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## **Diversity and assessment of potential risk factors of Gram-negative isolates associated with French cheeses**

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1           **Diversity and assessment of potential risk factors of Gram-**  
2           **negative isolates associated with French cheeses.**

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27 **Running title: Biodiversity & characterization of Gram-negative dairy isolates**

28

29 **Abstract**

30 **The goal of this study was to identify at the species level a large collection of Gram-**  
31 **negative dairy bacteria isolated from milks or semi-hard and soft, smear-ripened**  
32 **cheeses (cheese core or surface samples) from different regions of France. The isolates**  
33 **were then assessed for two risk factors, antibiotic resistance and volatile and non-**  
34 **volatile biogenic amine production *in vitro*. In total, 173 Gram-negative isolates were**  
35 **identified by *rrs* and/or *rpoB* gene sequencing. A large biodiversity was observed with**  
36 **nearly half of all Gram-negative isolates belonging to the *Enterobacteriaceae* family.**  
37 **Overall, 26 different genera represented by 69 species including potential new species**  
38 **were identified among the studied Gram-negative isolates for both surface and milk or**  
39 **cheese core samples. The most frequently isolated genera corresponded to *Pseudomonas*,**  
40 ***Proteus*, *Psychrobacter*, *Halomonas* and *Serratia* and represented almost 54% of the**  
41 **dairy collection. After *Pseudomonas*, *Chryseobacterium*, *Enterobacter* and**  
42 ***Stenotrophomonas* were the most frequently isolated genera found in cheese core and**  
43 **milk samples while *Proteus*, *Psychrobacter*, *Halomonas* and *Serratia* were the most**  
44 **frequently isolated genera among surface samples. Antibiotic resistance profiles**  
45 **indicated that resistances to the aminoglycosides, imipenem and quinolones were relatively low**  
46 **while over 50% of all tested isolates were resistant to antibiotics belonging to the**  
47 **monobactams, cephalosporins, fosfomycin, colistin, phenicol, sulfamides and some from the**  
48 **penam families. Thirty-six % of isolates were negative for *in vitro* biogenic amine**  
49 **production. Among biogenic amine-producers, cadaverine was the most frequently**  
50 **produced followed by isoamylamine, histamine and putrescine. Only low levels**  
51 **(<75mg/l) of tyramine were detected *in vitro*.**

52

53 **Keywords:** Gram-negative bacteria, diversity, dairy products, risk factors, antibiotic  
54 resistances, biogenic amines  
55

## 56 **1. Introduction**

57 Cheese production is based on the use of both defined starter cultures and the presence  
58 of undefined indigenous microbial populations (including diverse yeasts and moulds, Gram-  
59 positive and Gram-negative bacteria). In France, the annual production of cheese is estimated  
60 at 1.8 million tons of which close to 11% are produced from raw milk. Understanding the  
61 complex microbial communities and their dynamics during cheese production (from the raw  
62 milk or milk, milking environment, during fermentation and to the ripening) are key factors to  
63 ensure food safety and control the sensorial properties of the final products. In the cheese  
64 core, the dominant microflora usually corresponds to lactic acid bacteria (LAB) species while  
65 Gram-positive catalase-positive bacteria, yeasts, moulds and diverse Gram-negative bacteria  
66 (*Citrobacter* spp., *Enterobacter* spp., *Pseudomonas* spp.) constitute the subdominant  
67 microflora (Abriouel et al., 2008; Duthoit et al., 2003; Ercolini et al., 2001) or even co-  
68 dominant microflora (Larpin et al., 2011). On the surface of cheeses, high genus and species  
69 diversity is observed and the main microbial groups correspond to coagulase-negative  
70 staphylococci, *Actinobacteria* members (*Micrococcus*, *Corynebacterium*,...) (Brennan et al.,  
71 2002; Mounier et al., 2005; Rea et al., 2007; Valdes-Stauber et al., 1997) while subdominant  
72 microflora correspond to Gram-negative group – *Pseudomonas* spp., *Pseudoalteromonas*  
73 spp., *Halomonas* spp., *Psychrobacter* spp. and species belonging to the *Enterobacteriaceae*  
74 family such as *Hafnia alvei* (Chaves-Lopez et al., 2006; Morales et al., 2003; Mounier et al.,  
75 2005; Mounier et al., 2009; Rea et al., 2007; Sorhaug and Stepaniak, 1997). Gram-negative  
76 bacteria are frequently isolated from both the surface of cheeses (Larpin et al., 2011; Maoz et  
77 al., 2003; Mounier et al., 2005; Mounier et al., 2009) and the cheese core (Chaves-Lopez et  
78 al., 2006; Delbes et al., 2007; Tornadijo et al., 2001) and, in particular, *Enterobacteriaceae*  
79 counts can reach  $10^6$  to  $10^7$  CFU/g in the cheese core during the first days of ripening and  
80 then slowly decrease (Tornadijo et al., 2001). *Enterobacteriaceae* distribution in the cheese

81 core and on the surface is likely influenced by the physico-chemical conditions encountered  
82 during cheese production including pH increase due to yeast growth, oxygen availability,  
83 water activity ( $A_w$ ) and salt content due to washing procedures favoring the growth of  
84 halophilic species such as those belonging to *Halomonas*. Furthermore, bacteria from the  
85 ripening facility environment may also contaminate the product.

86 On one hand, the presence of some Gram-negative bacteria is often used as a marker  
87 for hygiene conditions, as coliforms are indicative of fecal contamination, and they are also  
88 considered as undesirable cheese contaminants (Bockelmann et al., 2005; Prado et al., 2001;  
89 Tornadijo et al., 2001). Morales et al. (2005) showed that *Pseudomonas* spp. were able to  
90 produce a wide range of volatile compounds which may negatively contribute to the sensory  
91 quality of cheese. In addition, defects in cheese texture and flavour or early blowing were  
92 attributed to the Gram-negative bacteria *Enterobacter aerogenes* and *Escherichia coli* (Luck  
93 and Dunkeld, 1981) while other *Enterobacteriaceae* family members such as *Serratia* spp.  
94 and *Kluyvera* spp. may affect cheese sensory quality via lipolytic and proteolytic activities  
95 (Chaves-Lopez et al., 2006; Morales et al., 2003). Moreover, some Gram-negative bacteria  
96 have been reported to produce volatile and non-volatile biogenic amines (BA) in foods, in  
97 particular in fish (Allen et al., 2005; Björnsdottir-Butler et al., 2010; Kanki et al., 2004; Kim  
98 et al., 2001; Lehane and Olley, 2000), but also in vegetables (Lavizzari et al., 2010) and  
99 cheese (Marino et al., 2000) which can be a concern in a food context due to their potential  
100 physiological impact (mainly for histamine and tyramine). BA are produced and can  
101 accumulate in foods containing free amino acids via intracellular bacterial catabolic pathways  
102 involving at least a decarboxylase or deiminase and a transporter responsible for the uptake of  
103 the amino acid and secretion of the amine. BA correspond to low molecular weight basic  
104 nitrogenous compounds and are formed at low levels within living cells. A few studies have  
105 also reported antibiotic resistance in Gram-negative bacteria isolated from foods such as

106 vegetables (Osterblad et al., 1999), minced meat (Osterblad et al., 1999) and Portuguese  
107 cheeses (Amador et al., 2009). Many natural resistances to antibiotics are already present in  
108 some Gram-negative bacteria. Moreover, members of the *Enterobacteriaceae* family are  
109 among the emerging antibiotic resistant bacteria transmitted to humans mainly via meat and  
110 other food of animal origin (Johnson et al., 2005). The wide use of antibiotics for the  
111 treatment of humans as well as the heavy use of antimicrobial agents in agriculture and  
112 animal/fish farming can lead to increased antibiotic dissemination in natural environments  
113 and enhanced resistances in environmental microorganisms such as Gram-negative bacteria  
114 and eventually in human pathogens (Cabello 2006; Cattoir et al., 2008; Hammerum 2009;  
115 Smith et al., 2005).

116 On the other hand, presence of some Gram-negative bacteria in cheese may play an  
117 important role in regards to cheese ripening and the production of aroma compounds and  
118 thereby the overall sensory qualities of the cheese (Dahl et al., 2000; Deetae et al., 2009;  
119 Morales et al., 2004; Morales et al., 2005). Among the Gram-negative bacteria previously  
120 isolated from cheeses, *Proteus vulgaris* has been shown to produce high concentrations of  
121 flavour compounds, in particular volatile sulphur compounds and branched-chain alcohols,  
122 during the ripening process in a model cheese (Deetae et al., 2009; Deetae et al., 2009).

123 The diversity and safety of the Gram-negative flora naturally present in milk and in  
124 cheese during processing is poorly documented. To gain knowledge on this microbial group,  
125 this study focused on characterizing Gram-negative bacteria most commonly found in dairy  
126 sources. A collection of 173 Gram-negative bacteria isolated from diverse French dairy  
127 sources was created in order to assess their biodiversity using molecular methods and  
128 characterize them in regards to the production of undesirable molecules (biogenic amines) and  
129 antibiotic resistances.

