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Short communication

Characterization of biocide-tolerant bacteria isolated from cheese and dairy small-medium enterprises

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ABSTRACT

A collection of 120 bacterial isolates from small medium enterprises involved in the production of cow milk and the manufacture of goat cheese were screened for sensitivity to biocides benzalkonium chloride (BC), cetrimide (CT), hexadecylpyridinium chloride (HDP), triclosan (TC), hexachlorophene (CF) and poly-(hexamethylen guanidinium) hydrochloride (PHMG). Nineteen isolates were selected according to biocide tolerance and identified by 16S rDNA sequencing as *Lactococcus* sp. (6) *Enterococcus* sp. (1), *Lactobacillus* sp. (4), *Bacillus* sp. (1) *Escherichia* sp. (5), *Enterobacter* sp. (1) and *Helicobacter* sp. (1). These were further characterised regarding antimicrobial resistance phenotype and genotype. Several isolates were multiply (3 or more) tolerant to biocides or resistant to antibiotics, but only two *Escherichia* sp. isolates and *Enterobacter* sp. were multiply resistant to biocides and antibiotics. Statistical analysis of biocide tolerance and antibiotic resistance revealed significant positive correlations between different biocides and between biocides and antibiotics. The biocide tolerance genes most frequently found were *qacEΔ1* and *qacA/B*. The sulfonamide resistance gene *sul1* was found in two *Escherichia* sp. isolates and in *Enterobacter* sp., all of which also carried *qacEΔ1*. Beta-lactam (bla_{CTX-M}, bla_{PSE}) and tetracycline resistance genes [*tet(A), tet(C)* and *tet(D)*] were detected. Efflux pump genes *acrB* and *mdfA* were found in most Gram-negative isolates. Results from the study suggest that exposure to biocides can indirectly select for antibiotic resistance.

1. Introduction

Biocides have become very popular in the non-specific control of microorganisms in a variety of environmental conditions, from the food industry to sanitation. Among quaternary ammonium compounds, benzalkonium chloride is widely used as disinfectant and cationic surface active agent for sanitation in food processing lines and surfaces in the food industry (Ueda and Kuwabara, 2007). Hexadecylpyridinium chloride was approved by the US-FDA for decontaminating raw poultry (Food and Drug Administration, 2004). The polymeric guanide polyhexamethyleneguanidine is used in different formulations for sanitizing surfaces of utensils and instruments in the food industry (Ueda and Kuwabara, 2007). The bis-phenol triclosan (TC) had a wide range of applications, from health care products to food industry materials (Yazdankhah et al., 2006). Its toxicity and endocrine-disrupting activity has prompted serious reconsideration or direct prohibition of its use, at least in cosmetics (European Union, 2016).

One concern about the extensive use of biocides is that bacterial adaptation upon exposure to biocides may increase antibiotic resistance (Ortega-Morente et al., 2013; SCENIHR, 2009). Furthermore, bacterial exposure to sub-inhibitory concentrations of decontaminants (trisodium phosphate, acidified sodium chlorite, citric acid, chlorine dioxide or peroxyacetic acid) also induced reduced susceptibility to various antibiotics (Alonso-Hernando et al., 2009). Small-medium enterprises are important in the production of traditional fermented foods including traditional fermented cheeses. Bacteria from the environment where the raw material is handled and where the cheeses are produced will eventually be present in the final product or even play a key role in the fermentation process. Therefore, it may be interesting to investigate their possible contribution in transmission of antimicrobial resistance in the food chain. The purpose of the present

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study was to determine biocide tolerance in bacterial isolates from local goat cheese and cow milk small-medium enterprises, and to analyse antibiotic resistance in the biocide-tolerant isolates.

2. Materials and methods

2.1. Sampling and bacterial isolation

Swab samples of two goat milk cheese SMEs and one cow's milk SME were taken from milk, milking equipment, tanks, cheese ripening chambers and sinks. Two samplings were performed on each SME within 3 months difference. Swab samples were resuspended in Brain Heart Infusion (BHI) broth (Scharlab, Barcelona, Spain) and spread on Trypticase Soy Agar (TSA, Scharlab). After 24–48 h incubation at 30 °C, colonies were purified by streaking on TSA, and the pure cultures were examined by Gram-staining and stored at −80 °C in Trypticase Soy Broth (TSB, Scharlab) supplemented with 20% glycerol.

