J. Dairy Sci. 97:789–797
http://dx.doi.org/10.3168/jds.2013-7070

Physicochemical factors differentially affect the biomass and bacteriocin production by bovine *Enterococcus mundtii* CRL1656

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ABSTRACT

Bovine *Enterococcus mundtii* CRL1656 (Centro de Referencia para Lactobacilos Culture Collection) produces an anti-*Listeria* and anti-*Streptococcus dysgalactiae* bacteriocin identified as mundticin CRL1656. The strain and its bacteriocin are candidates to be included in a beneficial product to prevent bovine mastitis as an alternative to antimicrobial agents. To optimize the production of biomass and mundticin CRL1656 by *E. mundtii* CRL1656, a complete 3×2^4 factorial design was applied. The effect of culture medium, initial pH, inoculum size, incubation temperature, and agitation conditions on biomass and bacteriocin production was evaluated simultaneously. Growth parameters were determined using the modified Gompertz model. A nonlinear model was used to estimate the effects of the variables on growth parameters. Bacteriocin production was analyzed using a linear mixed model. Optimal biomass and mundticin CRL1656 production by *E. mundtii* CRL1656 were obtained in different conditions. Maximal growth was recorded in autolyzed yeast, peptone, tryptone, Tween 80, and glucose or M17 broths, pH 6.5, 5.0% inoculum, 30°C, with agitation. However, bacteriocin titers were higher in autolyzed yeast, peptone, tryptone, Tween 80, and glucose or de Man-Rogosa-Sharpe (MRS) broths, pH 6.5, 30°C, both with or without agitation. Knowledge of the optimum conditions for growth and bacteriocin production of *E. mundtii* CRL1656 will allow the obtainment of high levels of biomass and mundticin CRL1656 as bioingredients of potential products to prevent bovine mastitis.
Key words: mundticin production, *Enterococcus mundtii*, bovine mastitis prevention, lactic acid bacterium

INTRODUCTION

Bovine mastitis (i.e., the inflammation of the bovine mammary gland) produces a negative economic effect on dairy farms (Huijps et al., 2008). This disease is mainly caused by pathogenic or environmental bacteria, such as *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, *Streptococcus agalactiae*, *Escherichia coli*, and *Streptococcus dysgalactiae*, that cause infection once they pass through the teat canal (Sears and McCarthy, 2003; Barkema et al., 2009; Taponen and Pyörälä, 2009). During the dry period, animals are more susceptible to infections, and dry cow therapies are applied to treat or prevent new cases of mastitis. Antimicrobial agents are administered without taking into account the fact that the overuse of these drugs could result in a selective pressure for antimicrobial-resistant organisms. Moreover, conventional antimicrobial therapy can also generate residues in the milk, which must then be discarded (Huijps et al., 2008).

Teat disinfection is an important strategy in mastitis control programs. Different substances, such as iodophors, lactic acid, FA, and nisin, have been tested as teat sanitizers (Boddie and Nickerson, 1992; Sears et al., 1992; Boddie et al., 2004). Beneficial microorganisms (Klostermann et al., 2008; Beecher et al., 2009; Frola et al., 2012) and bacteriocins from lactic acid bacteria (**LAB**), such as nisin (Sears et al., 1992; Cao et al., 2007), lacticin 3147 (Crispie et al., 2004; Klostermann et al., 2010) and macedocin ST91KM (Pieterse et al., 2008, 2010; Pieterse and Todorov, 2010), have been proposed as alternatives for the prevention or treatment of bovine mastitis. Products containing viable beneficial microorganisms, supplemented or not with bacteriocins, can be administered as intramammary treatments (to prevent or treat infections) or as external teat treatments applied to the udder skin (to prevent infections).

Bacteriocin-producing *Enterococcus mundtii* CRL1656 [from the Centro de Referencia para Lactobacilos (**CERELA**) Culture Collection, Tucumán, Argentina], an autochthonous strain isolated from bovine udder, was previously characterized and selected

Received May 27, 2013.
Accepted October 28, 2013.
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as a beneficial microorganism (Espeche et al., 2009). This strain produces mundticin CRL1656, a type II bacteriocin active against a bovine mastitis pathogen (*Strep. dysgalactiae*) and a food-borne pathogen (*Listeria monocytogenes* Scott A). Both autochthonous *E. mundtii* CRL1656 and its bacteriocin are potential candidates to be included in the design of veterinary products for mastitis prevention.