130



## 131 **2. Materials and Methods**

132

### 133 *2.1 Bacteria and culture media*

134

135 A working collection of 173 Gram-negative bacterial isolates isolated from milk  
136 (diverse sources) as well as soft, smear-ripened and semi-hard cheeses made from raw  
137 thermally treated or pasteurized milk (including at different ripening stages for some) from  
138 either the cheese surface or cheese core from different French regions was created. Fifty seven  
139 isolates originated from Munster cheese, 53 from Livarot cheese, 29 from milk or raw milk  
140 samples, 13 from St. Nectaire, 12 from Epoisses cheese, 2 from Pont l'Evêque, 2 from  
141 Camembert, 2 from Langres, 1 from Salers, 1 from Niolo and 1 from goat cheese. These  
142 isolates were provided by 4 different French laboratories: INRA Aurillac (URF 545), INRA  
143 Grignon (UMR 782 GMPA), University of Caen - Basse-Normandie (E.A. 3213 MILA) and  
144 Aérial in Illkirch. They were isolated either from selective (*Pseudomonas* agar base  
145 supplemented with cetrimide, fusidin and cephalosporin (CFC), Violet Red Bile Glucose  
146 Agar (VRBG), Plate Count Agar (PCA) supplemented with 1% crystal violet and 0.5%  
147 vancomycin to inhibit Gram-positive bacteria PCAI (Delbes et al., 2007)) or non-selective  
148 (PCA + 3% NaCl) culture media. They were initially assigned to Gram-negative bacteria  
149 genera or species by phenotypic or molecular (16S rRNA gene partial sequencing) methods  
150 (data not shown). At least one representative isolate of each different genera or species found  
151 in each culture collection was selected to constitute the working collection.

152 Isolates were cultivated either in Tryptic Soy Broth (TSB, AES, France) supplemented  
153 with 2.5g/l Yeast Extract (TSBYE) and 30 g/l NaCl (Labogros, France), in Brain Heart  
154 Infusion broth (BHI, AES, France) or on Tryptic Soy Agar (TSA, AES, France) supplemented  
155 with 2.5g/l Yeast Extract (TSAYE) and 30 g/l NaCl. Cultures were incubated under aerobic

156 conditions at 25°C for 24h. Isolates were then stored in cryotubes with 30% v/v glycerol at -  
157 80°C until use.

158

## 159 *2.2 Identification of Gram negative isolates by rrs and rpoB genes sequencing*

160

161 Total DNA was prepared from bacterial cultures by using the Promega Genomic DNA  
162 purification kit (Promega, Madison, WI, USA). Purified DNA samples were stored at -20°C.  
163 The *rrs* gene (encoding the 16S rRNA) was amplified with primers A and rJ (Harf-Monteil et  
164 al., 2004). Since the *rrs* gene is not always sufficient to distinguish closely related species,  
165 especially *Enterobacteriaceae*, the housekeeping gene *rpoB* (coding for the  $\beta$  subunit of the  
166 RNA polymerase) that has been shown to resolve phylogenetic relationships in various  
167 bacterial groups (Adékambi et al., 2009) was also used. PCR amplification of *rpoB* was  
168 performed with primers VIC4 and VIC6 (Tayeb et al., 2008).

169

## 170 *2.3 Sequencing and data analysis*

171

172 PCR products were analysed by the Platform 8 of the Pasteur Institute Genome Center  
173 for sequencing (Paris, France). Sequencing primers for *rpoB* were the same as those used in  
174 the PCR experiments. Additional sequencing primers for *rrs* fragments were E, RE and D  
175 (Harf-Monteil et al., 2004).

176 Concerning data analysis, partial *rpoB* and complete *rrs* gene sequences were edited  
177 using the Lasergene package (DNASTAR, Madison, WI, USA). Phylogenetic analysis was  
178 based on the *rrs* or *rpoB* sequence of the reference type strains. Genbank sequence  
179 comparisons were performed using BLAST (Altschul et al., 1990). The sequences were  
180 aligned using CLUSTAL V.

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#### *2.4 Antibiotic susceptibility testing*

Antimicrobial susceptibility of the 173 Gram-negative isolates used in this study was assessed for 24 antibiotics using the disk diffusion method on Mueller-Hinton agar (AES, France) selected according to guidelines for Gram-negative bacteria given by the AntibioGram Committee of the French Microbiology Society (CA-SFM, French equivalent to the Clinical and Laboratory Standards Institute –CLSI-) in 2008 and 2009. The following antimicrobial susceptibility test disks (BioRad, France) were used: amikacin 30µg (AN), gentamicin 15µg/10UI (GM), tobramycin 10µg (TM), imipenem 10µg (IPM), cefalotin 30µg (CF), cefotaxime 30µg (CTX), ceftazidime 30µg (CAZ), aztreonam 30µg (ATM), amoxicillin 25µg (AMX), amoxicillin/clavulanic acid 20+10µg (AMC), ampicillin 10µg (AM), mecillinam 10µg (MEC), piperacillin 75µg (PIP75), piperacillin/tazobactam 75+10µg (TZP85), ticarcillin 75µg (TIC), ticarcillin/clavulanic acid 75+10µg (TCC), colistin 50µg (CS50), ciprofloxacin 5µg (CIP), fosfomycin 50µg (FOS), chloramphenicol 30µg (C), nalidixic acid 30µg (NA), rifampicin 30µg (RA30), sulfamid 200µg (SSS200) and tetracycline 30µg (TE). Briefly, to determine antibiotic susceptibility, isolates were first cultivated in BHI broth for 24h under aerobic conditions (cultures agitated at 160 rpm) and incubated at 25°C. Cultures were then diluted in Tryptone-Salt (TS; AES, France) to obtain an optical density equivalent to 0.5 on the Mac Farland scale. For each isolate, 2 ml of diluted culture was used to fully immerse Mueller-Hinton agar plate. Excess culture was then removed and Petri dishes were incubated at 25°C for 15 min to dry the surface of the agar before applying the antibiotic discs. The inhibition zone surrounding the antibiotic disk was measured in millimeters and compared to the critical diameters (mm) values as defined by the CA-SFM. Interpretation for susceptibility status was based on the most recent criteria adopted by the CA-SFM (2008 and 2009 data).

206

207 *2.5 In vitro detection of volatile and non-volatile biogenic amine production*

208

209 All Gram-negative isolates were first tested for their potential to produce biogenic  
210 amines (BA) *in vitro* using a rapid screening method based on the “biogenic amine test”  
211 medium described by Bover-Cid & Holzapfel (Bover-Cid and Holzapfel, 1999). Briefly,  
212 experiments were carried out on 96 well microplates in 225 µl final volumes. Before  
213 inoculation into the “BA test” medium, three successive precultures for each isolate were  
214 carried out in BHI broth containing 4 amino acid precursors (L-lysine monohydrochloride, L-  
215 histidine monochloride monohydrate, L-ornithine monohydrochloride (Sigma, France) and L-  
216 tyrosine disodium salt (BioWest, France) at a concentration of 2.5 g/l each and adjusted to pH  
217 5.3. Cultures were incubated at 25°C with agitation for 24h. Five µl of each isolate was then  
218 inoculated into the “BA test” medium containing one amino acid precursor (2.5 g/l) at a time  
219 or without the target amino acid precursor to test for false-positives. Microplates were then  
220 incubated at 25°C under aerobic conditions for 1 to 5 days. BA production was characterized  
221 by a color change in the medium (from yellow to purple). Plate readings were carried out  
222 daily by visual observation to ensure proper growth of each isolate and to check for BA  
223 production.

224 Potential BA-producing isolates, as determined by screening isolates in the “BA test”  
225 medium, were further analyzed to determine actual BA production levels using high  
226 performance liquid chromatography (HPLC). Briefly, isolates were grown in 10 ml of “BA  
227 test” medium supplemented with each of the amino acid precursor and BA content was  
228 determined by HPLC using the dansyl chloride derivatisation of amines as previously  
229 described by Duflos et al. (Duflos et al., 1999) with a LUNA 5µ C18(2) 100A, 250mm x  
230 4,0mm column (Phenomenex, France).

231

## 232 *2.6 Statistical analysis of biogenic amine production by the Gram-negative isolates*

233

234 Volatile and non-volatile BA profiles from the different isolates were analysed by an  
235 ascending hierarchical classification (AHC) analysis (Statistica software version 6; Statsoft,  
236 Maisons-Alfort, France). The dendrogram obtained made it possible to significantly display 3  
237 groups of isolates at a threshold aggregation distance of 5000; the use of the K-means  
238 clustering allowed us to establish the amine profile of each group. The Chi-square test was  
239 performed to assess the differences between these groups.

240

## 241 **3. Results**

242

### 243 *3.1 Identification of Gram-negative isolates and biodiversity at the genus and species* 244 *levels*

245

246 In this study, the collection of Gram-negative dairy bacteria was created by isolating bacteria  
247 from milk and from diverse cheese types, in some cases at different ripening stages, using the  
248 described plating methods. The isolate collection was not exhaustive and cheese types were  
249 represented according to availability and the microflora present. Dairy isolate identifications  
250 at the species level were carried out based on the *rrs* and/or *rpoB* gene sequences obtained for  
251 all 173 Gram-negative bacteria isolated from the various French dairy sources (all sequences  
252 were uploaded into NCBI in alphabetical order by genus and species and accession numbers  
253 are as follows: JF710846 to JF710953 correspond to *rpoB* gene sequences and JF710954 a  
254 JF711018 correspond to *rrs* gene sequences). To do so, the *rrs* gene was first used to identify  
255 all bacterial isolates at either the genus or species level. The *rpoB* gene was also used to

256 identify isolates to a given species as this gene provides a higher level of discrimination. All  
257 sequences were compared with publically available databases (using the Blast resource at the  
258 National Center for Biotechnology Information, NCBI, and the Ribosomal Database Project,  
259 RDP). In total, 63 isolates were identified at the species level based on the *rrs* gene sequence  
260 and 110 based on the partial *rpoB* gene sequence. In the case of no more than 1% divergence  
261 in nucleotide sequences between a Gram-negative dairy isolate and the sequence of a valid  
262 species, they were considered as the same species. A sequence presenting more than 2%  
263 divergence with the most closely related and validated sequence was considered as a potential  
264 new species.

