Food Research International 105 (2018) 897–904

Contents lists available at ScienceDirect

Food Research International

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

Expression of genes associated with stress conditions by *Listeria monocytogenes* in interaction with nisin producer *Lactococcus lactis*

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A R T I C L E I N F O

Keywords:
Listeria monocytogenes
Lactococcus lactis
Nisin
Interaction
Stress

A B S T R A C T

The use of nisin producers in foods is considered a mitigation strategy to control foodborne pathogens growth, such as *Listeria monocytogenes*, due to the production of this bacteriocin *in situ*. However, when the bacteriocin does not reach an adequate concentration, the target bacteria can develop a cross-response to different stress conditions in food, such as acid, thermal and osmotic. This study aimed to evaluate the interaction of a nisin-producing strain of *Lactococcus lactis* DY-13 and *L. monocytogenes* in BHI and skim milk, and its influence on general (*sigB*), acid (*gadD2*), thermal (*groEL*) and osmotic (*gbu*) stress-related genes of the pathogen. *L. monocytogenes* populations decreased approximately 2 log in BHI and 1 log in milk after 24 h in co-culture with the nisin producer *L. lactis*, coherent with the increasing expression of *nisK*. Expression of stress-related genes by *L. monocytogenes* presented lower oscillation in BHI than in milk, indicating its better ability to survive in milk, despite the higher nisin production. Stress-related genes presented a varied expression by *L. monocytogenes* in the tested conditions: *sigB* expression remained stable or reduced over time; *gadD2* presented high expression in milk; *groEL* presented low expression in BHI when compared to milk, trending to decrease overtime; *gbu* expression in milk after 24 h was lower than in BHI. The presented study demonstrated the growth of a nisin producer *L. lactis* can affect the expression of stress-related genes by *L. monocytogenes*, and understating these mechanisms is crucial to enhance the conservation methods employed in foods.

1. Introduction

Lactococcus lactis subsp. *lactis* is a lactic acid bacterium widely used in the dairy industry, particularly, in cheese production (Ruggirello, Cocolin, & Dolci, 2016). Some *L. lactis* isolates are able to produce nisin, a bacteriocin with bactericidal and bacteriostatic action against several microorganisms, mainly Gram-positive bacteria (Zacharof & Lovitt, 2012). Thus, the use of a nisin-producing *L. lactis* as a starter culture in the production of food represents an additional safety advantage, considering it can inhibit possible contamination by potential foodborne pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus* spp. and *Clostridium* spp. (de Arauz, Jozala, Mazzola, & Penna, 2009). However, the successful application of this starter culture depends on its ability to synthesize nisin in the food matrix (Perin et al., 2015). Furthermore, the synthesis of low nisin concentrations in food may be insufficient to control pathogens and spoilage bacteria because, under these conditions, some microorganisms are able to adapt and increase their resistance against specific stress conditions (Wesche, Gurtler, Marks, & Ryser, 2009).

L. monocytogenes is a foodborne pathogen that possesses a substantial number of virulence factors which allow for host colonization, like internalins, listeriolysin O, phospholipases and the actin-assembly inducing protein ActA (Vázquez-Boland et al., 2001). Adverse conditions found in foods and in their production lead to physiological responses of *L. monocytogenes*. Expression of genes related to stress conditions enable this pathogen to survive this adverse conditions, making it possible for *L. monocytogenes* to remain in the food during its shelf life, and its inoculation to a host (Lado & Yousef, 2007). Variations in acidity, temperature and osmotic pressure are the most common stress situations found in food production and to which *L. monocytogenes* is subjected. In these situations, the expression of various genes, such as glutamate decarboxylase (*gadD*), *groEL* chaperone and glycine betaine transporter (*gbu*), is increased depending on the specific stress environments, allowing the pathogen to survive under sub-optimal conditions (Cotter, Gahan, & Hill, 2001; Hayer-Hartl, Bracher, & Hartl, 2016; Wemekamp-Kamphuis et al., 2002). The alternative sigma factor, sigma B (*sigB*), plays a key role in various stress conditions, allowing the expression of a variety of genes in response to environmental and growth changes (Wemekamp-Kamphuis et al., 2004). Modifying the expression of these stress-related genes may alter the

