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

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ABSTRACT

Twenty Gram-negative bacterial (GNB) strains were selected based on the biodiversity previously observed in French traditional cheeses and their safety was assessed considering various safety criteria. For the majority of tested GNB strains, only gastric stress at pH 2 (*vs* pH 4) resulted in low survival and no regrowth after an additional simulated gastro-intestinal stress. Presence of milk was shown to be rarely protective. The majority of strains was resistant to human serum and had a low level of adherence to Caco-2 cells. When tested for virulence in *Galleria mellonella* larvae, GNB strains had LD 50 values similar to that of safe controls. However, four strains, *Hafnia paralvei* 920, *Proteus* sp. (close to *P. hauseri*) UCMA 3780, *Providencia heimbachae* GR4, and *Morganella morganii* 3A2A were highly toxic to the larvae, which suggests the presence of potential virulent factors in these strains. Noteworthy, to our knowledge, no foodborne intoxication or outbreak has been reported so far for any of the GNB belonging to the genera/species associated with the tested strains. The role of multiple dynamic interactions between cheese microbiota and GIT barriers could be key factors explaining safe consumption of the corresponding cheeses.

1. Introduction

Cheese microbiota consists of diverse microorganisms, including yeasts, moulds, Gram-positive and -negative bacteria (Dugat-Bony et al., 2016; Gori et al., 2013; Imran et al., 2012; Irlinger and Mounier, 2009; Larpin-Laborde et al., 2011; Martín and Coton, 2017; Mounier et al., 2009, 2017; Wolfe et al., 2014). Microbial community diversity is important during cheese making as it is associated with cheese sensorial quality but can also contribute to ensure microbiological control and safety (Delbes et al., 2007; Delbès-Paus et al., 2012; Irlinger and Mounier, 2009). While literature data are abundant concerning yeasts/moulds and Gram-positive bacteria (GPB) in cheese, Gram-negative bacteria (GNB) have been seldomly studied. However, a wide diversity of GNB, found at relatively high levels in raw milk (around 3 to 4 Log CFU/mL), has been reported (Desmasures et al., 1997; Frétin et al., 2018; Kable et al., 2016). GNB usually represent from 18 to 60% of the bacteria isolated from the surface of European smear cheeses (Larpin-Laborde et al., 2011; Maoz et al., 2003; Montel et al., 2014; Mounier et al., 2005, 2017; Wolfe et al., 2014). Most of the microorganisms found in cheese originate from raw milk, (animal, milking machine, environment/air) (Desmasures et al., 1997; Frétin et al., 2018), processing steps, plant, transportation equipment, labor and cheese factory sources (Mounier et al., 2006). GNB present on the surface of the ripened soft cheese mainly belong to *Enterobacteriaceae, Moraxellaceae, Pseudoalteromonadaceae, Pseudomonadaceae, Sphingobacteriaceae* and *Vibrionaceae* families (Bockelmann et al., 2005; Chaves-Lopez et al., 2006; Maoz et al., 2003; Mounier et al., 2005; Tornadijo et al., 1993). Previous work, done between 2008 and 2010 aimed at studying GNB

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associated with French milk and corresponding cheeses. The obtained 173 isolates corresponded to at least 26 genera and 68 species, including potential new species. *Pseudomonas*, *Chryseobacterium*, *Enterobacter,* and *Stenotrophomonas* were the most frequent genera found in cheese core and milk samples, while *Proteus*, *Psychrobacter*, *Halomonas* and *Pseudomonas* were the most frequent genera isolated from cheese surface (Coton et al., 2012).

Some GNB species found in cheese were reported to include some non-foodborne strains associated with clinical cases (Delbès-Paus et al., 2012). Criteria to classify bacteria as human pathogens or non-pathogens depends on the presence or absence of virulence factors. Survival during gastro intestinal transit and then adhesion to enterocyte cell surfaces is often the first step in establishing potential bacterial disease. For extracellular pathogens, adhesion is a means to withstand mechanical cleaning. For intracellular pathogens, adhesion is often a prerequisite for invasion (dos Santos et al., 2015). The human adenocarcinoma cell line Caco-2, isolated from an adult human colon, which expresses several markers characteristic of normal small villi cells (Fogh et al., 1977; Pinto et al., 1983) has been extensively used to study bacterial adhesion mechanisms for pathogens and probiotic strains (Greene and Klaenhammer, 1994).