Physicochemical factors, such as pH, temperature, culture medium composition, and agitation conditions, influence biomass and bacteriocin production by different LAB strains (Nel et al., 2001; Juarez Tomás et al., 2002; Van den Berghe et al., 2006). Several authors have indicated that optimal culture conditions are strain dependent and should be adjusted for each microorganism (Mataragas et al., 2003; De Vuyst and Leroy, 2007; Settanni et al., 2008).

Some bacteriocin-producing *E. mundtii* strains isolated mainly from vegetables matrices have been described and proposed for the biopreservation of plant-related foods (Granger et al., 2005; Zendo et al., 2005; Settanni et al., 2008). *Enterococcus mundtii* CRL35, a strain isolated from an artisanal cheese, produces enterocin CRL35, which exerts an inhibitory effect on the growth of spoilage and pathogenic microorganisms (Salvucci et al., 2012; Vera Pingitore et al., 2012). The effect of different environmental factors on the production of bacteriocins synthesized by some *E. mundtii* strains has already been studied (De Kwaadsteniet et al., 2005; Zendo et al., 2005; Settanni et al., 2008; Todorov and Dicks, 2009). However, bacteriocin and biomass production by a bovine *E. mundtii* strain, which is proposed as a potential probiotic for mastitis prevention in this work, has not been reported to date. The aim of the current study was to determine the combined effects of culture medium, initial pH, inoculum size, incubation temperature, and agitation conditions on biomass and bacteriocin production by bovine *E. mundtii* CRL1656, applied using a complete 3×2^4 factorial design.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Enterococcus mundtii CRL1656 was previously isolated from a healthy dairy cow and deposited in the CERELA Culture Collection (Espeche et al., 2009). *Listeria innocua* 7 (provided by the Unité de Recherches Laitiéres et Génetique Appliqueé, INRA, France) was used as an indicator strain and cultured in autolyzed yeast, peptone, tryptone, Tween 80, and glucose (**LAPTg**; Raibaud et al., 1961) at 30°C. Stock cultures were maintained in milk-yeast extract at −70°C. Individual components for LAPTg were obtained from Britania Laboratories (Buenos Aires, Argentina).

Inoculum Preparation and Growth Experiments

Enterococcus mundtii CRL1656 was subcultured 3 times in LAPTg broth at 37°C. The active culture was centrifuged for 7 min at 10,000 × *g* at room temperature. The supernatant was discarded and the bacterial pellet was washed twice with sterile saline solution [0.85% (wt/vol) NaCl]. Optical density at 540 nm ($\mathbf{OD_{540}}$) was adjusted in sterile saline solution to 0.7 (approximately 10^9 cfu/mL) and this suspension was used as the inoculum for different culture conditions.

The initial pH of LAPTg, de Man-Rogosa-Sharpe (**MRS**) broth (de Man et al., 1960; Merck, Darmstadt, Germany), or M17 (Terzaghi and Sandine, 1975) broth (Biokar Diagnostics, Beauvais, France) was adjusted to 5.0 or 6.5 with 2 *M* HCl or 2 *M* NaOH before sterilization. *Enterococcus mundtii* CRL1656 was inoculated [2.5 (vol/vol) or 5.0% (vol/vol)] into tubes containing 4 mL of each culture medium. Then, aliquots for each of the conditions to be assayed were distributed into sterile tubes corresponding to each sampling time and incubated at a constant temperature of 30 or 37°C, with (50 oscillations/min; incubator Dubnoff Model; Vicking S.R.L., Buenos Aires, Argentina) or without agitation (0 oscillations/min) as appropriate.

The responses evaluated were OD_{540} (as a measure of bacterial growth) and bacteriocin titer. Samples were taken after 0, 3, 6, 9, 12, and 24 h from each culture condition and OD_{540} was recorded with a microplate reader (VersaMax, Molecular Devices LLC, Sunnyvale, CA,). Samples were centrifuged for 7 min at 10,000 × *g* at room temperature and supernatants were neutralized with sterile 2 *M* NaOH. Bacteriocin titers were determined by the well diffusion assay with *L. innocua* 7 as indicator strain and expressed in arbitrary units (**AU**)/mL. The relative amount of bacteriocin produced per unit of biomass [calculated as $(AU/mL)/OD_{540}$] was estimated for each culture condition for each time point.

Experimental Design and Statistical Analysis

A total of 48 different conditions were studied by applying a 3×2^4 full factorial design. The factors evaluated were culture medium (LAPTg, M17, and MRS), initial pH (5.0 and 6.5), inoculum size (2.5 and 5.0%), incubation temperature (30 and 37°C), and agitation level (0 and 50 oscillations/min). The complete experimental design was repeated twice on different days. The randomization was performed for each experimental day.