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Inhibitory activity of reuterin, nisin, lysozyme and nitrite against vegetative cells and spores of dairy-related *Clostridium* species

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ABSTRACT

The butyric acid fermentation, responsible for late blowing of cheese, is caused by the outgrowth in cheese of some species of *Clostridium*, resulting in texture and flavor defects and economical losses. The aim of this study was to evaluate the effectiveness of different antimicrobial compounds against vegetative cells and spores of *C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii* and *C. sporogenes* strains isolated from cheeses with late blowing defect. Minimal inhibitory concentration (MIC) for reuterin, nisin, lysozyme and sodium nitrite were determined against *Clostridium* strains in milk and modified RCM (mRCM) after 7 d exposure. Although the sensitivity of *Clostridium* to the tested antimicrobials was strain-dependent, *C. sporogenes* and *C. beijerinckii* generally had higher MIC values than the rest of *Clostridium* species. The majority of *Clostridium* strains were more resistant to antimicrobials in milk than in mRCM, and vegetative cells exhibited higher sensitivity than spores. Reuterin (MIC values 0.51–32.5 mM) and nisin (MIC values 0.05–12.5 μg/ml) were able to inhibit the growth of vegetative cells and spores of all assayed *Clostridium* strains in milk and mRCM. Strains of *C. tyrobutyricum* exhibited the highest sensitivity to lysozyme (MIC values < 0.20–400 μg/ml) and sodium nitrite (MIC values 18.75–150 μg/ml). These results suggest that reuterin and nisin, with a broad inhibitory activity spectrum against *Clostridium* spp. spores and vegetative cells, may be the best options to control *Clostridium* growth in dairy products and to prevent associated spoilage, such as late blowing defect of cheese. However, further studies in cheese would be necessary to validate this hypothesis.

1. Introduction

The genus *Clostridium* consists of a diverse group of obligately anaerobic, Gram-positive, endospore-forming microorganisms, which are distributed in all sorts of environments such as soil, water, plants and intestinal tracts of animals and humans (Johnson, 2009). Several species can grow in a wide range of foods, including dairy products, meat and poultry products and fresh or canned fruits and vegetables, causing food spoilage and/or food poisoning in humans (Peck et al., 2004). In semi-hard and hard cheeses, the butyric acid fermentation responsible for the late blowing defect (LBD) is one of the major causes of spoilage, resulting in texture and flavor defects (McSweeney and Fox, 2004) with an unfavorable economic impact. This defect has been attributed to the outgrowth in cheese of strains belonging to *Clostridium tyrobutyricum* capable of fermenting lactic acid with production of butyric acid, acetic acid, carbon dioxide and hydrogen (Klijn et al., 1995). However, other clostridial species such as *C. sporogenes*, *C. beijerinckii* and *C. butyricum* have also been shown to significantly contribute to the occurrence of LBD in cheese (Cocolin et al., 2004; Garde et al., 2011a; Le Bourhis et al., 2005, 2007).

Physical methods like bactofugation or microfiltration of the cheese milk (García et al., 2013; Tamime et al., 2006; Te Giffel and van der Horst, 2004) may be used to remove clostridial spores and avoid LBD, but they require specific equipment and large capital expenditures. In addition, these methods modify milk composition and the reduction in the number of spores achieved may not suffice to prevent LBD in cheese. On the other hand, microfiltration can only be applied to skim milk because the milk fat globules are too large to pass through the microfiltration membrane. Consequently, the addition of *Clostridium*-inhibitory substances to milk, such as nitrate and lysozyme (Lodi, 1990; Stadhouders, 1990; van den Berg et al., 2004; Wasserfall and Teuber, 1979), is preferred by cheese makers as they are easy to use, no specific equipment is required, and it is a non-costly procedure. The addition of nitrate (E251 and E252) is permitted at a maximum amount of 150 mg/kg (expressed as sodium nitrite) in the milk during manufacture (European Parliament, 2006). As the cheese ripens, nitrate is converted to nitrite by the enzyme xanthine-oxidase, present in the milk/curd and by some nitrate reducing bacteria present in cheese (Galesloot, 1961; Nieuwenhof, 1977; Stadhouders, 1990). The nitrite, which is the actual inhibitor of the development of the spores, is in turn slowly reduced in cheese during ripening. Nonetheless, the EFSA recommends minimizing the use of nitrate and nitrite as food additives in order to keep the level of potentially carcinogenic nitrosamines as

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low as possible (EFSA, 2010). In the case of lysozyme (E1105), obtained from hen egg-white, although its use is authorized at "*quantum satis*" (European Parliament, 1995) it may pose risks for allergic subjects (Fremont et al., 1997).