Another barrier in the human body against invading pathogens is the action of serum through a series of serum proteins interacting in a regulated sequential manner that eventually leads to bacterial death due to either lysis or opsonisation (Morgan et al., 2000). Furthermore, the safety status evaluation of a given microorganism must also be assessed by its actual pathogenicity on an animal model. Mammals have been used for a long time to evaluate microbial pathogen virulence but it is time consuming, labor intensive, and expensive in terms of purchasing animals, feeding, and housing. An alternative option is the use of an insect model (Kavanagh and Reeves, 2004) because the innate immune responses are similar. A useful model is *Galleria mellonella* (wax moth) larvae (Ramarao et al., 2012). It has been used to evaluate the pathogenicity of various GNB such as *Proteus mirabilis* (Morton et al., 1987), *Francisella tularensis* (Aperis et al., 2007), *Yersinia pseudotuberculosis* (Champion et al., 2009), *Stenotrophomonas maltophilia* (Nicoletti et al., 2011) and *Escherichia coli* (Walters and Ratcliffe, 1983). For example, *Pseudomonas aeruginosa* virulence or that of *Bacillus thuringiensis* and *Bacillus cereus* were correlated in *Galleria* larvae and in mice (Jander et al., 2000; Salamitou et al., 2000). In fact, the virulence of many pathogens is similar in wax moth larvae and mammals, including Humans (Desbois and Coote, 2012).

The present study is a continuation of the previous work reported by Coton et al. (2012) which focused on the diversity and potential risk factors (antibiotic resistance and biogenic amines production) of milk and cheese GNB isolates. It aimed at further evaluating the safety aspects of selected representative GNB strains from each genus identified in the cited study. Growth in conditions mimicking the gastrointestinal tract (GIT) transit, Caco 2 cell adhesion, survival in serum conditions and pathogenicity on insect larvae, were assessed to provide a more comprehensive view about the safety these GNB isolates.

2. Materials and methods

2.1. Strains and culture conditions

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

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

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

2.2. Growth at 37 °C

Each precultured strain (cf. §2.1) was isolated on three plates poured with Brain Heart Infusion agar (BHA, AES, France). For each strain, three plates were inoculated simultaneously: two plates were incubated at 37 °C respectively under aerobic and anaerobic (AnaeroGen Pack, Oxoid, France) conditions for 14 days and one plate was incubated at 25 °C under aerobic conditions. If growth appeared as expected in aerobiosis on at least one plate and for one temperature, results for growth/no growth at 37 °C under anaerobic conditions were considered.

2.3. Gastric and gastro-intestinal media and stress simulation

For gastro-intestinal stress simulation tests, bacterial strains were first precultured in J broth (JB) (5 g/L peptone, 15 g/L yeast extract, 2 g/L glucose, 3 g/L K_2HPO_4, adjusted to pH 7.2) (Clavel et al., 2004) and incubated at 25 °C for 24 h with shaking to obtain optimal growth. For all enumerations, bacteria were plated on J agar (JA) (JB supplemented with 15 g/L agar), incubated at 25 °C under aerobic conditions for 24 h.

Simulated gastric medium (GM) was prepared by adding one volume of a sterile (autoclaved 121 °C, 15 min) gastric electrolyte solution (Gänzle et al., 1999) containing 4.8 g/L NaCl, 1.56 g/L $NaHCO_3$, 2.2 g/L KCl and 0.22 g/L $CaCl_2$ to one volume of JB (GM-JB; autoclaved 121 °C, 15 min) or milk medium (100 g/L of half-skim milk powder; autoclaved 100 °C, 30 min) GM-milk (Clavel et al., 2004, 2007). After sterilization, the pH of the GM-JB or GM-milk media were adjusted to 2 or 4 with sterile 1 N HCl to simulate the acidic environment of human gastric fluids. Finally, media were supplemented with 500U/l of a filter sterilized (0.22 µm) pepsin (gastric juice enzyme) solution prepared in water (P6887; Sigma-Aldrich, France) just before use.

For gastric stress experiments, GM-JB and GM-milk media were inoculated with each strain at initial populations of $N_0 = 1.10^6$ CFU/ml and incubated at 37 °C with shaking (160 rpm) for 3 h to simulate human stomach conditions. Numerations were carried out during the gastric stress simulation at 0, 1.5 and 3 h.

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

Bacterial survival was expressed as the log (N/N_0) where N_0 is the initial population that was adjusted to 10^6 CFU/ml and N is the number of CFU/mL obtained after 3 h (gastric stress) or 31 h (gastro-intestinal stress). The two-fold dilution, due to changing the gastric stress medium to the gastro-intestinal stress medium, was taken into account.

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

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

[a] All strains grew at 25 °C in aerobic conditions.