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https://doi.org/10.1016/j.foodres.2017.12.030
Received 13 September 2017; Received in revised form 29 November 2017; Accepted 12 December 2017
Available online 13 December 2017
0963-9969/

ability of *L. monocytogenes* to survive under these conditions, including those found after ingestion by a host (Gahan & Hill, 2014).

In this context, the current study aims to evaluate the interaction of nisin-producing *L. lactis* subsp. *lactis* with *L. monocytogenes* in culture medium and milk by assessing their populations and the expression of genes related to stress conditions.

2. Material and methods

2.1. Microorganisms

L. monocytogenes Scott A (ATCC 49594) and the commercial nisin-producing strain *L. lactis* subsp. *lactis* DY-13 (Lyofast DY 13; Sacco, Cadorago, Italy) were used in this study. The strains were kept in brain and heart infusion broth (BHI; Oxoid, Basingstoke, Hampshire, England, for *L. monocytogenes*) and Man, Rogosa and Sharpe broth (MRS; Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA, for *L. lactis* subsp. *lactis*) with 20% (v/v) glycerol added, stored at − 20 °C. At the time of use, the strains were cultivated in their respective broths at 37 °C for 24 h.

2.2. L. lactis subsp. lactis growth in BHI broth and reconstituted skim milk

2.2.1. Treatments and incubation conditions

The growth of *L. lactis* subsp. *lactis* DY-13 was evaluated by its inoculation in BHI broth (Oxoid) and in reconstituted skim milk (BD). In the moment of inoculation, an aliquot was transferred to the growth medium for an initial concentration of 6 to 7 log colony-forming units (CFU)/mL. The media were then incubated at 20 and 30 °C for 24 h. Analyses were performed at time zero and every 3 h, for a total of 24 h.

2.2.2. Population and pH evaluation of the media

Samples of inoculated reconstituted skim milk were serially diluted in 0.85% (w/v) NaCl, followed by surface plating in duplicate in MRS agar (BD). Plates were incubated at 37 °C for 24 h, and results expressed as CFU/mL. Optical density analysis was performed for BHI broth (Oxoid) in a spectrophotometer (Bel Equipamentos Analíticos LTDA, Piracicaba, SP, Brazil) at a wavelength of 600 nm. When the optical density measurement was higher than 1.0, the sample was diluted with medium without inoculum, and the density measurement obtained was corrected by the dilution factor. pH measurement was performed using a potentiometer (Bel).

2.2.3. Bacteriocin production

Nisin production was detected by using the agar spot test, according to Todorov (2008), with modifications. After centrifugation of the sample at 10,000 × *g* for 10 min, the supernatant was collected and heat-treated at 80 °C for 10 min. To quantitatively assess nisin production, the treated supernatant was serially diluted in 10 mM phosphate buffered saline (PBS, Oxoid) pH 6.5. Aliquots of 10 μl were then applied to the surface of BHI agar (Oxoid, 12 g/L agar) containing 1% *L. monocytogenes* Scott A. Plates were incubated at 37 °C for 24 h. Observation of halos on the surface of the agar was considered to be positive for inhibition. The observation of halos at the highest dilution performed was envisaged for the determination of bacteriocin concentration in arbitrary units. Nisin production was expressed in arbitrary units per milliliter (AU/mL), calculated as $a^b \times 100$, where a corresponds to the dilution factor, and b corresponds to the last dilution that produced an inhibition halo (Schirru et al., 2012).