265 Overall, after molecular identification, the Gram-negative isolates were classified into  
266 13 different families within the Proteobacteria phylum (mainly from the gamma-  
267 proteobacteria group and to a lesser extent the  $\beta$ - and  $\alpha$ -proteobacteria groups) and 1 family  
268 within the Cytophaga-Flavobacterium-Bacteroides (CFB) complex (Table 1). Among  
269 proteobacteria, nearly half of all isolates (47.4%) belonged to the *Enterobacteriaceae* family  
270 (represented by 12 genera) followed by the *Pseudomonadaceae* (14.4%; represented by 1  
271 genus), *Moraxellaceae* (11 %; represented by 2 genera), *Halomonadaceae* (9.2%; represented  
272 by 1 genus) and *Alcaligenaceae* (6.4%; represented by 2 genera). Isolates belonging to the  
273 *Xanthomonadaceae* (2.9% of all isolates), *Oceanospirillaceae* (1.2% of all isolates),  
274 *Brucellaceae*, *Caulobacteraceae*, *Pseudoalteromonadaceae*, *Sphingomonadaceae* and  
275 *Vibrionaceae* families were more seldomly found (for each family <1% of all isolates).

276 In spite of a limited number of cheeses analyzed, high biodiversity at the genus and species  
277 levels was observed for both surface flora and cheese core and milk samples, with a total of  
278 26 different genera and 69 different species identified (Table 1). Approximately 47% of all  
279 the identified species were only represented by a single isolate while approximately 12% were  
280 represented by 5 or more isolates. Several isolates (Byrne-Bailey et al., 2009) potentially

281 corresponded to novel species within the *Pseudomonas*, *Halomonas*, *Marinomonas* and  
282 *Providencia* genera. However, further work would be required to determine whether the  
283 isolates belonging to these groups do correspond to new species. It is important to note that  
284 the distribution of cheese types as well as the repartition in numbers of surface, core and milk  
285 isolates is variable depending on cheese availability, microflora present in the tested samples,  
286 and plating methods used.

287 Overall, 7 genera were described in all sample types (*Pseudomonas*, *Serratia*,  
288 *Enterobacter*, *Citrobacter*, *Klebsiella*, *Raoultella* and *Acinetobacter*). Among surface isolates,  
289 at least 49 different species out of 134 surface isolates were identified, of which 42 were only  
290 found in cheese surface samples (Table 1). Besides *Pseudomonas* and *Serratia*, the 3 other  
291 most frequently isolated genera, *Proteus*, *Psychrobacter* and *Halomonas*, were only identified  
292 for surface flora. The most dominant species identified were *P. vulgaris*, *H. alvei*/*H. paralvei*,  
293 *A. faecalis*, *Halomonas* spp. and *Psychrobacter* spp.. Less frequently isolated surface isolates  
294 belonged to the genera *Citrobacter*, *Providencia* and *Morganella* and really low levels of  
295 surface isolates belonged to 8 other genera (*Raoultella*, *Klebsiella*, *Escherichia*,  
296 *Marinomonas*, *Tetrathiobacter*, *Brevundimonas*, *Kluyvera* and *Acinetobacter*).

297 For milk and cheese core isolates, 26 different species were identified for 39 isolates.  
298 Noteworthy, although a limited number of isolates were from cheese core samples (Ten Brink  
299 et al., 1990) in comparison to milk (Duflos et al., 1999), biodiversity in these core samples  
300 was surprisingly high as they were represented by 8 genera (*Pseudomonas*,  
301 *Chryseobacterium*, *Enterobacter*, *Stenotrophomonas*, *Citrobacter*, *Klebsiella*, *Raoultella* and  
302 *Serratia*) and 11 species. *Chryseobacterium* spp. and *Stenotrophomonas* spp. were only found  
303 among cheese core and milk samples.

304

305 *3.2 Antibiotic resistances*

306           Although antibiotic resistances of non-pathogenic Gram-negatives may not have direct  
307 public health consequences, world safety authorities are concerned about their dissemination.  
308 In this context, the antibiotic resistance profile of all 173 Gram-negative isolates was  
309 determined for 24 antibiotics belonging to 10 antibiotic families by the disk diffusion method.  
310 The spectrum of action of all 24 antibiotics against 173 Gram-negative dairy isolates was first  
311 evaluated and showed that over 50% of all tested isolates were resistant to the following 6  
312 antibiotics all belonging to the beta-lactam family (AMX, AM, MEC, ATM, CF, CTX) and to  
313 fosfomycin, colistin, phenicol and sulfamid (Table 2). The dominant antibiotic resistance  
314 targeted mecillinam (a penam) with almost 87% of resistant isolates and aztreonam (a  
315 monobactam) with almost 73% of resistant isolates. On the contrary, only a low number of  
316 antibiotics resistances for all Gram-negative isolates were observed for 7 antibiotics  
317 belonging to the  $\beta$ -lactam family, mostly penams (TCC, PIP75, TZP85, AMC, TIC, IPM) as  
318 well as one cephem (CAZ), and for all 3 tested aminosides (AN, GM, TM), both quinolons  
319 (NA, CIP), rifamycin (RA30) and tetracycline (TE) (Table 1). In particular, a limited number  
320 of resistances (only 15 to 35%) for all Gram-negative isolates were found against the three  
321 aminosides, the 2 quinolons and the 4  $\beta$ -lactams (ticarcillin/clavulanic acid, piperacillin,  
322 piperacillin/tazobactam and imipenem).

323           The antibiotic resistance profile for each genus was further evaluated (Figure 1).  
324 Profiles for low numbers of resistances were observed for two of the most frequently isolated  
325 surface flora identified in this study *Psychrobacter* (18 isolates and <12% overall antibiotic  
326 resistances) and *Halomonas* (14 isolates, <23% overall antibiotic resistances); all isolates of  
327 both genera were clearly susceptible to most tested antibiotics (Figure 2A). Fewer numbers of  
328 antibiotic resistances ranging from 15% to 50% were also observed for 10 other genera  
329 (*Enterobacter*, *Raoultella*, *Acinetobacter*, *Alcaligenes*, *Escherchia*, *Hafnia*, *Marinomonas*,  
330 *Brevundimonas*, *Kluyvera* and *Pantoea*) (Figure 2A).



331 On the contrary, high resistance profiles (>50% and up to 75% of all species and  
332 isolates within a given genus) were observed for several of the most frequently isolated  
333 genera found for both surface and cheese core/milk samples: *Pseudomonas* (24 isolates),  
334 *Proteus* (22 isolates), *Chryseobacterium* (8 isolates) and *Stenotrophomonas* (5 isolates)  
335 (Figure 2B). High resistance profiles were also found for *Serratia* , *Citrobacter* , *Morganella* ,  
336 *Providencia* , *Klebsiella*, *Ochrobactrum* , *Sphingobacterium*, *Tetrathiobacter* and *Vibrio*  
337 genera but the number of isolates for some genus was limited, only 1 isolate (Figure 2B).  
338 Overall, the level of antibiotic resistance varied intra-genus and intra-species

339

### 340 3.3 Volatile and non-volatile biogenic amine production in vitro

341 Characterizing food-related microorganisms for their potential to produce biogenic  
342 amines potentially having a toxicological impact on the consumer is of interest. In this  
343 context, the potential to produce the two main BA known to have detrimental physiological  
344 effects in humans (histamine and tyramine) as well as two possible potentiators (putrescine  
345 and cadaverine (Taylor, 1986), was evaluated in the 173 Gram-negative bacterial isolates of  
346 dairy origin. It was first carried out using the specific “BA-test” detection medium. Secondly,  
347 representative isolates of positive isolates on “BA-test” medium were further analyzed for  
348 actual BA production by HPLC which allowed for the detection and quantification of 11  
349 volatile or non-volatile BAs (volatile : methylamine, ethylamine, isoamylamine and non-  
350 volatile : spermine, spermidine, tryptamine, phenylethylamine, putrescine, cadaverine,  
351 histamine and tyramine).

352 Overall, potential decarboxylase activities (histidine decarboxylase; tyrosine  
353 decarboxylase; ornithine decarboxylase and lysine decarboxylase involved in histamine-,  
354 tyramine-, putrescine- and cadaverine-production, respectively) implicated in the production  
355 of at least one BA were found in 64% of isolates (111 isolates) after pre-screening isolates on

356 specific “BA-test” medium. More precisely, potential putrescine- and cadaverine-producers  
357 were the most frequently encountered with the “BA-test” medium in 69 and 67 isolates,  
358 respectively. Potential tyramine-producers (42 isolates) and histamine-producers (15 isolates)  
359 were observed to a lesser extent. Using the BA-test medium, no BA were produced among  
360 isolates belonging to *Chryseobacterium* spp. and *C. freundii* and only a relatively small  
361 number of isolates were positive (~35%) among the *Proteus* spp. and *Psychrobacter* spp.  
362 isolates.