2.4. Tissue culture and in vitro adhesion assay

The strains were tested for their adhesion ability *in vitro* on epithelial intestinal cells (Caco-2: colon adenocarcinoma human, ATCC, USA, Lot# 4129634). The intestinal cells were routinely cultured and used as already described (Tareb et al., 2013). Cultures were used at post-confluence after 15 days of culture (differentiated cells). To determine the number of Caco-2 cells in a monolayer, cells were detached for 2 min with Splittix and Splitstop (Bio Media) at ambient temperature and counted using a Thoma cell. Three non-pathogenic control strains were used: *E. coli* Nissle 1917 (obtained from Dr. Ulrich Sonnerborn from Ardeypharm, Germany GmbH), with a long history of safe use as a probiotic and large body of acquired knowledge (EFSA Panel on Biological Hazards (BIOHAZ), 2014; Wassenaar, 2016), *E. coli* K12 (ATCC 10798) and *Lactobacillus rhamnosus* GG strain. In addition, another control was used: *Escherichia coli* O157: H7 C267 (*stx*-, *eae* +), this strain is a shiga toxin negative mutant (*stx1*-and *stx2*-) which has kept the gamma-intimin (adherence protein) producing gene (*eaeA* +) (Vernozy-Rozand et al., 2000). Bacterial strains were cultured in TSB-YE incubated at 37 °C or 25 °C (for strains unable to grow at 37 °C) for 24 h under shaking (120 rpm) and then washed twice with PBS. Concentrations were adjusted to 2×10^8 cells/mL and cells were labeled with 0.2% aqueous solution of 4′, 6-diamidino-2-phenylindole (DAPI, Sigma) by incubating for 15 min. Three washing steps were performed with PBS (0.01 M, pH 7.4) to remove excess unbound DAPI, then cells were suspended in 0.2 mL Dulbecco's Modified Eagle Medium (DMEM) without antibiotics and put into contact with Caco-2 cells at a final concentration of 10^7 per 7×10^4 Caco-2 cells for 2 h under standard Caco-2 growing conditions (incubation at 37 °C, 5% CO_2, 95% humidity). Not adherent bacterial cells were removed by three washing steps with PBS solution and cell fixation was performed using a 3.7% (w/v) solution of formaldehyde in PBS.

Enumeration of adherent bacteria was performed using an epifluorescence microscope. Results were determined as an average of ten observations per assay. Adhesion was calculated by enumerating the adhered bacterial cells in 10 microscopic fields for each strain and was expressed as the average number of adhered bacterial cells per 100 Caco-2 cells. For selected strains, validation of bacterial adhesion was performed by using electron microscopy as routinely done (Tareb et al., 2013).

2.5. Human serum bactericidal assay

To determine the sensitivity of GNB strains to human serum, human serum type male, blood group: AB, HIV negative (Biowest, Nauille-France) was used. The bacterial strains were cultivated overnight in TSB under shaking at 30 or 37 °C, depending on the strain. GNB strains and negative/positive control strains (see below) were added to 50% human serum solution to obtain the initial cell density of 10^5 cells per mL (Jankowski et al., 1996). Each mixture was separated in two aliquots, one was plated onto Tryptic Soy Agar (TSA) in Petri plate and was incubated at 30 or 37 °C according to the tested strain. The other one was placed in a water bath for 3 h at 37 °C then plated onto TSA and incubated at 30 or 37 °C. The results were validated by using *Hafnia alvei* 56.85 (resistant strain) as a positive control and *Hafnia alvei* 31.86 (sensitive strain) as a negative control (Jankowski et al., 1996).

2.6. Pathogenicity evaluation

Galleria mellonella larvae were grown in medium containing 50.6% wheat flour, 19% honey, 19% glycerol, 7.7% brewer's yeast and 3.7% bee hive wax. All bacterial strains were grown as mentioned above, washed three times and cell concentrations were adjusted to $\sim 10^{10}$ cells/mL of PBS by optical density (OD 600 nm) determination using pre-enumeration data for each strain. The two non-pathogenic *Escherichia coli* K12 and *E. coli* Nissle 1917 strains were used as safe controls. Additionally, as above, *E. coli* C267, O157: H7 (*stx*1-, stx2-, *eaeA* +) was also used because of the presence of an adherence protein (intimin coding gene *eaeA*). *Galleria mellonella* larvae, in groups of ten, were inoculated by injection into the haemocoel through the last proleg by using a sterile syringe (Needle: 0.33 × 12 mm; 0.3 mL U-100 insulin- TERUMO, Belgium) with a needle diameter of 0.33 mm. Ten µl of sterile PBS (negative control) and 10 µl of a dose series corresponding to successive 10-fold dilutions of bacterial cell suspensions (ranging from 2 to 10 logs) were injected. After inoculation, larvae were placed in sterile Petri dishes and incubated at 30 °C for 72 h and up to the moth stage (10–15 days). Mortality rate was assessed by the lack of movement of larvae in response to stimulation and observation of concomitant melanization of the cuticle. LD 50 (cfu/g of larvae) was estimated at 72 h in two independent assays as the geometric mean of the doses for which the first 100% mortality and 0% mortality were found (Lorke, 1983).