2.2. Determination of biocide tolerance

A collection of 120 bacterial isolates (including 80 Gram-positives and 40 Gram-negatives) were screened for biocide sensitivity. Benzalkonium chloride (BC), cetrimide (CT), hexadecylpyridinium chloride (HDP), triclosan (TC) and hexachlorophene (CF) were from Sigma-Aldrich (Madrid, Spain). BC commercial solution contained 50% (wt/v) of the active compound. TC and CF were dissolved (10% wt/v) in 96% ethanol. HDP (5%) and CT (10%) were dissolved aseptically in sterile distilled water. Biocide solutions were stored at 4 °C for ≤7 days. Poly-(hexamethylen guanidinium) hydrochloride (PHMG) solution (containing 7.8% of PHMG, by weight) was a kind gift of Oy Soft Protector Ltd (Espoo, Finland). Minimal inhibitory concentrations (MIC's) to biocides were determined by the broth microdilution method on 96-well bottom microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ). Briefly, serial dilutions of each biocide were inoculated (1%, vol/vol) with overnight cultures of bacterial strains grown in TSB. Growth and sterility controls were included for each isolate. Microtiter plates were incubated at 30 °C for 24–48 h and optical density readings (OD 595 nm) were performed with an iMark Microplate Reader (BioRad, Madrid). All assays were done in triplicate.

As result from this preliminary screening, a collection of 19 isolates were selected according to their biocide tolerance for further studies as follows.

2.3. Determination of antibiotic resistance

Selected isolates were tested for antibiotic resistance by disk diffusion method (CLSI, 2014) on cation-adjusted Mueller-Hinton agar (Fluka, Sigma-Aldrich, Madrid, Spain). Ampicillin (AMP, 30 μg), ceftazidine (CFZ, 30 μg), cefotaxime (CTX, 30 μg), imipenem (IPM, 10 μg), streptomycin (SM, 10 μg), netilmicin (NET, 30 μg), tetracycline (TET, 30 μg), ciprofloxacin (CIP, 5 μg), nalidixic acid (NA, 30 μg) and trimethoprim/sulfamethoxazole (TMP/STX, 25/75 μg) were from Biomérieux (Madrid, Spain). Chloramphenicol (CMP, 30 μg) was from BBL (Madrid, Spain).

2.4. Determination of strain sensitivity to carvacrol, thymol, and chemical preservatives

Strain sensitivity to other antimicrobials was tested on TSB by microdilution in 96-well microtiter plates as described for biocides. Briefly, overnight cultures were inoculated on TSB (0.1%, vol/vol) supplemented with different concentrations of carvacrol (Sigma), thymol (Sigma), sodium lactate (SL, Sigma) and trisodium phosphate (TSP, Sigma) or lysozyme-EDTA combinations prepared as follows. Solutions containing 100 mg/l lysozyme and 5 mM EDTA (both from Sigma) in TSB were combined in different proportions to yield the following final concentrations: A, 30 mg/l lysozyme plus 3.5 mM EDTA; B, 50 mg/l lysozyme plus 2.5 mM EDTA; C, 70 mg/l lysozyme plus 1.5 mM EDTA. Growth was determined spectrophotometrically at 595 nm after 24 h incubation at 30 °C.

2.5. Identification of biocide-tolerant isolates

Selected isolates were identified by conventional tests (Gram staining, catalase and oxidase tests) and 16S rDNA sequencing. DNA was extracted with a bacterial genomic DNA extraction kit (GenElute™, Sigma-Aldrich, Madrid). 16 S rDNA was amplified as described by Abriouel et al. (2005), and sequences were analysed with the BLAST algorithm available at the National Centre for Biotechnology Information (NCBI, USA).

2.6. Investigation of biocide and antibiotic resistance genes

QAC resistance genes were determined by PCR amplification according to Noguchi et al. (2005) for *qacA/B* and Smith et al. (2008) for *qacC* (*smr*), *qacG*, *qacH* and *qacJ*. The presence of *qacE* and *qacEΔ1* genes and their association with Class I integrons was investigated as described by Chuanchuen et al. (2007), by using forward primer qacEF in combination with reverse primers qacER, qacEΔ1R and sulR, respectively. The integrase gene *intI1* was investigated with intF and intR primers (Chuanchuen et al., 2007).

