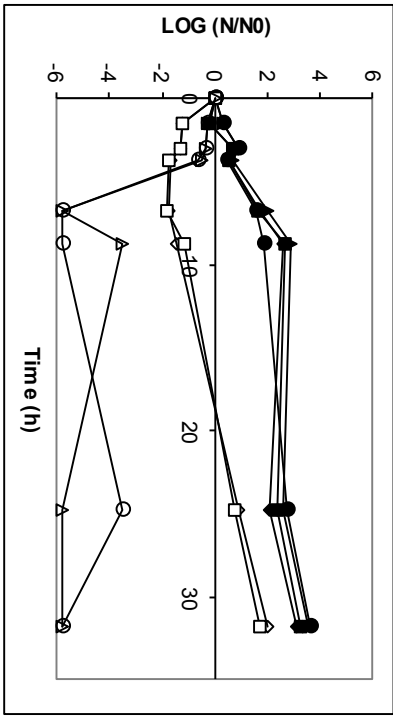
List of Figures Captions

Figure 1. Changes in counts of *Halomonas venusta/alkaliphila/hydrothermalis* 4C1A (A), *Chryseobacterium* sp. close to *C. bovis* Pi18 (B), and *Protetis* sp. (close *P. hauseri*) UCMA 3779 (C), during gastric (from T0h primary adjusted to pH 2 or 4 → T3h) and gastrointestinal stress (from T3h with addition of new media initially adjusted pH 6.5 → T31h), GIM-JB (closed symbols) or GIM-milk (open symbols) media primary adjusted to pH 4.0 with 0 g/l bile salts (◆, ◇) and 1.5 g/l bile salts (■, □) or primary adjusted to pH 2.0 with 0 g/l bile salts (▲, Δ) and 1.5 g/l bile salts (●, ○). Experimental replicate for these 4 representative strains gave similar results.

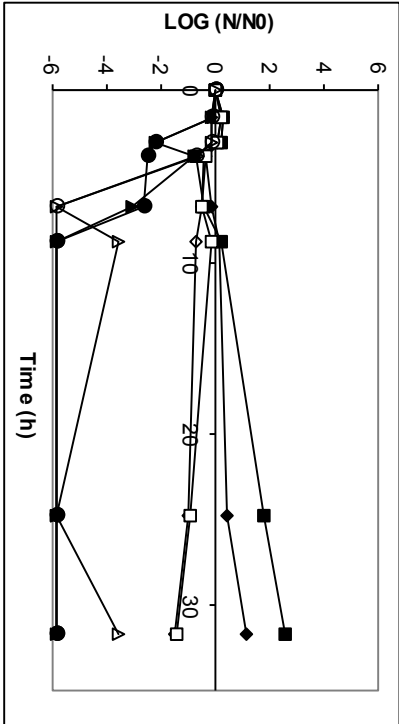
Figure 2: Scheme of main body barriers observed during human GIT transit of fermented cheese (1; A to F). For each barriers, beneficial (+) or adverse (-) effects involved in safety of ingested food during its GIT transit were highlighted (2; A to F).