2.7. Statistical analysis

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

3. Results and discussion

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

3.1.1. Growth at 37 °C

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

3.1.2. Gastric and gastrointestinal stress simulation

To get closer to *in vivo* conditions, survival of 20 GNB dairy strains was evaluated successively in simulated gastric fluids and gastrointestinal stress environments over time, in the presence or absence of milk (to mimic ingestion of a dairy product), *in vitro*. For all strains, growth data after 3 h gastric stress and 28 h gastrointestinal stress at 37 °C were plotted together and are presented in Table 2. Additionally, changes in counts for three strains representative of the observed behaviours during the 31 h gastric and gastrointestinal stress simulation are presented in Fig. 1 A to C.

The effects of *in vitro* simulated gastric stress were assessed over a 3 h period, for the 20 selected GNB dairy isolates, in the presence of gastric juices (containing 500 U/l pepsin), and at pH 2 and pH 4, supplemented or not with milk. A good survival, corresponding to stable or increased population counts, was observed for the majority of the 20 GNB isolates in gastric juices initially adjusted to pH 4, both in the presence or absence of milk, except for *Alcaligenes faecalis* 904 and to a lesser extent for *Pseudomonas putida* VRBG37.3, and in GM-milk for *Halomonas venusta/alkaliphila/hydrothermas* 4C1A and *Sphingobacterium* sp. PCAi F2.5 (Table 2). At pH 2.0 in the absence of milk, a decrease in survival for the majority (14 strains) of strains was reported. Six strains actually grew or presented relatively stable populations in these conditions: *Acinetobacter* sp. PCAi E6.10, *Hafnia paralvei* 920, *Halomonas venusta/alkaliphila/hydrothermas* 4C1A (Fig. 1A), *Pantoea agglomerans* Q6.3, *Providencia heimbachae* GR4, and *Sphingobacterium faecium* F2.5. In the presence of milk, a decrease in survival was also mainly observed for 13 strains. In these conditions, populations remained relatively stable for only 7 strains (log (N/N0) > -0.5): *Chryseobacterium bovis* Pi18 (Fig. 1B), *Hafnia paralvei* 920, *Halomonas venusta/alkaliphila/hydrothermalis* 4C1A (Fig. 1A), *Halomonas* sp. B39, *Morganella morganii* 3A2A, *Providencia heimbachae* GR4 and *Pseudomonas putida* CV30.6 (Table 2).

These results showed that under simulated gastric stress conditions (no bile salts added), pH 2 had a different effect on growth than pH 4; presence of milk had only a slight impact on growth for both tested pH values.

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

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

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

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

3.2. Adhesion analyses

The observed adhesion capacities are presented in Table 3 as a mean of two biological replicates. Results for all tested strains varied from 5 to 363 bacterial cells per 100 Caco-2 cells. Value for attachment of

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 3 h gastric stress (GM-3) followed by 28 h 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 + 3 h							
		3	31	3	31	3	31	3	31
Acinetobacter (close to *A.* genospecies 3) 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
Alcaligenes faecalis 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
Chryseobacterium (close to *C. bovis*) 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
Citrobacter freundii 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
Hafnia alvei (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
Hafnia paralvei 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
Halomonas venusta/alkaliphila/hydrothermas 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
Halomonas 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
Klebsiella oxytoca 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
Morganella morganii 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
Pantoea agglomerans 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
Proteus sp. (close *P. hauseri*) 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
Proteus sp. (close *P. hauseri*) 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
Providencia heimbachae 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
Pseudomonas group *putida* 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
Pseudomonas group *putida* 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
Pseudomonas stutzeri 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
Psychrobacter celer 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
Sphingobacterium sp. (close to *S. faecium*) 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
Stenotrophomonas maltophilia/rhizophila 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

Lactobacillus rhamnosus GG to Caco-2 cells were similar to that previously published (Gopal et al., 2001), thus confirming the validity of the present results. The results for adherence of the two Gram negative species used as safe control strains were low (< 100 cells) as well for the control *E. coli* O157: H7 C267 (*stx*1-, *stx*2-, *eae*+) and twelve other strains. The other remaining eight strains exhibited attachment > 100 cells and the most adherent strain was *Halomonas venusta/alkaliphila/hydrothermalis* 4C1A with 363 cells per 100 Caco-2 cells, followed by 200, 152, 145, 141, 138, 114, 100 microbial units for *Alcaligenes faecalis* 904, *Proteus* sp (close to *P. hauseri*) UCMA 3779, *Psychrobacter celer* 91, *Pseudomonas* group *putida* VRBG 37.3, *Klebsiella oxytoca* 927, *Pseudomonas stutzeri* UCMA 3883 and *Citrobacter freundii* UCMA 4217, respectively. Adherence is a clue for probiotic potential but is also the first step leading to potential cytotoxicity (dos Santos et al., 2015; Pavlov et al., 2004; Pogačar et al., 2015). In the present study, adhesion capacity was overall low for most of the tested strains and it was lower than previously tested strains of the same species present in drinking water (Pavlov et al., 2004). The low level of adherence was also confirmed by scanning electron microscopy (data not shown).