The beta-lactamase resistance genes investigated were bla_{TEM} (Sáenz et al., 2004), bla_{PSE} (Chiu et al., 2006), bla_{CTX-M} and $bla_{CTX-M-2}$ (Bertrand et al., 2006). Aminoglycoside resistance gene *aac(6′)-Ib-cr* was investigated according to Park et al. (2006). Tetracycline resistance genes *tet(A), tet(B), tet(C), tet(D), tet(F)* and *tet(G)* were investigated according to Ng et al. (2001). Sulfonamide and trimethoprim resistance genes *sul1*, *dfrA12* and *dfrA15* were determined according to Guerra et al. (2001). The presence of efflux pump genes *acrB* and *mdfA* was studied according to Swick et al. (2011). The *oxqA* gene of the OqxAB multidrug efflux pump was investigated according to Hansen et al. (2005).

2.7. Statistical analysis

The percentages of resistance obtained for each biocide and antibiotic were used for calculation of Pearson correlation coefficients (r) and Principal component analysis by using IBM SPSS Statistics 22 (IBM Corporation, Armonk, New York, USA) and Mystat statistics and graphics package (Systat Software, Hounslow, London, UK; evaluation version 2015.1). Positive correlations were defined as very weak (0.00–0.19), weak (0.20–0.39), moderate (0.4–0.59), strong (0.60–0.79) or very strong (0.80–0.99), with a P significances of <0.05 or 0.01.

3. Results

3.1. Incidence of biocide tolerance in bacterial isolates

A total of 127 isolates (including 85 Gram-positives and 42 Gram-negatives) were obtained after the sampling procedure. A few isolates yielded very small colonies or grew very slowly and were discarded, leaving round numbers of 80 Gram-positives and 40 Gram-negatives for further study.

According to MIC distributions, several Gram-positive isolates were considered to be biocide-tolerant (Table 1): 3.75% for BC (MIC > 7.5 mg/l) and for CT (MIC > 10 mg/l), 8.75% for TC

Table 1
Minimum inhibitory concentrations for biocides in Gram-positive bacterial isolates from dairy industry.

Biocide	Biocide minimum inhibitory concentration (mg/l)[a]									
	2.5	5	7.5	10	25	50	75	100	250	500
BC	18	49	10	1	1	1				
CT		23	30	24	1	1	1			
HDP			13	67						
TC	2	24	47		3	1	1	1	1	
CF		7	9	64						
PHMG			8	20	47	1	3	1		

BC, benzalkonium chloride; CT, cetrimide; HDP, hexadecylpyridinium chloride; TC, triclosan; CF, hexachlorophene; PHMG, poly-(hexamethylen guanidinium) hydrochloride.
[a] The numbers of isolates for each minimum inhibitory concentration are shown. Total number of Gram-positive isolates tested: 80.

(MIC > 7.5 mg/l), and 6.25% for PHMG (MIC> 25 mg/l). None of isolates were be tolerant to HDP or CF. Most isolates were tolerant to only one biocide, except for two isolates that were tolerant to three different biocides and one that was able to tolerate four.

A 15.0% of Gram-negative isolates were considered to be tolerant to BC (MIC > 75 mg/l), 5.0% to CT (MIC > 50 mg/l), 2.5% for HDP (MIC > 10 mg/l), 5.5% to TC (MIC > 5 mg/l), and 12.5% to CF (MIC > 50 mg/l) (Table 2). A 17.5% of isolates had MICs above 25 mg/l for PHMG. Most isolates were able to tolerate three or more biocides, and one isolate tolerated all six biocides assayed.

3.2. Characterization of biocide-tolerant isolates

3.2.1. Identification of isolates

Eleven of the Gram-positive isolates were identified as lactic acid bacteria (mainly of the genera *Lactococcus* and *Lactobacillus*), and one as *Bacillus* sp. (Table 3). Six Gram-negative isolates were identified as members of Fam. *Enterobacteriaceae* (mainly *Escherichia* sp.), and one as *Helicobacter* sp. (Table 3).

3.2.2. Antibiotic resistance

All isolates were sensitive to CIP. However, most of them (12 out of 19) were resistant to AMP, and in addition six of them were also resistant to CFZ and CTX (Table 3). Resistance to TET and to TMP/STX, were also found frequently among the selected isolates, most of which were also resistant to beta-lactams. A few isolates were multiply resistant to antibiotics and biocides (Table 3), specially *Enterobacter* sp. and *Escherichia* sp. UJAv9m and UJAv18m. Among the Gram-positive isolates, two *Lactococcus* sp. were tolerant to three biocides and resistant to two antibiotics.