To solve some of the aforementioned disadvantages, new control strategies are necessary to prevent LBD. The use of antimicrobial compounds produced by lactic acid bacteria may be an alternative strategy to inhibit *Clostridium* growth in dairy products. Thus, the addition the bacteriocin nisin (E234) is allowed at a maximum amount of 12.5 mg/kg of cheese, but it may be also present naturally in cheese as a result of fermentation processes. In this regard, nisin-producing *Lactococcus lactis* strains have been successfully used to control LBD in cheese (Garde et al., 2011b; Rilla et al., 2003). Reuterin (3-hydroxypropionaldehyde) is an antimicrobial compound produced by *Lactobacillus reuteri* as an intermediate step in the conversion of glycerol to 1,3-propanediol (Lüthi-Peng et al., 2002). Reuterin is active against a wide range of Gram-positive and Gram-negative bacteria belonging to *Bifidobacterium*, *Enterococcus*, *Eubacterium*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Collinsella*, *Listeria*, *Staphylococcus*, *Bacillus*, *Clostridium*, *Escherichia*, *Salmonella*, *Yersinia*, *Aeromonas*, *Shigella* and *Campylobacter* genera (Arqués et al., 2004; Axelsson et al., 1989; Bian et al., 2011; Cleusix et al., 2007; El-Ziney and Debevere, 1998; Spinler et al., 2008). In addition, reuterin is water-soluble, active at a wide range of pH values and resistant to proteolytic and lipolytic enzymes. Therefore, reuterin has a high potential as a preservative against food microbial spoilage, including that caused by clostridia.

In this study, we investigated the sensibility of spores and vegetative cells of *C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii* and *C. sporogenes* reference strains and isolates from LBD cheeses, to reuterin, nisin, lysozyme and nitrite, in broth and milk.

2. Material and methods

2.1. Bacterial strains and growth conditions

L. reuteri INIA P572 (INIA culture collection) was used as the reuterin-producing strain (Rodríguez et al., 2003). The strains of *C. tyrobutyricum* (INIA 68 and INIA 69), *C. butyricum* (INIA 66 and INIA 67), *C. beijerinckii* (INIA 63 and INIA 65) and *C. sporogenes* (INIA 70 and INIA 71) were isolated from Manchego cheeses with pronounced late blowing defect (Garde et al., 2011a, 2012a) and selected because they presented different pulsotypes represented by several isolates which produced high amounts of gas and butyric acid in milk and in Bryant and Burkey broth (containing sodium lactate) (Garde et al., 2011a, 2012b). Type strains used were *C. beijerinckii* LMG 5716 (Belgian Coordinated Collections of Microorganisms/Laboratorium voor Microbiologie en microbiële Genetica; BCCM/LMG, Gent, Belgium) and *C. butyricum* CECT 361, *C. sporogenes* CECT 892, *C. tyrobutyricum* CECT 4011, and *Escherichia coli* K12 CECT 433 from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT, Valencia, Spain).

L. reuteri INIA P572 was cultured in MRS broth (Biofile, Milano, Italy) at 37 °C for 18 h, in anaerobic conditions. Clostridia were grown in Reinforced Clostridial Medium (RCM, Difco, Detroit, USA) and incubated at 37 ºC for 48 h in anaerobic conditions. All anaerobic incubations in this study were carried out in jars with an H_2 plus CO_2 generating kit (AnaeroGen, Oxoid, Basingstoke, UK), with an anaerobic indicator within the jars to monitor anaerobiosis. *E. coli* K12 CECT 433 was propagated in Tryptic Soy Broth (TSB, Biolife) at 37 °C for 18 h. Bacteria were stored at −80 °C as stock cultures in their corresponding culture media supplemented with 5% glycerol, and subcultured twice before their use in experiments.