3.3. Bactericidal effect of human serum

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

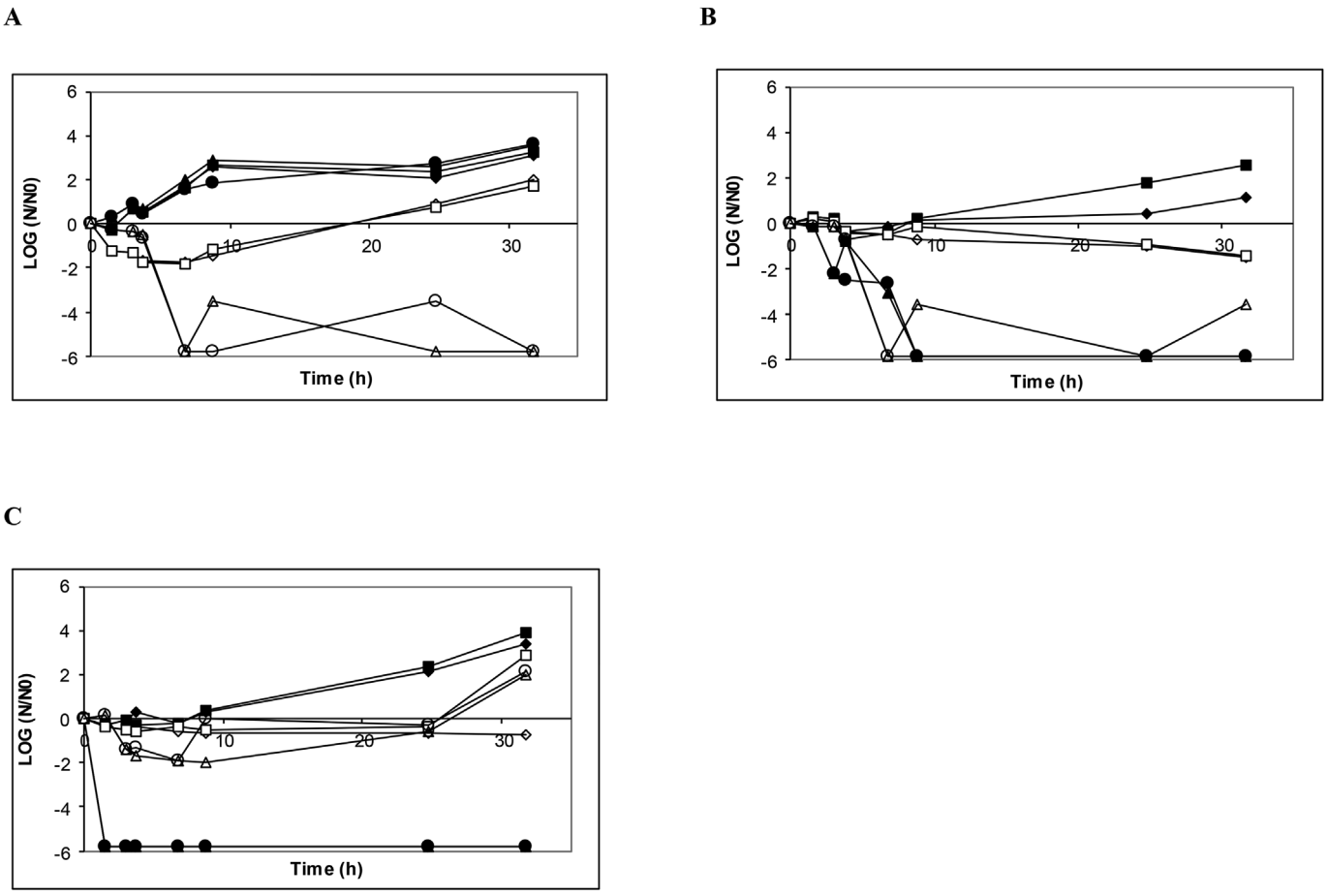

Fig. 1. Changes in counts of *Halomonas venusta/alkaliphila/hydrothermalis* 4C1A (A), *Chryseobacterium* sp. close to *C. bovis* Pi18 (B), and *Proteus* sp. (close *P. hauseri*) UCMA 3779 (C), during gastric (from T0h primary adjusted to pH 2 or 4→T3h) and gastrointestinal stress (from T3h with addition 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.