Table 2
Minimum inhibitory concentrations for biocides in Gram-negative bacterial isolates from dairy industry.

Biocide	Biocide minimum inhibitory concentration (mg/l)[a]									
	2.5	5	7.5	10	25	50	75	100	250	500
BC					3	22	9	5	1	
CT				3	11	24		1	1	
HDP				39		1				
TC	16	22		1	1					
CF					23	12	4		1	
PHMG			1	5	27	1	3	2	1	

BC, benzalkonium chloride; CT, cetrimide; HDP, hexadecylpyridinium chloride; TC, triclosan; CF, hexachlorophene; PHMG, poly-(hexamethylen guanidinium) hydrochloride.
[a] The numbers of isolates for each minimum inhibitory concentration are shown. Total number of Gram-positive isolates tested: 40.

3.2.3. Tolerance to chemical preservatives and to lysozyme

Only a few LAB were more tolerant to carvacrol and thymol, including the *Lactococcus* sp. isolate UJAq7e tolerant to three biocides and resistant to two antibiotics (Table 3). All Gram-negative isolates were inhibited by carvacrol and thymol at 0.4%, regardless of their biocide-antibiotic resistance phenotype (Table 3). Eight of the LAB isolates were tolerant to 3% TSP, and six of them also required a high concentration of SL (6%) for inhibition. Most Gram-negatives were also inhibited by 4.5% SL, except the multi-resistant *Enterobacter* sp. and *Escherichia* sp. UJAv18m. The lysozyme-EDTA combinations having a higher proportion of EDTA were more effective (Table 3). Among LAB isolates tolerant to all lysozyme-EDTA combinations and to TSP, one (UJAq9e) was tolerant to 4 biocides and resistant to ampicillin, and another (UJAq3e) was tolerant to TC and resistant to three antibiotics.

3.2.4. Genetic determinants of resistance

The efflux pump genes *acrB* and *mdfA* were found in most Gram-negative isolates (Table 3). The biocide tolerance genes most frequently found were *qacEΔ1* and *qacA/B*. Since *qacEΔ1* is frequently linked to integrons, the integrase gene *intl1* was also investigated by PCR. Nevertheless, *intl1* was found only in *Escherichia* sp. UJAv9m. The sulfonamide resistance gene *sul1* (also linked to integrons carrying *qacEΔ1*) was found in two *Escherichia* sp. isolates and in *Enterobacter* sp., all of which also carried *qacEΔ1*. PCR with a forward primer for *qacE* and a reverse primer for *sul1* yielded positive results only for *Helicobacter* sp., indicating an adjacent location of both genes (Table 3). Four isolates resistant to beta-lactams carried $bla_{\text{CTX-M}}$ and another one carried bla_{PSE}. Tetracycline resistance genes were detected at low frequency. Other antibiotic resistance genes investigated were not detected.

3.2.5. Correlations between biocide tolerance and antibiotic resistance

Among the biocide-tolerant LAB, a statistically-significant positive correlation (Fig. 1A, Supplementary Table 1) was found between BC and CT. Both biocides correlated positively with AMP resistance. IMP, CM, TET and NAL resistances were all positively correlated. SM was negatively correlated with CFZ and with NET, while CFZ and NET were positively correlated. For Gram-negative bacteria, statistically-significant positive correlations (Fig. 1B, Supplementary Table 2) were found between BC and CF, and between TC and CT. Positive correlation was detected for antibiotics CTX with CFZ, and AMP with TM-STX. Positive correlations were also observed for AMP and TM-STX with PHMG and for SM with HDP. Antibiotic resistance to CTX and CFZ showed statistically-significant negative correlations with SM resistance and also with HDP tolerance. All other high positive correlations detected were not statistically significant ($P > 0.05$).

4. Discussion

Results from the present study revealed the presence of biocide-tolerant bacteria in SMEs involved in the production of goat cheese or cow milk. A previous study showed that Gram-positive bacteria differ from Gram-negatives in biocide tolerance (Morrissey et al., 2014), hence both groups were studied separately in the present study. A large fraction of the biocide-tolerant isolates belonged to lactic acid bacteria (LAB), as expected from the sampled environment. Most of the LAB isolates were tolerant to only one biocide, and only a few seemed to be multiply-tolerant. Arioli et al. (2013) also found no systematic co-tolerance between two or more of the biocides tested in LAB. However, a few LAB isolates from the present study showed co-resistance to biocides and antibiotics. These results raise concerns that biocides could indirectly select

Table 3
Characterization of selected biocide-tolerant isolates.