363 *In vitro* BA-production for 57 representative isolates (positive isolates as well as a few  
364 “BA-test” non-producer isolates) was analyzed by HPLC using the same culture medium  
365 (Table 3). Overall, cadaverine concentration ranged from undetected to 1559 mg/l. Its  
366 production was often associated with isoamylamine production (from undetected to 1458  
367 mg/l), in particular for isolates belonging to the species *E. hormaechei*, *M. morgani* and  
368 *Serratia* spp. while this was not observed for *K. oxytoca* and *S. maltophilia* isolates.  
369 Histamine levels varied from undetected to 1706 mg/l with highest levels produced *in vitro* by  
370 *M. morgani*, *P. heimbachae* and *Serratia* spp. Only 7 putrescine-producers were detected *in*  
371 *vitro*, with detected concentration ranging from 100 to 155 mg/l except for one *Proteus* sp.  
372 nov. isolate that produced 1083 mg/l. Tyramine producers were detected at levels less than 74  
373 mg/l. Finally, 7 isolates were found to produce phenylethylamine (154 to 261 mg/l detected)  
374 and 4 were tryptamine-producers (108 to 425 mg/l detected). The other BAs analyzed were  
375 detected at levels below 100 mg/l and did not contribute to the classification. The 57 Gram-  
376 negative isolates clustered in three classes after ascending hierarchical classification based on  
377 their amine content profiles (Classes given in Table 3). Class 1 was comprised of the lowest  
378 number of isolates (12 isolates), which produced up to 6 amines simultaneously and were  
379 characterized by the highest cadaverine (average value of 1327 mg/l), histamine (944 mg/l),  
380 isoamylamine (827 mg/l) and phenylethylamine (133 mg/l) production capacities. It was

381 comprised of all the three tested isolates of *M. morganii*, as well as several isolates of the  
382 genera *Halomonas* and *Serratia*. Class 2 counted 19 isolates, characterized by high cadaverine  
383 (1224 mg/l) and isoamylamine (626 mg/l) production capacities. It comprised all the tested  
384 isolates of *E. hormaechei* (4 isolates) and *S. maltophilia* (2 isolates), as well as several isolates  
385 of the genera *Pseudomonas* and *Serratia*. Class 3 counted the highest number of isolates (27  
386 isolates), characterized by low or sporadic BA-production. It comprised all the tested isolates  
387 of the genus *Proteus* (10 isolates), as well as several isolates of the genera *Pseudomonas*,  
388 *Providencia* and *Halomonas*.

389

## 390 **Discussion**

391 In this study, the biodiversity as well as the existence of potential risk factors *in vitro*  
392 was evaluated for 173 Gram-negative isolates originating from milk and cheese core or cheese  
393 surface from various French cheese-producing regions.

394 Concerning the biodiversity aspects, for the 173 Gram-negative isolates identified in  
395 this study, 26 genera and 68 species were identified and classified among 14 families clearly  
396 highlighting the wide diversity and the potential importance of the presence of Gram-negative  
397 bacteria in complex cheese microbial ecosystems during production. Although all the isolates  
398 were not typed, the diversity of their origins in regards to geography, cheese technological  
399 practices and cheese factories argue in favour of their potential genetic diversity. The most  
400 frequently isolated genera identified in the selected cheeses included *Pseudomonas*, *Proteus*,  
401 *Psychrobacter*, *Halomonas* and *Serratia* as well as *Chryseobacterium* for cheese core.  
402 Although a variety of selective and non-selective media were used for Gram-negative bacteria  
403 isolation, this study is obviously not exhaustive. Moreover, in most cases, the concentration of  
404 the Gram-negative isolates identified in the milks/cheese core or on the cheese surface was  
405 not determined and dairy sample types and numbers varied; therefore, it is not possible to

406 provide information on their dominance or sub-dominance in the complex cheese microbial  
407 ecosystems studied. However, for Livarot cheese isolates used in this study, the Gram-  
408 negative flora (isolated in PCA + 3% NaCl) were part of the dominant flora (data not shown)  
409 and reached levels equivalent to Gram-positive bacteria (data not shown). Finally, some  
410 genera (i.e. *Halomonas*) have already been identified at high levels in soft, smear-ripened  
411 cheeses (Mounier et al., 2009).

412 *Pseudomonas* strains have been previously shown to be part of raw milk (Ercolini et  
413 al., 2009; Giannino et al., 2009) and cheese ecosystems (Leriche et al., 2004) and to play a  
414 role in cheese alteration (yellow or brown coloration, flavor and texture defects due to  
415 proteolysis and lipolysis) (Leriche et al., 2004; Luck and Dunkeld, 1981), yet it is important  
416 to note that *Pseudomonas* spp. are frequently encountered in foodstuffs and are widespread in  
417 the environment. A predominance of the *Enterobacteriaceae* family (12 genera and 28  
418 species representing 47% of all Gram-negative isolates) was observed. *Enterobacteriaceae*  
419 have been frequently encountered during cheese production (Chaves-Lopez et al., 2006;  
420 Morales et al., 2003; Mounier et al., 2005; Sorhaug and Stepaniak, 1997), although they have  
421 been considered as a negative flora (ex. marker for hygiene, cheese texture defects, blowing  
422 and off-flavours) and can be associated with raw milk samples from mastitic animals (Nam et  
423 al., 2010). Among *Enterobacteriaceae*, *Serratia* strains have been described in milk  
424 (Tornadijo et al., 1993) and cheese (Martin-Platero et al., 2009) and have been shown to affect  
425 cheese sensory quality due to high proteolytic activities and dimethyl sulfide production  
426 (Chaves-Lopez et al., 2006; Morales et al., 2003) while *Proteus* strains have been shown to  
427 positively impact cheese flavour by the production of aromatic compounds (Deetae et al.,  
428 2009; Deetae et al., 2009). On the other hand, little is known about the impact of *Halomonas*  
429 spp. and *Chryseobacterium* spp. strains during cheese production. *Halomonas* spp. have been  
430 reported in milk (Fricker et al., 2010) and cheese (Bockelmann et al., 2003; Maoz et al., 2003;

431 Mounier et al., 2005; Mounier et al., 2009; Ogier et al., 2004), while *Chryseobacterium* spp.  
432 have been frequently described in raw milk (Franciosi et al., 2010; Giannino et al., 2009;  
433 Hantsis-Zacharov and Halpern, 2007) and seldomly in cheese (Delbes et al., 2007). The origin  
434 of the Gram-negative isolates in raw milk could correspond to bovine teats and possibly from  
435 faeces (Kagkli et al., 2007) but the environment, water, and the cheesemaking processing  
436 steps can certainly contribute to the contamination of the product and may even be a major  
437 source. The presence of *Hafnia alvei* may be associated to use of strains as a starter. That of  
438 *Halomonas* spp. might correspond to the use of salt (brine or dry salt) during the cheese  
439 process, while the origin of *Psychrobacter* spp. strains were probably already present in raw  
440 milk as they are frequently isolated from this product just like *Stenotrophomonas* spp.  
441 (whereas *Halomonas* is almost always undetected).

442         Concerning potential risk factors, antibiotic resistance and ability to form biogenic  
443 amines *in vitro* were considered. The antibiotic resistance of the 173 Gram-negative isolates  
444 was first evaluated. Although the ability of non-pathogenic bacteria to resist to an antibiotic  
445 does not constitute a direct risk for human health upon consumption, world public health  
446 authorities are more and more concerned by the dissemination of antibiotic resistances from  
447 environmental microorganisms to human pathogenic bacteria (Cabello 2006; Cattoir et al.,  
448 2008; Hammerum 2009; Smith et al., 2005). Many natural resistances to antibiotics are  
449 already present in some Gram-negative bacteria. For example, many *Enterobacteriaceae*  
450 members are naturally resistant to aminopenicillins and first and/or second generation  
451 cephalosporines. On the other hand, the majority of antibiotic resistance genes acquired  
452 through horizontal gene transfer (HGT) in human pathogens are now considered to have  
453 originated in environmental microorganisms (Martinez, 2008). In this context, food-related  
454 Gram-negative strains could constitute a potential reservoir for antibiotic resistance genes. In  
455 this study, antibiotic-resistance was widespread in Gram-negative dairy-related isolates. This