3.4. Virulence of bacteria to Galleria mellonella

The 20 selected GNB and four control strains were tested for virulence in *G. mellonella* (Table 3). All PBS-injected control larvae grew until the moth stage. Control strains, *E. coli* Nissle 1917, *E. coli* K12 ATCC 10798, and *Lactobacillus rhamnosus* GG had LD 50 from around 10^7 to > 10^8, respectively. This range was considered as the reference non-toxic range, since it was obtained for the three safe control strains. Thirteen GNB strains showed no virulence score within this range. Among them *Hafnia alvei* (biogroup 1) B16 was found safe which is reassuring as strains from this species are used as commercial ripening cultures for many cheeses (Irlinger et al., 2012). Three strains, including *K. oxytoca* 927, *P. stutzeri* UCMA 3883, *Sphingobacterium* sp (close to *S. faecium*) PCAi F2.5 had intermediate LD 50 around 6.5 10^6 as observed for *E. coli* O157:H7 (*stx*1-, *stx*2-, *eae*+) C267 which was used as a control. Two strains, *H. paralvei* 920 and *Proteus* sp (close to *P. hauseri*) UCMA 3780, were toxic to larvae with a lower LD 50 around 10^5. Two other strains were even more virulent, with LD50 value lower than 10^4, and corresponded to *Providencia heimbachae* GR4 and *Morganella morganii* 3A2A.

Assuming that *G. mellonella* larvae injection experiments are commonly used to detect bacterial strains presenting virulence factors and that the virulence of many pathogens is similar in wax moth larvae and mammals, including Humans (Desbois and Coote, 2012), it was deduced that only few strains contained efficient virulent factors and could act as direct or indirect pathogens. In the literature, a recent study showed that *Galleria mellonella* can also provide significant insights into virulence mechanisms and that this can be applied to the study of opportunistic human pathogens (Wand et al., 2013). Virulence clearly depended here on the considered strain but not on its origin as two *Proteus* sp. (close to *P. hauseri*) strains, 3779 and 3780, having the same origin, were very different in their virulence towards the wax moth larvae. Only *Proteus* sp. (close to *P. hauseri*) UCMA 3780, *H. paralvei* 920, and even more *M. morganii* 3A2A and *P. heimbachae* GR4 strains required the lowest concentrations to kill larvae and can be considered as harbouring efficient virulence factors. Overall, the majority of the strains tested for virulence in *Galleria mellonella* larvae were safe for this organism when compared with the three control strains known to be safe for use in Humans through oral absorption.

3.5. Data analysis for safety assessment of GNB associated with traditional French cheeses

A compilation of selected results obtained in the present work (Table S1) provides a global view about main safety characteristics of the tested GNB during simulated GIT transit after oral ingestion of milk products and their potential virulence. In this table, the individual strains were classified from safe to virulent based on the results obtained using the insect model *Galleria mellonella* larvae. Then, *in vitro* results are presented by following the GIT transit progression described in Fig. 2 for cheese ingestion.

From the *in vivo* data, it was concluded that *Proteus* sp (close to *P. hauseri*) UCMA 3780, *M. morganii* 3A2A, *H. paralvei* 920 and *P. heimbachae* GR4 strains presented some efficient virulence factors. Among

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			
Lactobacillus rhamnosus GG (Gram +)	200	nd	$7.00\ 10^{8}$
Escherichia coli Nissle 1917	23	nd	$1.70\ 10^{7}$
Escherichia coli K12 ATCC 10798	65	nd	$3.16\ 10^{7}$
Strain with intimin			
Escherichia coli O157:H7 C267 (*eae*$^{+}$, *stx*$^{-}$)	35	nd	$1.46\ 10^{6}$
Dairy strains			
Chryseobacterium sp (close to *C. bovis*) Pi18	73	R	$3.71\ 10^{8}$
Psychrobacter celer 91	145	S	$1.41\ 10^{8}$
Alcaligenes faecalis 904	200	R	$9.83\ 10^{7}$
Proteus sp. (close to *P. hauseri*) UCMA 3779	58	R	$9.78\ 10^{7}$
Acinetobacter sp. (close to *A.* genospecies 3) PCAi E6.10	33	R	$8.82\ 10^{7}$
Halomonas venusta/alkaliphila/hydrothermalis 4C1A	363	S	$6.06\ 10^{7}$
Pantoea agglomerans PCA Q6.3	63	R	$5.91\ 10^{7}$
Halomonas sp. nov. B39	30	S	$2.54\ 10^{7}$
Citrobacter freundii UCMA 4217	100	R	$2.18\ 10^{7}$
Pseudomonas group *putida* VRBG 37.3	141	S	$1.91\ 10^{7}$
Stenotrophomonas maltophilia/rhizophila PCAi D6.5	70	R	$1.33\ 10^{7}$
Pseudomonas group *putida* CV30.6	5	R	$1.25\ 10^{7}$
Hafnia alvei biogroup 1 B16	43	R	$9.48\ 10^{6}$
Pseudomonas stutzeri UCMA 3883	114	R	$6.57\ 10^{6}$
Sphingobacterium sp. (close to *S. faecium*) PCAi F2.5	45	S	$6.57\ 10^{6}$
Klebsiella oxytoca 927	138	R	$6.13\ 10^{6}$
Hafnia paralvei 920	36	R	$2.80\ 10^{5}$
Proteus sp. (close to *P. hauseri*) UCMA 3780	152	R	$9.63\ 10^{4}$
Providencia heimbachae GR4	49	R	$< 1.6\ 10^{4}$
Morganella morganii 3A2A	54	R	$< 1.16\ 10^{4}$

