International Journal of Food Microbiology 163 (2013) 1–5

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Assessment of the susceptibility of lactic acid bacteria to biocides

Stefania Arioli [a,*], Marina Elli [b], Giovanni Ricci [a], Diego Mora [a]

[a] DeFENS Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy
[b] AAT-Advanced Analytical Technology, Piacenza, Italy

ARTICLE INFO

Article history:
Received 2 July 2012
Received in revised form 10 January 2013
Accepted 8 February 2013
Available online 16 February 2013

Keywords:
Biocides
Lactic acid bacteria
Lactobacillus
Lactococcus garvieae
Streptococcus salivarius
Streptococcus thermophilus

ABSTRACT

Biocides are antimicrobial compounds that are widely used in the food industry and medical environments. In this study, we determined the Minimum Inhibitory Concentrations (MICs) by the microdilution method of four different biocides for a large collection of LAB of different origins. The tested isolates belong to 11 species of the *Lactobacillus* genus and to *Streptococcus thermophilus*, *Streptococcus salivarius* and *Lactococcus garvieae*. The results obtained in this study indicate that low susceptibilities to benzalkonium chloride (BC), triclosan (Tr), chlorhexidine (Ch), and sodium hypochlorite (SH) are not frequent among LAB. Moreover, no systematic co-tolerance between two or more tested biocides was found; that is, strains displaying high MIC values and thus low sensitivity to one of the biocides did not show higher MIC values for the other biocides.

1. Introduction

Biocides are antimicrobial molecules that play an essential and effective role in limiting the spread of infection and disease and are important to the food industry as an aid to control the microbial contamination of the environment (Condell et al., 2012; McBain et al., 2002). To improve hygiene measures and ensure food safety, the food industry has increased the use of biocides and chemical-based disinfectants to control the microbial ecology of the production environment (Lansgrud et al., 2003). Moreover, disinfectants are largely present in cosmetic, hygiene, and personal care products, such as eyewash/artificial tears, facial cleansers, creams and lotions, and hand sanitisers (Buffet-Bataillon et al., 2012; Gilbert and Moore, 2005). Unlike antibiotics, which are selectively toxic and act at specific sites within the bacterial cell (Gilbert and McBain, 2003), most biocides do not have a distinct bacterial cell target upon which to act (Condell et al., 2012). Only for triclosan (McMurry et al., 1998) and for chlorhexidine (Komljenovic et al., 2010) have defined mechanisms of action been proposed and demonstrated; these biocides block lipid synthesis in *Escherichia coli* and interact with the bacterial plasma membrane, respectively.

LAB are a group of anaerobic Gram-positive bacteria, that are non-sporulating and acid tolerant and can ferment hexose sugars and a variety of nutrients to produce primarily lactic acid (Klaenhammer et al., 2005). LAB are common microorganisms in foods and represent a large amount of the natural intestinal microbiota of humans and most animals (Tannock, 1995). Among LAB, *Streptococcus thermophilus* and several *Lactobacillus* species are largely employed in the dairy industry as starter or non-starter Lactic Acid Bacteria (NSLAB). Such microorganisms are responsible for matrix acidification due to their metabolic and biochemical activities and are involved in a number of biological modifications, allowing food transformation and thus contributing to the quality of finished products (Comunian et al., 2010; Bourdichon et al., 2012). Given their long history of consumption in traditional fermented foods, most species of LAB have been awarded the status of "Generally Recognized as Safe" (GRAS) by the American Food and Drug Agency (Ammor et al., 2007) and proposed for QPS status (Qualified Presumption of Safety) by EFSA (EFSA, 2007, 2008a, 2008b), whereas *Streptococcus salivarius* and *Lactococcus garvieae*, which are currently classified in Risk Group 2 (according to the German legislation, Committee on Biological Agents – ABAS – www.baua.de/abas), the safety status has not yet defined and is limited to only a few strains of each species. In fact, some *S. salivarius* strains are used as probiotics while others are considered opportunistic pathogens (Guglielmetti et al., 2010). *L. garvieae* is one of the most important bacterial fish pathogens, and it is found in dairy products produced from raw milk, such as artisanal cheeses (Vendrell et al., 2006; Alegria et al., 2009; Ricci et al., 2012). The objective of this study was to determine the Minimum Inhibitory Concentrations (MICs) of four biocides (triclosan, chlorhexidine, benzalkonium chloride, and sodium hypochlorite) that are commonly used in the food industry and/or in products for personal care (hand washing, toothpaste, cream) for a large collection of LAB: 135 strains belonging to *S. thermophilus*, 44 strains of *S. salivarius*, 42 strains of *L. garvieae*, and 245 strains belonging to the *Lactobacillus* genus.