456 finding, in and of itself, is not surprising since the intrinsic resistance of many organisms to  
457 antibiotics is well documented (Quintiliani et al., 1999). The obtained results indicated that  
458 some of the species presenting the highest number of antibiotic resistances belonged to the  
459 *Chryseobacterium* genus (from 13 to 22 antibiotic resistances) except for *C. jejuense* (5  
460 resistances). In this genus, the only reported antibiotic resistances in strains associated with  
461 food corresponded to the presence of *Chryseobacterium meningosepticum* (species not found  
462 in this study) in beverages obtained from soda fountain machines (White et al., 2010);  
463 however, the existence of metallo-beta-lactamase was observed in clinical isolates of  
464 *Chryseobacterium indologenes* (Lin et al., 2008). For the other dominating bacterial species,  
465 the strains exhibiting the highest number of antibiotic resistances generally belonged to the  
466 *Enterobacteriaceae* family especially within the *Proteus* (13 to 16 antibiotic resistances  
467 according to the considered species), *Serratia* (7 to 15 according to the considered species),  
468 *Morganella* (11 to 17 according to the considered species) and *Klebsiella* (10 to 19 according  
469 to the considered species). For *Proteus* spp., the existence of multiresistant strains of *P.*  
470 *mirabilis* in meat retail products has been described (Kim et al., 2005), while multiresistant  
471 *Klebsiella* spp. (mainly *K. pneumoniae*, species not isolated during this study) and *Serratia*  
472 spp. strains were observed in mastitic raw milk samples (Bengtsson et al., 2009; Nam et al.,  
473 2010). Within each species, the results were compatible with the intrinsic antibiotic  
474 resistances described by the CA-SFM (French equivalent to the CLSI). Concerning the  
475 *Pseudomonas* genera, a certain variability was observed, with for the most dominant species  
476 *Pseudomonas* sp. close to *P. putida*, seven antibiotic resistances that suggest that they could  
477 correspond to natural resistances as 100% of the 11 tested strains are presenting resistances to  
478 MEC, AM, CF, CTX, NA, CS50 and C, while the tested *P. aeruginosa* strain presented 20  
479 antibiotic resistances. Moreover, according to the CA-SFM, *P. aeruginosa* is known to  
480 possess natural resistances to aminopenicillins, cefotaxime and chlroamphenicol as well as at

481 least 8 others. Strains belonging to this species and associated with cow mastitis (Swartz et  
482 al., 1984) or retail meat products and food processing environments (Kim and Wei, 2007)  
483 have been shown to be extremely resistant to antibiotics; *Tetrathiobacter kashmirensis*  
484 presented 13 antibiotic resistances. Interestingly, isolates belonging to the *Halomonas* and  
485 especially the *Psychrobacter* genus presented only few antibiotic resistances. For *Halomonas*  
486 spp., the observed antibiotic resistances ranged from 2 for *Halomonas* sp. to 7 for *H. venusta*.  
487 Resistance to MEC seemed to be common to most strains belonging to this genus (86 %).  
488 Little information is available in the literature; however, Fernandez-Castillo et al. (1992)  
489 showed that isolates of *H. elongata* and *H. halmophila* (species not found in this study)  
490 harboured a plasmid carrying genetic determinants that mediate resistance to kanamycin,  
491 tetracycline, and neomycin. For *Psychrobacter* spp., the number of antibiotic resistances  
492 ranged from 0, for *Psychrobacter* sp. nov., to 3, for *Psychrobacter faecalis*. The most  
493 encountered antibiotic resistances corresponded to SSS200, FOS 50 and MEC, (67%, 67%  
494 and 50% of *Psychrobacter* spp. isolates, respectively). To date, only sulfonamide resistance in  
495 bacterial isolates from manured agricultural soils and pig slurry has been reported (Byrne-  
496 Bailey et al., 2009).

497 Finally, the second studied risk factor corresponded to the potential to produce  
498 biogenic amines *in vitro* as these molecules are known to have physiological impact  
499 especially for people deficient in the detoxification systems (Caston et al., 2002; Lehane and  
500 Olley, 2000). No clear toxicity threshold for biogenic amines has been defined in foods, in  
501 general, except for histamine content of fish species associated with a high amount of  
502 histamine (100 mg/kg, EU regulation 2073/2005) and some data for histamine and tyramine  
503 toxicity levels in cheese (histamine toxicity level considered as 8-1000 mg (Adékambi et al.,  
504 2009) and tyramine as 10-80 mg (Blackwell and Mabbit, 1965)). Moreover, it depends on  
505 multiple factors including individual acceptability and the presence of other components as

506 toxicity potentiators (Halasz et al., 1994; Lehane and Olley, 2000; Silla Santos, 1996; Ten  
507 Brink et al., 1990). In regards to sensory impact, the relation between the presence of 564.02  
508 mg/kg cheese of cadaverine in Camembert cheese and bitter taste was not clearly  
509 demonstrated (Kubichova and Grosch, 1998).

510         The ability to form biogenic amines was widely spread among the collection isolates  
511 as 64% of strains were able to produce at least one BA, mainly putrescine and cadaverine,  
512 after pre-screening strains on specific “BA-test” medium. When considering the two main  
513 amines implicated in health problems, namely histamine and tyramine, the number of BA-  
514 producing strains was much lower. Presence of histamine producers belonging to the genera  
515 or species *M. morganii*, *H. alvei*, *Klebsiella* spp., *Enterobacter* spp. and *R. planticola* was  
516 consistent with previous results as these species have been reported to be the dominant  
517 species involved in histamine fish poisoning associated to fish consumption (Gingerich et al.,  
518 1999; Kim et al., 2001; Lehane and Olley, 2000; Okuzumi et al., 1982). Tyramine producers  
519 belonged to the *Enterobacter*, *Proteus* and *Pseudomonas* genera. *Enterobacteriaceae* and  
520 *Pseudomonadaceae* isolated from spinach have also been shown to produce *in vitro*  
521 histamine, cadaverine, and putrescine (Lavizzari et al., 2010). It is important to note that the  
522 ability to form biogenic amines *in vitro* does not reflect the actual production in cheese as  
523 environmental (temperature, water activity, pH, precursor availability) as well as  
524 microbiological factors (flora competition) can influence BA-production; therefore *in situ*  
525 confirmation of production is necessary (Bunkova et al., 2010). In conclusion, this work  
526 highlighted the high biodiversity of Gram-negative bacteria associated with cheese production  
527 in France. The characterization of the collection isolates for the presence of various risk  
528 factors may indicate the existence of different groups potentially presenting more or less  
529 potential risks. Strains with potential risks included *M. morganii*, *C. freundii*, *E. hormaechei*,  
530 *S. maltophilia* as well as all *Serratia* spp. isolates. They were multi-resistant to the tested



531 antibiotics, were strong BA-producers, producing multiple BAs simultaneously *in vitro*.  
532 Moreover, all these species have also been reported in numerous human clinical cases (Choi  
533 et al., 2007; Falagas et al., 2009) but are not necessarily associated to food intoxications.  
534 However, *M. morgani* has been implicated in food intoxication cases due to high levels of  
535 histamine production in particular in fish (Lehane and Olley, 2000). On the contrary, a low  
536 number of risk factors was identified in numerous species including those belonging to the  
537 two dominant surface flora *Psychrobacter* and *Halomonas*. Indeed, all *Psychrobacter* species  
538 isolates identified were only resistant to a maximum of eight antibiotics, potentially produced  
539 a relatively low number of BA and to date, species belonging to this genus have never been  
540 documented in human clinical cases. For the *Halomonas* species isolates, except for those  
541 belonging to the *Halomonas venusta/alkaliphila/hydrothermalis* group, a maximum of only  
542 11 antibiotic resistances were observed, some strains produced BA *in vitro* (0-3 BA detected)  
543 and no human clinical cases related to isolates belonging to this genus have been reported to  
544 date.

545 In conclusion, concerning the Gram-negative isolates that exhibited potential risks in  
546 this study, further investigation (i.e. strain survival during gastric and gastrointestinal  
547 simulation, microbial adhesion to human cell lines, pathogenicity in adapted model, biogenic  
548 amine production during cheese making) should be carried out. Moreover, based on the tests  
549 performed in this study *in vitro*, we were able to show that for the large biodiversity of Gram-  
550 negative dairy isolates identified, some isolates were free of all tested risk factors. Clearly,  
551 these isolates should be further studied for their technological potential during cheese making.  
552 For some isolates, certain risk factors were detected.

553 In either case, an *in situ* approach would allow to confirm their risk-free or potential  
554 risk status. Indeed, risk-free or low risk Gram-negative strains will be further investigated in  
555 terms of impact during cheese making and in cheese from the microbiological (i.e. barrier

556 effect against pathogens) and sensorial point of views (impact on cheese texture and aroma).  
557 Moreover, for Gram-negative strains showing technological traits of interest (organoleptic  
558 characteristics, barrier effect against pathogens) and provided that a safety evaluation has  
559 been performed, they could be considered for use as potential adjuncts for cheese making.  
560 This is already the case with *H. alvei* strains on the market for use as starter cultures (personal  
561 communication).