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

Table 4
Impact of human serum on Gram-negative bacteria (GNB) strains after none and 3 h 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	*Chryseobacterium* sp. (close to *C. bovis)* Pi18 (number of colonies)	*Pseudomonas putida* VRBG 37.3 (number of colonies)	Control sensitive (number of colonies)	Control Resistant (number of colonies)
At 0 h				
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

these four strains, *M. morganii* 3A2A and *H. paralvei* 920 survived well in aerobic simulated gastric conditions (with milk and bile salts) while the two others survived to a lesser extent. Only one strain, *H. paralvei* 920, did not grow at 37 °C under anaerobic conditions. PCA analysis (Fig. S2) of the quantitative results given in Table S1, for pH 2 as primary gastric stress, showed some negative correlation ($r = -0.42$) between LD50 in wax moth larvae and survival in GIM-milk at pH 6.5 with bile salt (1.5 g/L). So, the more sensitive the strain to pH 2 (Fig. S2A), the higher the value of LD50 (= the less toxic it was); at pH4 (Fig. S2B), this negative correlation value was weaker ($r = -0.29$) and it could be explained by a lower number of strains sensitive to this less aggressive pH. Furthermore, at pH4, although adherence to Caco-2 cells and survival were opposed on the F1 axis, no negative correlation appeared ($r = -0.21$) between them. Qualitative factors were not specifically associated with any of the quantitative factors studied in Table S1.

3.6. Safety assessment of GNB strains belonging to genera/species of strains assessed in this study

To assess the safety status of GNB strains of dairy origin studied here, a bibliographic review on the potential human pathogenicity of strains belonging to the corresponding species, was conducted and is listed in Table S3. Four bacteria, namely *Chryseobacterium* sp., *Psychrobacter celer, Sphingobacterium* sp., and *Stenotrophomonas maltophilia,* were so far never reported in any clinical cases. Only individual clinical cases were reported for the GNB species studied in Table S3 but not associated with food vectors. The only one exception was for *Proteus* sp. that caused biogenic amine (BA) related intoxications (after eating BA contaminated fish). BA could pose a safety risk for human health. Fortunately, the corresponding clinical effect appeared to be very moderate except in rare cases and it could explain that there is no regulation for BA in food, except for histamine levels in fish products. Furthermore, a study (Delbès-Paus et al., 2012) involving eleven strains used *in vitro* in the present study, reported that only negligible biogenic amine amounts were produced *in situ* (in model cheeses), contrary to BA production observed *in vitro* by Coton et al. (2012). Nevertheless, as BA production is a strain-dependant trait, strain effect and cheese type must also be considered.

4. General discussion and conclusion

The safety status as well as contribution of most GNB to the cheese process is poorly documented. In previous years, GNB were classically considered as indicators of hygienic problems (Bockelmann et al., 2005; Tornadijo et al., 1993) and responsible for defects in cheese texture and flavor due to the production of extracellular proteolytic and lipolytic enzymes (Amato et al., 2012; Martins et al., 2006). For example, dairy related *Pseudomonas* spp. strains have been shown to produce volatile compounds such as ethyl esters and alcohols that may negatively affect cheese sensory characteristics (Arslan et al., 2011; Morales et al., 2005). The idea that GNB species can be normal and interesting elements of cheese microbiota is more recent (Larpin-Laborde et al., 2011). In this