Isolate	Biocide tolerance	Antibiotic resistance	Genetic determinants	Carvacrol (MIC, %)	Thymol (MIC, %)	Na-Lactate (MIC, %)	TSP[a] (MIC, %)	Lysozyme-EDTA (A)[b]	Lysozyme-EDTA (B)[b]	Lysozyme-EDTA (C)[b]
Lactococcus sp. UJAq3e	TC	AMP, CTX, IMP	bla_{CTX-M}	0.5	0.25	6.0	>3.0	R	R	R
Lactococcus sp. UJAq4e	TC		*qacEΔ1*	0.5	0.25	6.0	>3.0	S	R	R
Lactococcus sp. UJAq7e	BC, CT, TC	AMP, CM	*qacEΔ1*	>0.5	0.5	6.0	>3.0	S	R	R
Lactococcus sp. UJAv8c	PHMG	TET, NAL, TM/STX	*qacA/B, tet(D)*	0.4	0.25	4.5	3.0	S	S	S
Lactococcus sp. UJAv2t	PHMG	TET, CM, SM, NAL, TM/STX	*qacA/B, tet(C)*	0.4	0.25	4.5	3.0	S	S	S
Lactococcus sp. UJAq5e	BC, CT, TC	AMP, TET	*qacEΔ1*	0.5	0.1	6.0	>3.0	S	R	R
Enterococcus sp. UJAq9m	TC	AMP, SM		>0.5	0.25	4.5	>3.0	S	S	S
Lactobacillus sp. UJAq2ef	TC	NET	*qacEΔ1*	>0.5	0.5	6.0	>3.0	R	R	R
Lactobacillus sp. UJAq9e	BC, CT, TC, PHMG	AMP	*qacEΔ1*	0.5	0.25	6.0	>3.0	R	R	R
Lactobacillus sp. UJAv14c	PHMG	AMP, CTX, CFZ, TM/STX	*qacA/B,* bla_{CTX-M}	0.4	0.25	4.5	1.0	S	S	S
Lactobacillus sp. UJAv19t	PHMG	AMP, CTX, CFZ, TM/STX	bla_{PSE}	0.4	0.4	4.5	>3.0	S	S	S
Bacillus sp. UJAv17t	BC	AMP, CTX, CFZ, TM/STX	bla_{CTX-M}	0.25	0.25	3.0	1.5	S	S	S
Escherichia sp. UJAv9m	BC, CF, PHMG	AMP, TET, TM/STX	*qacA/B, qacEΔ1, sul1, intl1, acrB, mdfA tet(A)*	0.25	0.4	4.5	3.0	S	S	R
Escherichia sp. UJAv10me	BC, TC, CF, PHMG		*qacA/B, qacEΔ1, acrB, mdfA*	0.4	0.25	4.5	3.0	S	S	S
Escherichia sp. UJAv12t	BC, CF, PHMG		*qacEΔ1, acrB, mdfA*	0.4	0.4	4.5	3.0	S	S	R
Escherichia sp. UJAv13me	BC, CF, PHMG		*qacEΔ1, acrB, mdfA*	0.4	0.4	4.5	3.0	S	S	R
Escherichia sp. UJAv18m	BC, CT, PHMG	AMP, CTX, CFZ, TET, TM/STX	*qacA/B, qacEΔ1, sul1, acrB, mdfA, tet(A)*	0.4	0.4	6.0	3.0	S	R	R
Enterobacter sp. UJAv11me	BC, CT, HDP, TC, CF, PHMG	AMP, SM, TET, TM/STX	*qacEΔ1, sul1, acrB, mdfA, tet(A)*	0.4	0.4	6.0	3.0	S	R	R
Helicobacter sp. UJAv20me	PHMG	AMP, CTX, CFZ, TM/STX	*qacEΔ1, qacEF/sulR,* bla_{CTX-M}	0.4	0.4	4.5	3.0	S	S	S

BC, benzalkonium chloride; CT, cetrimide; HDP, hexadecylpyridinium chloride; TC, triclosan; CF, hexachlorophene; PHMG, poly-(hexamethylen guanidinium) hydrochloride; AMP, ampicillin; CTX, cefotaxime; CFZ, ceftazidime; IMP, imipenem; SM, streptomycin; CIP, ciprofloxacin; CM, chloramphenicol; TET, tetracycline; NAL, nalidixic acid; NET, netilmicin; TM/STX, trimethoprim-sulfametoxazol.