2.2. Reuterin production

A fresh culture of reuterin-producing *L. reuteri* INIA P572 was inoculated at 1% in 1 l of MRS broth and incubated anaerobically at 37 °C overnight. After growth, cells were harvested by centrifugation (4500 ×*g*, 5 min) and gently washed in sterile aqueous solution of glycerol (100 mM). In order to produce reuterin from glycerol, the obtained cell biomass was resuspended into 250 ml sterile aqueous solution of glycerol (100 mM), and resting cells were incubated under anaerobic conditions at 37 ºC for 3 h. After centrifugation (6600 × *g*, 5 min), the resulting supernatant was collected, filter-sterilized (0.22 μm) and maintained at −40 °C for subsequent experiments. The concentration of reuterin in the supernatant was determined by a colorimetric method as described below.

2.3. Reuterin quantification and inhibitory activity

The concentration of reuterin (3-hydroxypropionaldehyde) in the cell-free supernatant of *L. reuteri* INIA P572 was determined following the colorimetric method described by Lüthi-Peng et al. (2002). Acrolein (Fluka; Sigma-Aldrich Quimica SA, Madrid, Spain) was used for obtaining the standard curve, since 3-hydroxypropionaldehyde dehydrates in equimolar concentrations to acrolein. Standards were made diluting acrolein in distilled water. Supernatants containing reuterin were diluted with distilled water if necessary before the colorimetric reaction. All determinations were carried out in triplicate.

The antimicrobial activity of reuterin in cell-free supernatant was determined with the modified minimal inhibitory concentration (MIC) assay of Chung et al. (1989), using *E. coli* K12 CECT 433 as the indicator strain, in sterile 96-well microtiter plates with lid. *E. coli* K12 CECT 433 was grown overnight and diluted to approximately 10^4 cfu/ml in double strength TSB and 150 μl was added to wells containing 150 μl of reuterin supernatant serially two-fold diluted in sterile water. Microplates were incubated at 37 °C for 24 h and growth was confirmed visually. A 100 mM water–glycerol solution without reuterin was used as control. Reuterin arbitrary units (AU) were defined as the reciprocal of the highest two-fold dilution that did not allow the growth of the indicator strain.

2.4. Nisin, lysozyme, and sodium nitrite solutions

Stock solutions of nisin (Sigma, St. Louis, Mo., USA; 2.5% pure nisin, potency of 10^6 IU/g) were prepared at concentrations of 0.25 mg/ml, in 0.02 N HCl (for MIC assays in RCM) or in distilled water (for MIC assays in milk). Lysozyme (Fluka Biochemica, Buchs, Switzerland ~70,000 U/mg) was dissolved in distilled water to yield a stock solution of 8 mg/ml (5.6×10^5 U/ml). Sodium nitrite (Sigma) stock solution of 40 mg/ml was also prepared. All solutions were filter-sterilized (0.22 μm) and stored at −40 °C until use.

2.5. MICs of antimicrobial compounds on vegetative cells

The MIC of reuterin supernatant, nisin, lysozyme and sodium nitrite were determined as the lowest concentration that showed a complete inhibition of the growth of the assayed *Clostridium* strains. Basic media for MIC analysis were litmus milk (Biolife) and modified RCM (mRCM) broth, which does not contain either cysteine hydrochloride or agar, as cysteine could counteract the effects of reuterin (Schaefer et al., 2010) and enhance the inhibitory effect of nitrite (Johnston and Loynes, 1971). Serial two-fold dilutions of the antimicrobial compounds were prepared in distilled water to obtain final concentration range of 32.50 to 0.06 mM for reuterin, 25 to 0.03 μg/ml (7.45×10^{-3} to 8.95×10^{-6} mM) for pure nisin, 400 to 0.20 μg/ml (2.80×10^{-2} to 1.40×10^{-5} mM) for lysozyme and 300 to 0.59 μg/ml (4.35 to 8.55×10^{-3} mM) for sodium nitrite. Then, 150 μl of each dilution was added into wells of sterile 96-well microplates with lid. Clostridial strains were grown overnight and diluted to approximately 10^6 vegetative cells/ml in double strength mRCM broth or litmus milk, and then 150 μl was pipetted into the wells containing the antimicrobial compounds, in duplicate. Positive clostridia growth controls