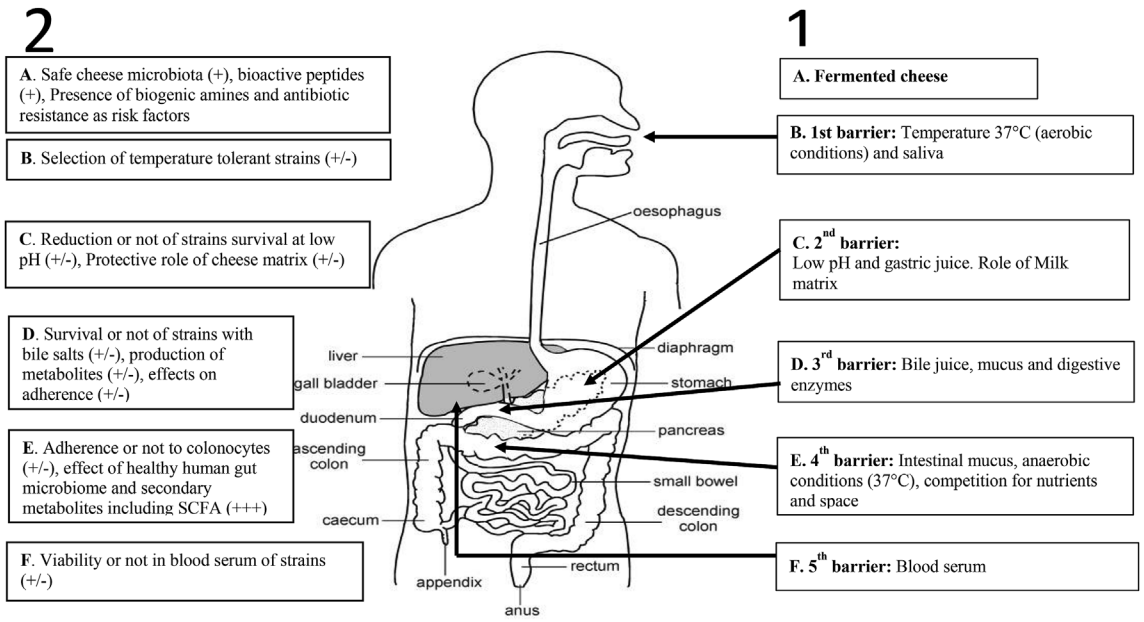

Fig. 2. Scheme of main body barriers observed during human GIT transit of fermented cheese (1; A to F). For each barriers, beneficial (+) or adverse (−) effects involved in safety of ingested food during its GIT transit were highlighted (2; A to F).

sense, for example, *Proteus* sp. (close to *P. hauseri*) and *Psychrobacter* sp. have been shown to significantly contribute to flavor production in smear cheeses (Deetae et al., 2007; Irlinger et al., 2012) raising questions about the potential beneficial effects of GNB in general. Recently, it was confirmed that some GNB strains have interesting specific technological properties (Schmitz-Esser et al., 2018).

In this context, to delve deeper into the knowledge about GNB, it was necessary to assess the safety status of these species. The present study was designed to evaluate the potential health risks, if any, associated with the consumption of GNB present in cheeses (and coming from raw milk, milk, or the milking environment), in simulated body conditions. However, risk analysis is complex. You need to know, successively, the danger and its characteristics, the exposure level and to set a safe threshold value, then, and only in that case, risk assessment can be performed. In this study, from the present data and by using a general exposure context, only part of risk analysis (i.e. the qualification of the danger) was done through an *in vivo* study of pathogenicity in *Galleria mellonella* larvae and by using *in vitro* determinations of bacterial growth in the simulated GIT, on Caco-2 cells and in the presence of human serum. The presence of virulent factors was suggested here for some specific GNB strains but their virulence in human gut also depends on their active dose which is modulated by the effect of different gut barriers, as reported in Fig. 2.

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

Up until now, the absence of cheese foodborne outbreaks, might be explained by the positive bioactive impact of the cheese microbial community, as previously demonstrated in a model cheese ecosystem against *Listeria monocytogenes* (Imran et al., 2013). Additionally, it was also recently shown that *Hafnia alvei* B16 had an inhibitory effect in a model cheese ecosystem against *Escherichia coli* O26:H11 (Callon et al., 2016; Delbès-Paus et al., 2013). Multiple dynamic interactions between GIT microbiota, cheese matrix and its initial associated microbiota (detailed in Fig. 2) must be taken into account. Among these barriers, and of special importance is the gut microbiota which was shown to play a crucial role for protection against some pathogenic bacteria (Ubeda et al., 2017). This dense resident microbial community, referred as the commensal microbiota, has a major function of protection against colonization of pathogens and this ability to restrain pathogen growth include competitive metabolic interaction, localization to intestinal niches, and induction of host immune responses (Kamada et al., 2013).

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

In silico studies could of help to complete our understanding of these interactions, but it is missing for the GNB studied here. Till now only the genome sequence of several other cheese-related GNB, which include the strain *P. heimbachae* GR4, was determined in a subsequent study (Almeida et al., 2014). It indicated that this strain, studied in this work, is not closely related to the reference strain; an adaptation to the dairy environment could be supposed. For the future, *in vivo* approaches

using wax worm could be further combined with *in silico* approaches to seek for pathogenicity factors and their potential mobility, in the genome sequence of these strains.

To conclude, presumption of safety of most GNB strains studied here was successfully established, while some could harbour virulence factors. These factors (including some antibiotic resistances) could be considered as a direct or indirect risk when dissemination to other bacteria of the human gut occurred. The role of multiple dynamic interactions between cheese microbiota and GIT barriers including gut microbiota could be key factors explaining safe consumption of the corresponding cheeses. However, the present study, which was partly limited to *in vitro* studies of selected individual strains, cannot replace *in vivo* studies involving actual cheese and gut microbiota ecosystem. There is always a gap between microbial behavior in *in vitro* and *in vivo* conditions, especially taking into account the complexity of the considered systems (cheese, alimentary bolus, human body). The present approach is a first step for future studies aiming at elucidating the role of each microbial component (GNB or others) within such complex microbial communities.

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

Declarations of interest conflict

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.11.001.

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