[a] TSP, trisodium phosphate.

[b] Lysozyme-EDTA combinations: A) 30 mg/l lysozyme plus 3.5 mM EDTA; B, 50 mg/l lysozyme plus 2.5 mM EDTA; C, 70 mg/l lysozyme plus 1.5 mM EDTA. R, resistant; S, sensitive.

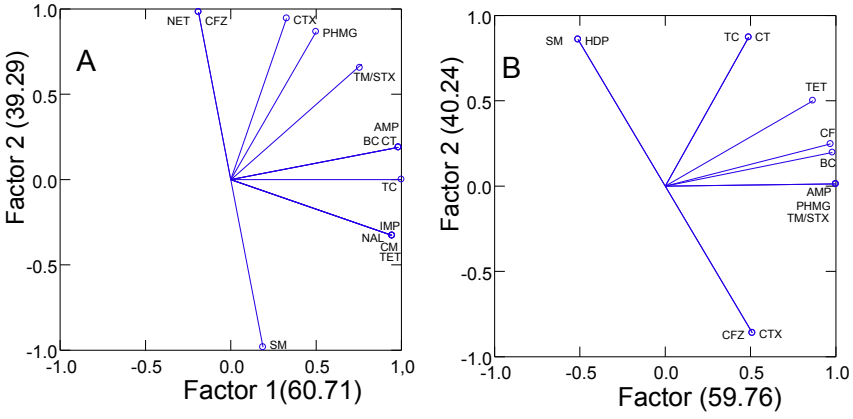

Fig. 1. Factor loading plots for biocide tolerance and antimicrobial resistance in lactic acid bacteria (**A**) and Gram-negative bacteria (**B**) from cheese and dairy environments. Abbreviations: BC, benzalkonium chloride; CT, cetrimide; HDP, hexadecylpyridinium chloride; TC, triclosan; CF, hexachlorophene; PHMG, poly-(hexamethylen guanidinium) hydrochloride; AMP, ampicillin; CTX, cefotaxime; CFZ, ceftazidime; IMP, imipenem; SM, streptomycin; CIP, ciprofloxacin; CM, chloramphenicol; TET, tetracycline; NAL, nalidixic acid; NET, netilmicin; TM/STX, trimethoprim-sulfametoxazol.

multiply antibiotic resistant LAB in the food chain, thus increasing the risks of prevalence and transmission of antibiotic resistance.

Biocide tolerance is often mediated by efflux pumps with broad sustrate specificity (Poole, 2007). All biocide-tolerant *Escherichia* sp. isolates as well as *Enterobacter* sp. carried efflux pump genes *acrB* and *mdfA*. Cells expressing MdfA are substantially more resistant to a diverse group of cationic or zwitterionic lipophilic compounds and to chemically unrelated, clinically important antibiotics (Edgar and Bibi, 1997). In the present study, *qacEΔ1* was found in five LAB isolates. It also was the most frequent biocide resistance gene among Gram-negative isolates, associated with *acrB* and *mdfA* in six isolates. Several Gram-negative isolates carried *sul1* gene in addition to *qacEΔ1*. This combination is typically associated to Class I integrons (White et al., 2001). Furthermore, one of these isolates also tested positive for *intl1* gene. In the *Helicobacter* sp. isolate, *qacEΔ1* seemed to be located close to *sul1*. Most gene cassettes in class 1 integrons confer resistance to various antibiotic classes. Remarkably, the multi-resistant isolate *Enterobacter* sp. carried several resistance genes and an antibiotic resistance profile resembling the ASSuT (ampicillin, streptomycin, sulfonamide, tetracycline) phenotype widely disseminated among *Salmonella enterica* (Briggs and Fratamico, 1999). Kücken et al. (2000) reported that *E. cloacae* carrying *qacEΔ1* gene was multiply antibiotic resistant. Antibiotic resistance and specially the presence of beta-lactamase genes in biocide-tolerant isolates from the studied dairy environments is a matter of concern. The genetic background of biocide-tolerant antibiotic-resistant Gram-negative isolates described here deserves to be investigated in depth.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.10.008.

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