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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