2.3. L. lactis subsp. lactis and L. monocytogenes interaction

2.3.1. Treatments and incubation conditions

BHI (Oxoid) and reconstituted skim milk (BD were inoculated with *L. monocytogenes* Scott A (initial population between 7 and 8 log CFU/mL) and *L. lactis* subsp. *lactis* DY-13 (initial population between 6 and 7 log CFU/mL) and incubated at 20 and 30 °C for 24 h. Samples were obtained at time zero, and every 3 h for plate count and nisin production analysis. In addition, media inoculated only with *L. monocytogenes* Scott A were used as controls, which were incubated and sampled under the same conditions.

2.3.2. Population enumeration

Plate count analysis for the populations was conducted for BHI and reconstituted skim milk as described in Section 2.2.2 for *L. lactis* subsp. *lactis* in reconstituted skim milk, but for *L. monocytogenes* the plating was performed on BHI agar (Oxoid) supplemented with 10 g/L lithium chloride (Merck KGaA, Darmstadt, Germany).

2.3.3. Bacteriocin production

Analysis of nisin production was performed as described in Section 2.2.3 for *L. lactis* subsp. *lactis* in pure culture.

2.4. Gene expression analysis

2.4.1. RNA sampling and extraction

Considering the results obtained for the interaction between *L. monocytogenes* Scott A and *L. lactis* subsp. *lactis* DY-13 in BHI broth and milk, 0, 9, 18 and 24 h were selected for sampling and analysis of gene expression. RNA extraction was performed using Trizol reagent (Ambion, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol, with modifications. The changes were as follows: 1) dilution of the inoculum of reconstituted skim milk in 2% sodium citrate solution at 1:1 (v/v), to facilitate the differentiation of the pellet after the centrifugation; 2) the introduction of a Gram-positive cell wall lysis step, according to the Wizard Genomic DNA Purification Kit protocol (Promega Co., Madison, WI, USA). After extraction, RNA was quantified using a Nanodrop Lite spectrophotometer (Thermo Scientific, Madison, WI, USA) and stored at − 80 °C until use.

2.4.2. DNase treatment and cDNA synthesis

The extracted RNA was treated with DNase to remove contaminating DNA, using Ambion's DNA-free kit (Ambion). The cDNA synthesis was performed from DNase-treated RNA. A 1 μl aliquot of dNTP solution (Invitrogen, Life Technologies, Carlsbad, California, USA), containing 10 mM dATP, dGTP, dCTP and dTTP, and 1 μl of random primer (Invitrogen) were added to 10 μl of the treated RNA and the mixture incubated in a thermocycler at 65 °C for 5 min and 4 °C for 5 min. To this same tube, 4 μl of 5 × First-Strand Buffer, 2 μl of DTT (0.1 M) and 1 μl of RNase OUT (Invitrogen) were added, and then the mixture was incubated in the thermocycler at 37 °C for 2 min. Next, 1 μl (200 U) of M-MLV RT enzyme (Invitrogen) was added to the tube, and the cDNA synthesis was performed in a thermocycler with a cycle of 25 °C for 10 min, 37 °C for 50 min and 70 °C for 20 min. The cDNA was then stored at − 80 °C until use.

2.4.3. Primers

Table 1 shows the primers used in this study. The primers for the *rplD*, *tuf*, *gadD2*, *groEL* and *gbu* genes were obtained from the primer BLAST tool, using the genomes deposited in the NCBI (*L. monocytogenes* Scott A, access code CM001159 and *L. lactis* subsp. *lactis* Il1403, access code AE005176). The primers for the *sigB* and *nisK* genes were based on Werbrouck et al. (2009) and Trmčić, Monnet, Rogelj, and Matijašić (2011), respectively.

2.5. Relative expression of nisK by L. lactis subsp. lactis in interaction with L. monocytogenes

The expression of the *nisK* gene, in relation to time was evaluated in *L. lactis* subsp. *lactis* when interacting with *L. monocytogenes*. The *tuf* gene was used as the endogenous control (Ulve et al., 2008). The relative expression of the *nisK* gene was evaluated using the initial (0 h)