562

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569

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- 757
- 758

759 **Table 1.**

Phylum	Family	Genus – Species	Dairy sources	# surface strains	# milk/core strains
alpha-P	<i>Brucellaceae</i>	<i>Ochrobactrum</i> sp.	Salers milk	0	1
alpha-P	<i>Caulobacteriaceae</i>	<i>Brevundimonas diminuta</i>	Munster	1	0
alpha-P	<i>Sphingomonadaceae</i>	<i>Sphingobacterium</i> sp. close to <i>S. faecium</i>	Salers milk	0	1
beta-P	<i>Alcaligenaceae</i>	<i>Alcaligenes faecalis</i>	Livarot, Munster	8	0
beta-P	<i>Alcaligenaceae</i>	<i>Alcaligenes</i> grp <i>A. faecalis/aquatilis</i>	Livarot	1	0
beta-P	<i>Alcaligenaceae</i>	<i>Tetrathibacter kashmirensis</i>	Munster	2	0
C-F-B	<i>Flavobacteriaceae</i>	<i>Chryseobacterium jejuense</i>	Milk 2.1	0	2
C-F-B	<i>Flavobacteriaceae</i>	<i>Chryseobacterium joostei</i>	Salers milk	0	1
C-F-B	<i>Flavobacteriaceae</i>	<i>Chryseobacterium shigense</i>	Salers milk	0	2
C-F-B	<i>Flavobacteriaceae</i>	<i>Chryseobacterium</i> sp. close to <i>C. bovis</i>	Saint Nectaire	0	1
C-F-B	<i>Flavobacteriaceae</i>	<i>Chryseobacterium</i> sp. close to <i>C. indologenes</i>	Salers milk	0	1
C-F-B	<i>Flavobacteriaceae</i>	<i>Chryseobacterium</i> sp. close to <i>C. vrystaatense</i>	Salers milk	0	1
gamma-P	<i>Enterobacteriaceae</i>	<i>Citrobacter braakii</i>	Munster	1	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Citrobacter freundii</i>	Livarot, Munster	4	1
gamma-P	<i>Enterobacteriaceae</i>	<i>Enterobacter amnigenus</i>	Salers milk, milk 2.1	0	5
gamma-P	<i>Enterobacteriaceae</i>	<i>Enterobacter hormaechei</i>	Munster	3	1
gamma-P	<i>Enterobacteriaceae</i>	<i>Escherichia coli</i>	Livarot	2	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Hafnia alvei</i>	Livarot, Munster	5	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Hafnia paralvei</i>	Livarot, Munster	4	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Klebsiella oxytoca</i>	Munster	2	1
gamma-P	<i>Enterobacteriaceae</i>	<i>Klebsiella terrigena</i>	Saint Nectaire	0	1
gamma-P	<i>Enterobacteriaceae</i>	<i>Kluyvera intermedia</i>	Munster	1	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Morganella morganii</i>	Munster	5	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Morganella</i> sp. close to <i>M. psychrotolerans</i>	Munster	1	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Pantoea agglomerans</i>	Salers milk	0	1
gamma-P	<i>Enterobacteriaceae</i>	<i>Proteus</i> close to <i>hauseri</i>	Livarot	4	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Proteus heimbachae</i>	Munster	3	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Proteus rettgeri</i>	Munster	1	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Proteus</i> sp. close to <i>vulgaris</i>	Saint Nectaire	1	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Proteus</i> sp. Nov.	Munster	1	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Proteus vulgaris</i>	Livarot, Munster, Saint Nectaire	12	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Providencia heimbachae</i>	Livarot, Epoisses	3	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Providencia</i> sp. nov.	Epoisses	2	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Raoultella planticola</i>	Livarot, Munster	3	1
gamma-P	<i>Enterobacteriaceae</i>	<i>Serratia grimesii</i>	Livarot	1	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Serratia liquefaciens</i>	Livarot, Munster	2	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Serratia marcescens</i>	Munster	1	0
gamma-P	<i>Enterobacteriaceae</i>	<i>Serratia proteomaculans</i>	Munster, Saint Nectaire	3	0

gamma-P	<i>Enterobacteriaceae</i>	<i>Serratia rubideae</i>	Munster	1	2
gamma-P	<i>Enterobacteriaceae</i>	<i>Serratia</i> sp. close to <i>S. grimesii</i>	Epoisses	3	0
gamma-P	<i>Halomonadaceae</i>	<i>Halomonas alkaliphila</i>	Munster	2	0
gamma-P	<i>Halomonadaceae</i>	<i>Halomonas</i> sp. close to <i>H. alkaliphila</i>	Livarot	1	0
gamma-P	<i>Halomonadaceae</i>	<i>Halomonas</i> sp. nov.	Livarot	5	0
gamma-P	<i>Halomonadaceae</i>	<i>Halomonas venusta</i>	Munster	1	0
gamma-P	<i>Halomonadaceae</i>	<i>Halomonas venusta/alkaliphila/hydrothermalis</i>	Livarot, Munster	7	0
gamma-P	<i>Moraxellaceae</i>	<i>Acinetobacter</i> close to genospecies 3	Salers milk	0	1
gamma-P	<i>Moraxellaceae</i>	<i>Acinetobacter johnsonii</i>	Livarot	1	0
gamma-P	<i>Moraxellaceae</i>	<i>Psychrobacter celer</i>	Livarot, raw milk Camembert	4	0
gamma-P	<i>Moraxellaceae</i>	<i>Psychrobacter cibarius/immobilis</i>	Epoisses	2	0
gamma-P	<i>Moraxellaceae</i>	<i>Psychrobacter faecalis</i>	Livarot	1	0
gamma-P	<i>Moraxellaceae</i>	<i>Psychrobacter namhaensis</i>	Langres, Camembert raw milk	2	0
gamma-P	<i>Moraxellaceae</i>	<i>Psychrobacter</i> sp.	Livarot, Niollo	4	0
gamma-P	<i>Moraxellaceae</i>	<i>Psychrobacter</i> sp. grp <i>P.celer/pacifisensis</i>	Epoisses, Pont l'évêque	4	0
gamma-P	<i>Oceanospirillaceae</i>	<i>Marinomonas</i> sp. nov.	Livarot	2	0
gamma-P	<i>Pseudoalteromonadaceae</i>	<i>Pseudoalteromonas haloplanktis/nigrifaciens</i>	Epoisses	1	0
gamma-P	<i>Pseudomonaceae</i>	<i>Pseudomonas</i> sp. grp <i>P.alcaligenes / piechaudii</i>	Saint Nectaire	1	0
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas aeruginosa</i>	Salers milk	0	1
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas fragi</i>	Livarot	2	0
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i> grp. <i>P. florescens</i>	Salers milk	0	2
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i> grp. <i>P. putida</i>	Livarot, Munster	6	1
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas lundensis</i>	Salers milk	0	1
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas putida</i>	Milk 2.1	0	3
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i> sp. close to <i>P. taetrolens</i> grp <i>P. fluorescens</i>	Munster	1	0
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i> sp. nov.	Livarot, Munster	4	2
gamma-P	<i>Pseudomonadaceae</i>	<i>Pseudomonas stutzeri</i>	Livarot	1	0
gamma-P	<i>Vibrionaceae</i>	<i>Vibrio</i> sp.	Livarot	1	0
gamma-P	<i>Xanthomonadaceae</i>	<i>Stenotrophomonas maltophilia</i>	Salers milk, milk 2.1	0	3
gamma-P	<i>Xanthomonadaceae</i>	<i>Stenotrophomonas rhizophila</i>	Saint Nectaire	0	1
gamma-P	<i>Xanthomonadaceae</i>	<i>Stenotrophomonas</i> sp. close to <i>maltophilia</i>	Saint Nectaire	0	1
		<b>Total</b>		<b>134</b>	<b>39</b>

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761

762 **Table 2.**

763

<b>Family</b>	<b>Antibiotic</b>	<b>% resistant strains</b>
<b>aminosids</b>	<b>AN</b>	15
	<b>GM</b>	20
	<b>TM</b>	32
<b>penams</b>	<b>TCC</b>	25
	<b>PIP 75</b>	29
	<b>TZP 85</b>	31
	<b>AMC</b>	41
	<b>TIC</b>	47
	<b>AMX</b>	<b>51</b>
	<b>AM</b>	<b>65</b>
<b>MEC</b>	<b>87</b>	
<b>imipenems</b>	<b>IPM</b>	23
<b>monobactams</b>	<b>ATM</b>	<b>73</b>
<b>cephems</b>	<b>CAZ</b>	48
	<b>CF</b>	<b>67</b>
	<b>CTX</b>	<b>67</b>
<b>fosfomycins</b>	<b>FOS 50</b>	<b>65</b>
<b>quinolons</b>	<b>NA</b>	21
	<b>CIP</b>	30
<b>colistin</b>	<b>CS 50</b>	<b>62</b>
<b>phenicols</b>	<b>C</b>	<b>56</b>
<b>tetracyclins</b>	<b>TE</b>	46
<b>rifamycins</b>	<b>RA 30</b>	34
<b>sulfamids</b>	<b>SSS 200</b>	<b>56</b>