* Corresponding author at: DeFENS, University of Milan, Via Celoria 2, 20133, Milano, Italy. Tel.: +39 0250319237; fax: +39 0250319238.
E-mail address: stefania.arioli@unimi.it (S. Arioli).

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.02.002

2. Materials and methods

2.1. Bacterial strains

A total of 466 strains from 3 genera of LAB of different origins were included in this study. Specifically, this study included 135 strains belonging to *S. thermophilus*; 245 strains belonging to the *Lactobacillus* genus, including *L. coryniformis* ($n=3$), *L. fermentum* ($n=4$), *L. acidophilus* ($n=4$), *L. bulgaricus* ($n=6$), *L. amylovorus* ($n=7$), *L. rhamnosus* ($n=9$), *L. brevis* ($n=13$), *L. helveticus* ($n=39$), *L. reuteri* ($n=42$), *L. plantarum* ($n=43$), and *L. paracasei* ($n=75$); 44 *S. salivarius* clinical isolates provided by Quotient Bioresearch (UK); and 42 *L. garvieae* strains. Some strains of *L. plantarum*, *L. paracasei*, *L. rhamnosus*, *L. brevis*, and *L. coryniformis* that were isolated from traditional Italian cheeses and have been recognized as not susceptible to antibiotics (project acronym ARAFOA, DM 662/7303/2003, funded by Ministero delle Politiche Agricole, Alimentari e Forestali, MiPAAF, IT) were kindly provided by Dr. Roberta Comunian of the Dipartimento per la Ricerca nelle Produzioni Animali (DiRPA) of AGRIS Sardegna (Sassari, IT), Dr. Chiara Devirgilis from INRAN (Rome, IT), Dr. Giorgio Giraffa from CRA-Istituto Sperimentale Lattiero Caseario (Lodi, IT), and Prof. Lorenzo Morelli from Istituto di Microbiologia of Università Cattolica del Sacro Cuore (Piacenza, IT).

Bacteria were maintained at −80 °C and resuscitated in M17 broth (Difco) containing 2% lactose or glucose (w/v) for *S. thermophilus*, *S. salivarius*, and *L. garvieae* and in MRS broth (Difco) for all *Lactobacillus* species. The cultures were incubated at 37 °C and 30 °C for thermophilic and mesophilic species, respectively. The identification of isolates at the species level was performed by means of 16S rDNA gene sequencing. The sequences of the primers used for the amplification of the *16S rDNA* gene were P0 and P6, and the PCR protocol described by the authors was used (Di Cello and Fani, 1996). *S. thermophilus* and *S. salivarius* strains were identified by *secY* gene sequencing (Pombert et al., 2009). The PCR products were purified using an UltraClean DNA Purification Kit (MO BIO). Finally, sequence comparison was performed with Genetics Computer Group sequence analysis software using BLAST.