Figure 1

A



B



C

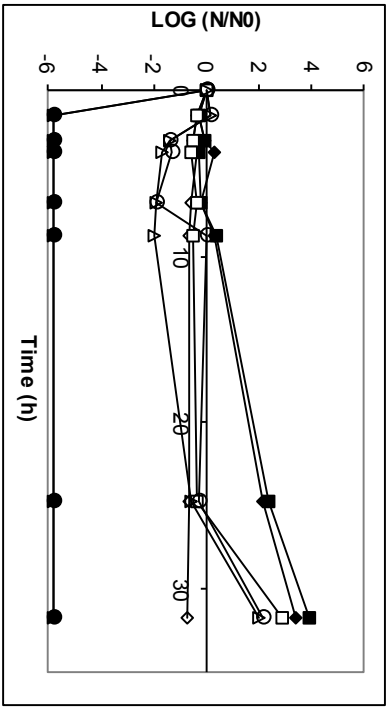
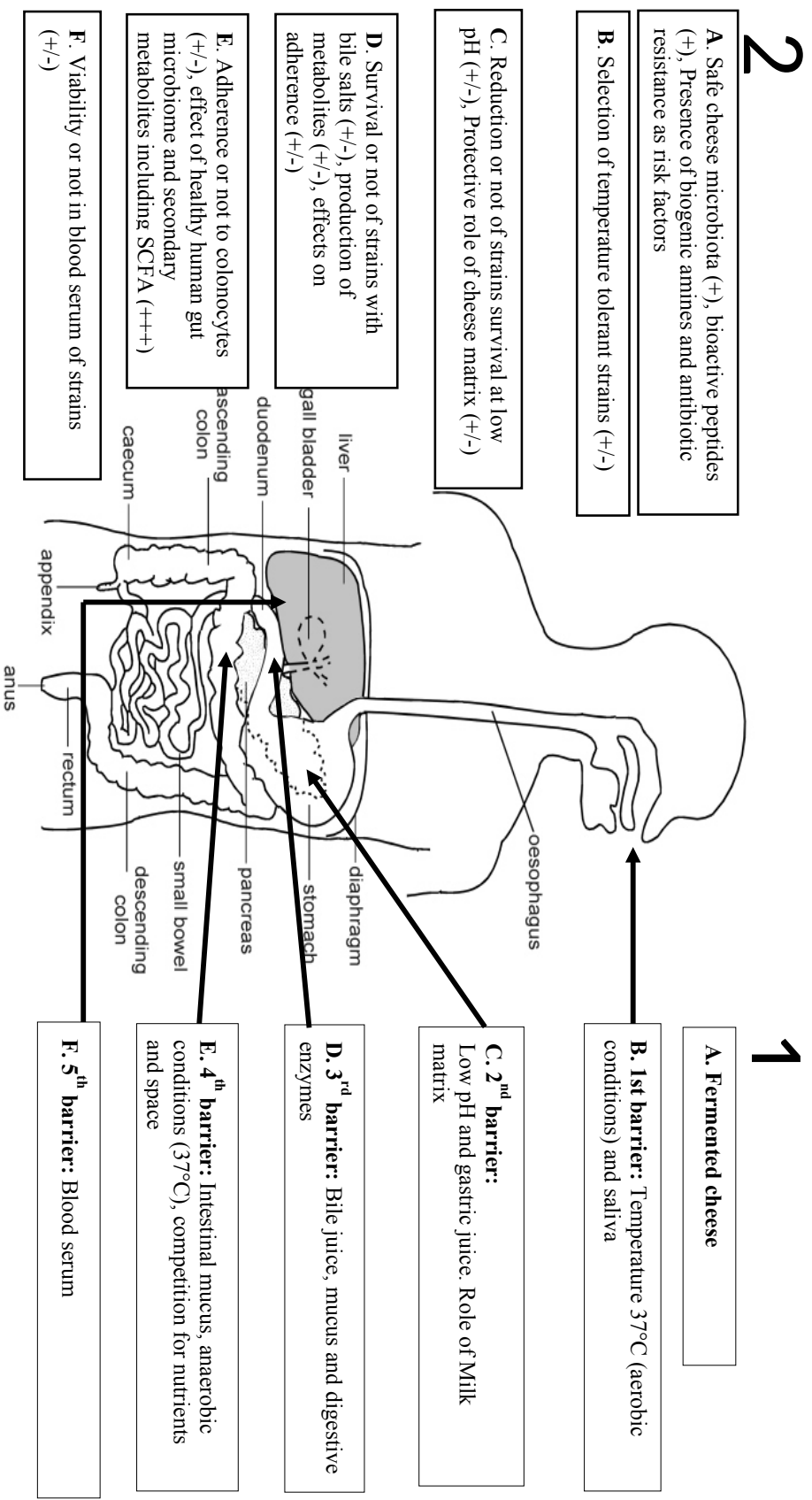


Figure 2



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Supplemental Table S1: Comprehensive view of the obtained safety data showing main characteristics of Gram negative bacteria of dairy origin *in vivo* and *in vitro* for each barrier points in simulated body GIT conditions.

Gram negative strains	LD 50 in wax moth worm model (72h; 30°C) (this study)	Anaerobic growth at 37°C (this study)	Survival (log N/N ₀) in GIM-milk at pH 6.5 with bile salt (1.5g/L) ¹ (this study)	Adherence to Caco-2 cells (this study)	Sensitivity to human serum ² (this study)	Antibiotic Resistance number (Coton et al, 2012)	Biogenic Amine production ³ (Coton et al, 2012)
<i>Chryseobacterium</i> sp. (close to <i>C. bovis</i>) P1i8	3.71 10 ⁸	+	-5.9/-1.5	73	R	15	-
<i>Psychrobacter celer</i> 91	1.41 10 ⁸	-	-5.7/+3.6	145	S	1	+
<i>Alcaligenes faecalis</i> 904	9.83 10 ⁷	-	-5.2/ -4.8	200	R	6	+
<i>Proteus</i> sp. (close to <i>P. hauseri</i>) UCMA 3779	9.78 10 ⁷	-	-6.7/+2.1	58	R	3	+
<i>Acinetobacter</i> sp. (close to <i>A. genospecies 3</i>) PCAi E6.10	8.82 10 ⁷	+	-2.8/+2.6	33	R	13	-
<i>Halomonas venusta/alkaliphila/hydrothermalis</i> 4C1A	6.06 10 ⁷	+	-5.8/+1.7	363	S	15	+
<i>Pantoea agglomerans</i> PCA Q6.3	5.91 10 ⁷	+	-0.9/+3.2	63	R	4	+
<i>Halomonas</i> sp. nov. B39	2.54 10 ⁷	-	+3.8/+3.4	30	S	0	+
<i>Citrobacter freundii</i> UCMA 4217	2.18 10 ⁷	+	-2.6/+2.9	100	R	13	+
<i>Pseudomonas putida</i> VRBG 37.3	1.91 10 ⁷	+	-4.2/-3.3	141	S	13	+
<i>Stenotrophomonas maltophilia/hizophila</i> PCAi D6.5	1.33 10 ⁷	+	-5.1/-2.8	70	R	22	+
<i>Pseudomonas</i> group <i>putida</i> CV30.6	1.25 10 ⁷	-	-6.3/+3	5	R	14	+
<i>Hafnia alvei</i> biogroup 1 B16	9.48 10 ⁶	+	-3/+3.1	43	R	11	+
<i>Pseudomonas stutzeri</i> UCMA 3883	6.57 10 ⁶	-	-5.8/+4.5	114	R	8	+
<i>Sphingobacterium</i> sp. (close to <i>S.faecium</i>) PCAi F2.5	6.57 10 ⁶	+	-1.7/+2.7	45	S	15	+
<i>Klebsiella oxytoca</i> 927	6.13 10 ⁶	+	+1.2/+3.9	138	R	22	+
<i>Hafnia parvhei</i> 920	2.80 10 ⁵	-	+3/+3.5	36	R	5	+
<i>Proteus</i> sp. (close to <i>P. hauseri</i>) UCMA 3780	9.63 10 ⁴	+	+2.2/+2.9	152	R	13	+
<i>Providencia heimbachae</i> GR4	<1.6 10 ⁴	+	-2.9/+1	49	R	15	+
<i>Morganella morganii</i> 3A2A	<1.16 10 ⁴	+	+0.2/-0.1	54	R	17	+