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765



766 **Table 3.**

Species	Dairy source	Strain #	Class	Tryptamine	Phenylethylamine	Putrescine	Isoamylamine	Cadaverine	Histamine	Tyramine
<i>Citrobacter freundii</i>	Livarot	UCMA 4217	1	32	154	52	780	1539	640	15
<i>Hafnia alvei</i> 1	Livarot	B16	1	52	179	60	734	1490	671	14
<i>Halomonas</i> sp. nov.	Livarot	B39	1	65	210	75	786	1559	729	14
<i>Halomonas venusta</i>	Munster	3D7M	1	N.D.	24	41	715	827	735	6
<i>Halomonas venusta/alkaliphila/hydrothermalis</i>	Munster	4C1A	1	96	199	100	593	1242	826	12
<b>Lb. saerimneri (ODC+, HDC+, LDC+)</b>	<b>control strain</b>	<b>L30a</b>	1	N.D.	13	N.D.	833	994	905	3
<i>Morganella morganii</i>	Munster	3A2A	1	108	261	124	753	1470	983	13
<i>Morganella morganii</i>	Munster	3A5A	1	N.D.	43	66	1137	1304	1202	8
<i>Morganella morganii</i>	Munster	3D4A	1	N.D.	59	155	1279	1529	1706	20
<i>Providencia heimbachae</i>	Epoisses	GR4	1	50	159	85	699	1419	945	47
<i>Serratia liquefaciens</i>	Munster	1B4F	1	112	260	119	727	1462	936	11
<i>Serratia</i> sp. (close <i>S. grimesii</i> )	Epoisses	GB3	1	N.D.	41	66	895	1094	1057	7
<i>Chryseobacterium shigense</i>	cow milk Salers	PCAi B2.3	2	50	N.D.	N.D.	673	1424	44	16
<i>Enterobacter hormaechei</i>	Munster	271	2	N.D.	N.D.	22	859	1442	N.D.	6
<i>Enterobacter hormaechei</i>	Munster	380	2	N.D.	N.D.	N.D.	1402	1158	12	3
<i>Enterobacter hormaechei</i>	Munster	272	2	N.D.	N.D.	N.D.	1458	1253	6	2
<i>Enterobacter hormaechei</i>	Salers	INRA 1439	2	N.D.	3	N.D.	895	450	8	9
<i>Hafnia paralvei</i>	Munster	920	2	N.D.	N.D.	N.D.	582	1319	41	16
<i>Klebsiella oxytoca</i>	Munster	927	2	N.D.	17	N.D.	35	1236	N.D.	11
<i>Pseudomonas</i> grp <i>putida</i>	milk	CV 30.6	2	N.D.	N.D.	19	674	1221	33	27
<i>Pseudomonas</i> grp <i>putida</i>	milk	VRBG 37.3	2	61	10	55	627	1240	18	22
<i>Pseudomonas</i> grp <i>putida</i>	milk	CFC 25.5	2	N.D.	N.D.	N.D.	28	2	N.D.	N.D.
<i>Pseudomonas lundensis</i>	cow milk Salers	PCAi D2.2	2	N.D.	N.D.	7	42	1368	476	14
<i>Pseudomonas stutzeri</i>	Livarot	UCMA 3883	2	N.D.	N.D.	N.D.	648	1042	25	22
<i>Psychrobacter celer</i>	Camembert raw milk	91	2	N.D.	N.D.	21	591	1315	26	9
<i>Raoultella planticola</i>	Munster	924	2	N.D.	N.D.	N.D.	82	946	533	1
<i>Serratia grimesii</i>	Livarot	UCMA 3895	2	N.D.	N.D.	N.D.	1396	1416	13	N.D.
<i>Serratia marescens</i>	Munster	448	2	N.D.	6	1	1305	1491	25	N.D.
<i>Serratia proteomaculans</i>	Munster	1C2F	2	N.D.	3	N.D.	607	753	336	N.D.
<i>Sphingobacterium</i> sp. (close <i>S. faecium</i> )	cow milk Salers	PCAi F2.5	2	N.D.	N.D.	23	21	1287	8	14
<i>Stenotrophomonas maltophilia</i>	cow milk Salers	PCAi I2.2	2	425	N.D.	N.D.	N.D.	1494	N.D.	20
<i>Stenotrophomonas maltophilia/rhizophila</i>	cow milk Salers	PCAi D6.5	2	N.D.	N.D.	3	7	1417	N.D.	23
<i>Acinetobacter</i> sp. (close genospecies 3)	cow milk Salers	PCA E6.10	3	35	N.D.	31	N.D.	N.D.	1	50
<i>Alcaligenes faecalis</i>	Livarot	UCMA 4164	3	N.D.	N.D.	N.D.	3	N.D.	N.D.	10
<i>Alcaligenes faecalis</i> 1	Munster	904	3	N.D.	N.D.	N.D.	867	73	12	27
<i>Chryseobacterium</i> sp. (close <i>C. bovis</i> )	St. Nectaire	Pi 18	3	N.D.	10	46	17	1	3	74
<i>Halomonas alkaliphila</i>	Munster	2B8N	3	N.D.	N.D.	1	N.D.	1	N.D.	N.D.
<i>Halomonas venusta/alkaliphila/hydrothermalis</i>	Livarot	B19b	3	N.D.	N.D.	N.D.	N.D.	1	N.D.	13
<b>Lb. brevis (TyrDC+, Agdi+)</b>	<b>control strain</b>	<b>IOEB9809</b>	3	N.D.	9	N.D.	1	1	1	664
<b>Lb. buchneri (HDC+)</b>	<b>control strain</b>	<b>DSM5987</b>	3	N.D.	N.D.	1	19	1	570	7
<i>Ochrobactrum</i> sp.	cow milk Salers	Ti 2.7	3	N.D.	N.D.	4	4	1	N.D.	N.D.
<i>Pantoea agglomerans</i>	cow milk Salers	PCA Q6.3	3	N.D.	N.D.	N.D.	14	194	N.D.	31
<i>Proteus</i> grp. <i>vulgaris</i>	Munster	929	3	N.D.	4	111	N.D.	1	N.D.	13
<i>Proteus</i> grp. <i>vulgaris</i>	Munster	932	3	N.D.	N.D.	N.D.	N.D.	1	N.D.	14
<i>Proteus</i> grp. <i>vulgaris</i>	Munster	941	3	N.D.	6	59	N.D.	1	N.D.	N.D.

<i>Proteus</i> grp. <i>vulgaris</i>	Munster	942	3	N.D.	3	88	3	1	N.D.	11
<i>Proteus heimbachae</i>	Munster	943	3	N.D.	10	N.D.	1	2	N.D.	28
<i>Proteus heimbachae</i>	Munster	945	3	N.D.	6	9	1	1	3	16
<i>Proteus rettgeri</i>	Munster	947	3	N.D.	9	13	N.D.	1	N.D.	18
<i>Proteus</i> sp. (close <i>P. hauseri</i> )	Livarot	UCMA 3779	3	<b>112</b>	17	<b>111</b>	8	N.D.	N.D.	27
<i>Proteus</i> sp. (close <i>P. hauseri</i> )	Livarot	UCMA 3780	3	<b>110</b>	12	92	44	8	9	21
<i>Proteus</i> sp. nov.	Munster	944	3	N.D.	2	<b>1083</b>	N.D.	12	N.D.	20
<i>Providencia</i> sp. nov.	Epoisses	GM2	3	N.D.	5	71	N.D.	3	N.D.	28
<i>Providencia</i> sp. nov.	Epoisses	GB1	3	N.D.	9	33	N.D.	2	5	37
<i>Pseudoalteromonas haloplanktis/nigrifaciens</i>	Epoisses	Pr 1	3	N.D.	26	N.D.	0	N.D.	N.D.	13
<i>Pseudomonas</i> grp <i>fluorescens</i>	St. Nectaire	PCA38	3	N.D.	N.D.	N.D.	7	N.D.	N.D.	1
<i>Pseudomonas</i> grp <i>putida</i>	Livarot	UCMA 3896	3	N.D.	N.D.	28	N.D.	N.D.	N.D.	6
<i>Pseudomonas</i> grp <i>putida</i>	milk	CFC25.4	3	N.D.	N.D.	N.D.	18	2	1	1

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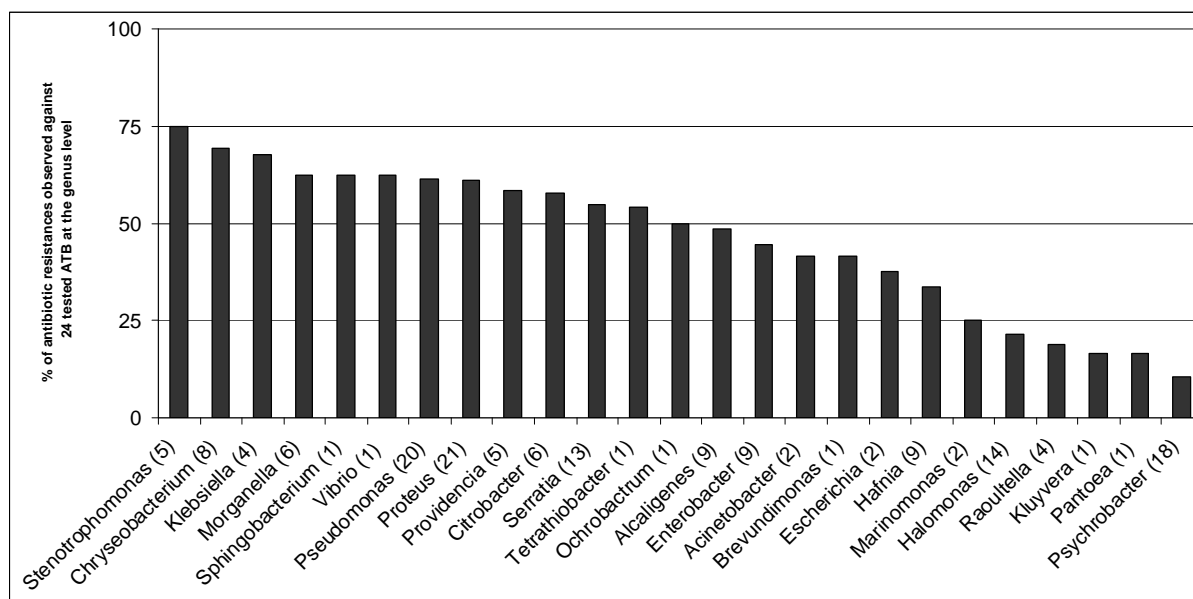
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780 **Figure 1.**