2.2. Setup of the microdilution method for biocide susceptibility determination

Biocide susceptibility tests and the determination of the Minimum Inhibitory Concentrations (MICs) of triclosan, benzalkonium chloride, chlorhexidine, and sodium hypochlorite (all purchased from Sigma-Aldrich, Italy) were performed using the broth microdilution method. The phenotypic antimicrobial susceptibility to each biocide tested was used to determine the MIC, defined as the lowest biocide concentration that resulted in no visible growth. The MICs were determined by the broth microdilution method using standardized LAB susceptibility test medium (LSM) broth (as recommended by EFSA for antibiotic sensitivity, EFSA, 2012), which ensured adequate growth of the test organisms. LSM consists of a mixture of IsoSensitest broth medium (Oxoid) (90%) and MRS or M17 broth medium (10%) adjusted to pH 6.7 as previously described by Klare et al. (2005). MIC microtiter tests were performed in 384-well plates that were filled with an automatic liquid handling system (EpMotion, Eppendorf, Italy) to a final volume of 80 μl. The bacteria were incubated in the absence (positive control) and in the presence of each biocide at six different concentrations. Each strain included in this study was exposed in triplicate to each biocide concentration tested at a final inoculum density of 10^5 bacteria/ml starting from cultures grown overnight to the stationary phase. The bacterial cell concentration of the overnight culture was determined microscopically by use of an improved Neubauer counting chamber (Marienfeld GmbH, Lauda-Königshofen, Germany). To ensure anaerobic incubation, each well was covered with 20 μl of sterile mineral oil (Sigma-Aldrich, Italy). The 384-well plates were

Table 1
Biocide MIC values for *Lactobacillus* species.

Lactobacillus species	Number of strains	BC MIC μg/ml	Tr MIC μg/ml	Ch MIC μg/ml	SH MIC mg/ml
L. coryniformis	3	1 (2), 2 (1)	64 (3)	1 (3)	2.048 (1), 4.096 (2)
L. fermentum	4	0.25 (1), 1 (1), 2 (2)	16 (1), 64 (3)	0.25 (1), 1 (3)	0.512 (3), 1.024 (1)
L. acidophilus	4	1 (3), 2 (1)	16 (4)	0.5 (1), 1 (2), 2 (1)	0.512 (3), 1.024 (1)
L. bulgaricus	6	1 (3), 2 (2), 4 (1)	8 (1), 16 (5)	1 (2), 2 (4)	0.512 (4), 1.024 (2)
L. amylovorus	7	0.5 (4), 1 (3)	64 (4), 256 (3)	0.25 (1), 0.5 (2), 1 (1), 2 (3)	0.128 (1), 0.512 (6)
L. rhamnosus	9	1 (5), 2 (2), 4 (2)	8 (1), 16 (4), 32 (1), 64 (3)	1 (2), 2 (5), 4 (2)	0.512 (2), 1.024 (2), 2.048 (3), 4.096 (2)
L. brevis	13	0.5 (8), 1 (4), 2 (1)	16 (3), 32 (4), 64 (6)	0.5 (4), 1 (7), 2 (2)	0.256 (2), 0.512 (6), 1.024 (2), 2.048 (1), 4.096 (2)
L. helveticus	39	0.25 (3), 1 (30), 2 (6)	2 (4), 4 (31), 8 (4)	0.5 (1), 1 (4), 2 (12), 4 (16), 8 (6)	0.256 (2), 0.512 (23), 1.024 (69), 2.048 (8)
L. reuteri	42	0.06 (4), 0.125 (5), 0.25 (12), 0.5 (11), 1 (5), 2 (5)	8 (1), 16 (2), 32 (11), 64 (22), 128 (3), 256 (3)	0.125 (7), 0.25 (12), 0.5 (11), 1 (7), 2 (3), 4 (2)	0.064 (1), 0.128 (3), 0.256 (10), 0.512 (28)
L. plantarum	43	0.5 (8), 1 (17), 2 (16), 4 (2)	16 (8), 32 (4), 64 (18), 128 (1), 256 (12)	0.5 (4), 1 (17), 2 (5), 4 (14), 8 (2), 16 (1)	0.256 (6), 0.512 (21), 1.024 (3), 2.048 (8), 4.096 (5)
L. paracasei	75	0.5 (10), 1 (23), 2 (20), 4 (22)	2 (14), 4 (29), 16 (15), 32 (7), 64 (9), 256 (1)	0.5 (1), 1 (4), 2 (18), 4 (20), 8 (22), 16 (10)	0.256 (3), 0.512 (36), 1.024 (31), 2.048 (3), 4.096 (2)