Initial pH for GM: pH 2/pH 4

² Resistance (R) or Sensitivity (S)

³ (+) : >100mg/L; (-) : <100mg/L

Supplemental Table S3: Bibliographical assessment of pathogenicity in Human for GNB strains belonging to genera/species of strains assessed in this study.

Species	Human clinical cases/food vector implication	References
<i>Acinetobacter</i> sp. (close to <i>A. genospecies 3</i>)	Yes/No	(Horrevorts et al., 1995) (Webster et al., 1999)
<i>Alcaligenes faecalis</i>	Yes/No	(Khokhar et al., 2002; Shimizu et al., 2002) (Bizet and Bizet, 1997; Ju et al., 2016; Khajuria et al., 2013; Pereira et al., 2000)
<i>Chryseobacterium</i> sp. (close to <i>C. bovis</i>)	No/No	(Hantisis-Zacharov et al., 2008; Iwai et al., 2012)
<i>Citrobacter freundii</i>	Yes/No	(Chen et al., 2008; Dervisoglu et al., 2008; Leski et al., 2016; Samonis et al., 2009)
<i>Hafnia alvei</i> biogroup 1	Yes/No	(Benito et al., 2008; Donato et al., 2008; Redondo et al., 2005; Rodriguez-Guardado et al., 2005)
<i>Hafnia paralvei</i>	Yes/No	(Abbott et al., 2011; Osuka et al., 2011)
<i>Halomonas venusta/alkaliphila/hydrothermalis</i>	Yes (fish bice)/No	(Berger et al., 2007; Stevens et al., 2009; von Graevenitz et al., 2000)
<i>Halomonas</i> sp.		(Berger et al., 2007; von Graevenitz et al., 2000)
<i>Klebsiella oxytoca</i>	Yes/No	(Bleich et al., 2008; Mohamed et al., 2016; Oishi et al., 2008; Shibasaki et al., 2016; Tsanaktisidis et al., 2003)
<i>Morganella morganii</i>	Yes/No	(Demiray et al., 2016; Fernandez Fuentes et al., 2014; Nitia et al., 2016; Salen and Eppes, 1997; Tsanaktisidis et al., 2003; Williams et al., 1983)
<i>Pantoea agglomerans</i>	Yes/No	(Bachmeyer et al., 2007; Cruz et al., 2007; Fernandez Fuentes et al., 2014; Uche, 2008)
<i>Proteus</i> sp. (close to <i>P. hauseri</i>)	Yes/Yes (Eating fish poisoned by biogenic amines)	(Cetre et al., 2005; Dybas et al., 2005; Fernandez-No et al., 2010; Nowakowska et al., 2004; Schaffer and Pearson, 2015)
<i>Providencia heimbachae</i>	Yes/No	(Ineid et al., 2016; Mohr O'Hara et al., 1999)
<i>Pseudomonas</i> group <i>putida</i>	Yes/No	(Carpenter et al., 2008; Lombardi et al., 2002; Miron et al., 2007; Yang et al., 1996; Zhang et al., 2012)
<i>Pseudomonas stutzeri</i>	Yes/No	(Carvalho-Assef et al., 2010; Lin et al., 2014; Miron et al., 2007; Wang et al., 2015)

<i>Psychrobacter celer</i>	No/No	(Yoon et al., 2005)
<i>Sphingobacterium</i> sp. (close to <i>S. faecium</i>)	No/No	(Mariko and Akira, 1992; Tronel et al., 2003)
<i>Stenotrophomonas maltophilia</i>	No/No	(Bin Abdulhak et al., 2009; Falagas et al., 2009; Liu et al., 2016; Spicuzza et al., 2009)

Annexe Table S3

References used in supplemental Table S3

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