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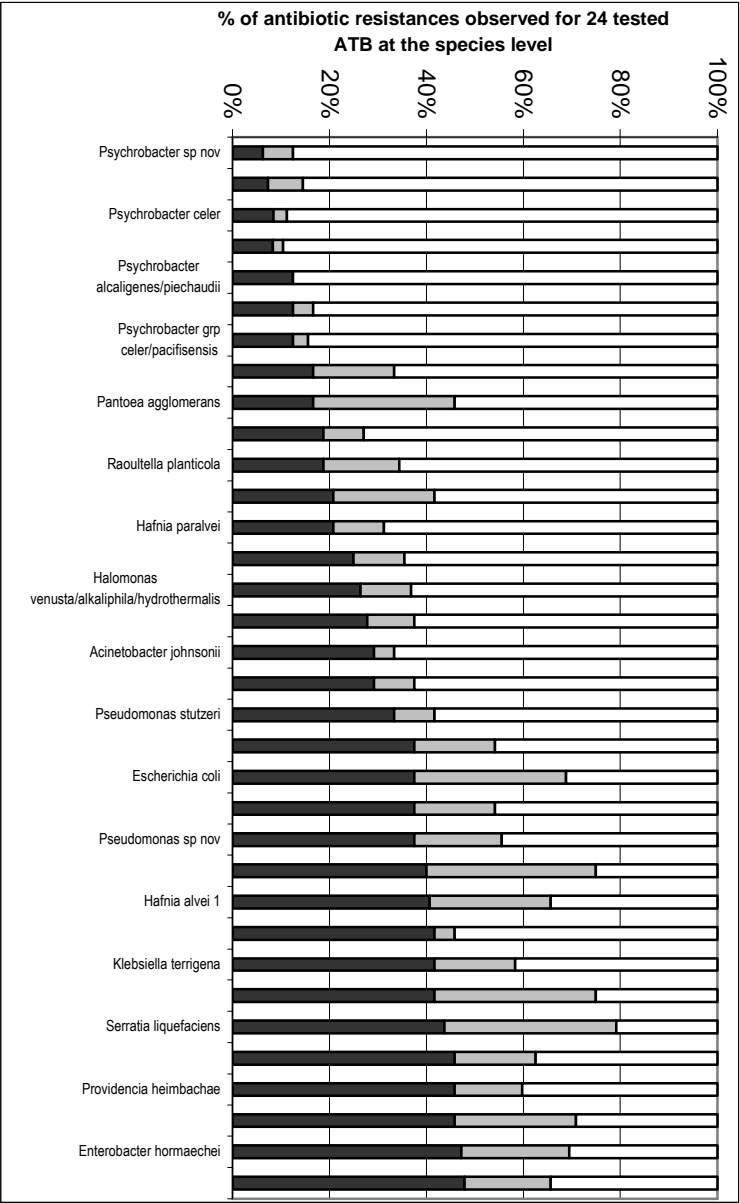
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790 **Figure 2A.**

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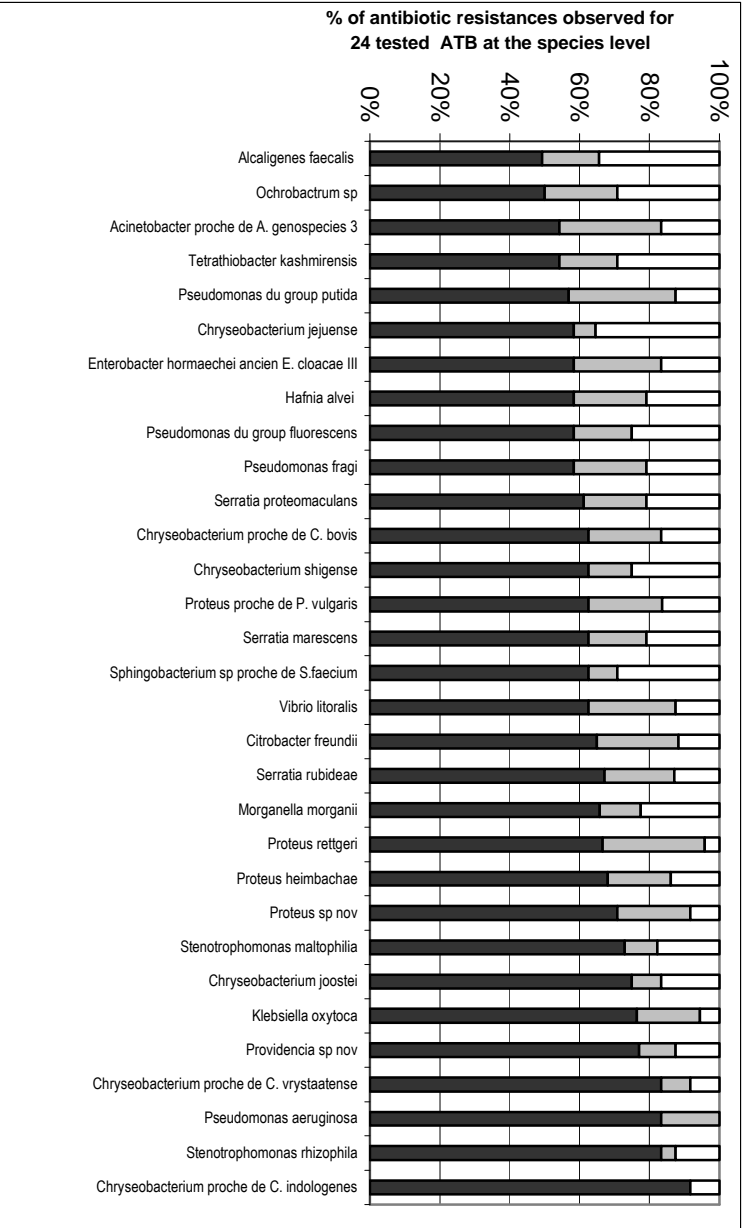
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**Figure 2B.**

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815 **Figure 1.** Spectrum of action of 24 antibiotics against a panel of 173 Gram-negative dairy  
816 isolates. Results are presented as the number of strains per genus (indicated in brackets) that  
817 were resistant to the tested antibiotics. Antibiotics used : amikacin, AN; gentamicin, GM;  
818 tobramycin, TM; imipenem IPM; cefalotin, CF; cefotaxime, CTX; ceftazidime, CAZ;  
819 aztreonam, ATM; amoxicillin, AMX; amoxicillin/clavulanic acid, AMC; ampicillin, AM;  
820 mecillinam, MEC; piperacillin, PIP75; piperacillin/tazobactam, TZP85; ticarcillin, TIC;  
821 ticarcillin/clavulanic acid, TCC; colistin, CS50; ciprofloxacin, CIP; fosfomycin, FOS;  
822 chloramphenicol, C; nalidixic acid, NA; rifampicin, RA30; sulfamid, SSS200; tetracycline,  
823 TE.

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825 **Figure 2A.** Incidence of antibiotic resistance (% of strains resistant ■, intermediate ■ and  
826 sensitive □) with an overall resistance below 50% of strains towards 24 antibiotics in Gram-  
827 negative dairy strains. Analysis at the species level.

828

829 **Figure 2B.** . Incidence of antibiotic resistance (% of strains resistant ■, intermediate ■ and  
830 sensitive □) with an overall resistance above 50% of strains towards 24 antibiotics in Gram-  
831 negative dairy strains. Analysis at the species level.

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842 **Table 1.** Overall repartition of surface and cheese milk/core isolates according to phylum,  
843 family, genus and species level identification. Phylum designations : gamma-P, gamma-  
844 Proteobacteria; alpha-P, alpha-Proteobacteria; beta-P, beta-Proteobacteria; C-F-B, Cytophaga-  
845 Flavobacterium-Bacteriodes of the superfamily V. In the case of more than 1% divergence in  
846 nucleotide sequences between a Gram-negative dairy isolate with known species sequences,  
847 Gram-negative isolates were identified as being most closely related to one of the already  
848 described and valid species names within a genus. A sequence presenting more than 2%  
849 divergence with validated sequences are considered as potential new species.

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851 **Table 2.** Spectrum of action of 24 antibiotics against a panel of 173 Gram-negative dairy  
852 isolates. Numbers in bold signify that >50% strains were resistant. Antibiotics used :  
853 amikacin, AN; gentamicin, GM; tobramycin, TM; imipenem IPM; cefalotin, CF;  
854 cefotaxime, CTX; ceftazidime, CAZ; aztreonam, ATM; amoxicillin, AMX;  
855 amoxicillin/clavulanic acid, AMC; ampicillin, AM; mecillinam, MEC; piperacillin, PIP75;  
856 piperacillin/tazobactam, TZP85; ticarcillin, TIC; ticarcillin/clavulanic acid, TCC; colistin,  
857 CS50; ciprofloxacin, CIP; fosfomycin, FOS; chloramphenicol, C; nalidixic acid, NA;  
858 rifampicin, RA30; sulfamid, SSS200; tetracycline, TE.

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860 **Table 3.** *In vitro* production of biogenic amines by food-related Gram-negative strains as  
861 determined by HPLC. Only trace amounts of methylamine, ethylamine, spermidine and  
862 spermine were detected for all strains, therefore, results are not presented in this table.  
863 Standard deviations were <1.5 mg/l for all samples with BA productions up to 500mg/l and  
864 were <13.8 mg/l for all samples above 500mg/l BA produced.