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Ultrasound attenuation of lactobacilli and bifidobacteria: Effect on some technological and probiotic properties

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A B S T R A C T

Attenuation can be regarded as a tool to modulate the metabolism of probiotic bacteria and, consequently, a strategy to reduce the acidification of the active drinks. Attenuation can be done through chemical and physical approaches and ultrasound (US) is a possibility, previously tested to modulate the metabolism of lactic acid bacteria inoculated in a rice drink, but no data are available on the effect of this treatment of the overall profile of probiotic bacteria. Therefore, the main topic of this paper was to study the effect of US-attenuation on some properties of *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Bifidobacterium longum*, and *Bifidobacterium infantis* (survival at pH 2, and 2.5, and with 0.3% of bile salt added, hydrophobicity, acidification, and growth at different temperatures, pH or in presence of 7% NaCl). A preliminary screening was done by using 3 power levels (40, 60, and 80%) and 3 different treatment times (2, 4, and 6 min); immediately after sonication, acidification and viable count were tested. The best combination to avoid post-acidification was the following one: power, 60%; time, 6 min; pulse, 2 s. The effect of this combination on the overall profile of the test strains (functional and technological properties) was studied. This combination exerted a positive effect on the hydrophobicity and adhesion to Caco-2 cells of *L. reuteri*, although the growth at pH 4 was negatively affected. In the other strains, there a was negative effect on acid and bile resistance.

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1. Introduction

Probiotic food products are categorized as functional foods, and represent a significant portion of this product category (BCC Research, 2014). However, the supplementation of probiotics requires special technologies because of their active metabolism, which could lead to significant changes in food flavor and rheology (Dave and Shah, 1997; Han et al., 2012). Therefore, many times it is important to control the metabolism of probiotic and starter cultures in foods, without adversely affecting their viability and functional properties (Bandiera et al., 2013; Ferdousi et al., 2013). A possible way to overcome this problem is the attenuation of probiotics through a physical method. Attenuation has been traditionally defined as a technological method to enhance the total pool of intracellular enzymes released into the matrix, positively influencing flavor and quality of the final product (Dahroud et al., 2016; Di Cagno et al., 2012). Petterson and Sjöström (1975) proposed the use of attenuated starters to accelerate the ripening of Svecia, a Swedish semi-hard cheese, by thermal treatments (e.g., 69 °C for 15 s) and a similar approach can be found in many other papers (Di Cagno et al., 2012; Lanciotti et al., 2007). In this paper, a different idea of attenuation has been proposed, i.e. as a tool to avoid acidification.

Besides thermal treatments, other methods have been studied. According to Yarlagadda et al. (2014) these techniques can be generally divided as follows: i) chemical treatments, such as the use of hexadecyltrimethylammonium bromide (CTAB), ethylenediaminetetraacetic acid (EDTA), isopropyl alcohol (IPA), sodium dodecyl sulphate (SDS), lysozyme, or n-butanol (Doolan and Wilkinson, 2009; Law et al., 1976; Ristagno et al., 2012; Yarlagadda et al., 2014); ii) physical treatments, including heat or freeze shocking, and/or mechanical treatments such as sonication, bead mill, high-pressure homogenization and microfluidization (Bevilacqua et al., 2016; Exterkate, 2006; Geciova et al., 2002; Klein and Lortal, 1999; Lanciotti et al., 2007; Yarlagadda et al., 2014). One of the emerging technologies is ultrasound (US). Sonication is generally applied to impart positive effects in food processing such as improvement in mass transfer, food preservation, assistance of thermal treatments and manipulation of texture and food analysis (Knorr et al., 2011). It increases the cell lysis, and the degradation of enzymes by heat denaturation, thus ultrasonic waves have the potential to influence the microorganisms and living cells (Tabatabaie and Mortazavi, 2010). Bevilacqua et al. (2016) studied the use of US-attenuated *Lactobacillus plantarum*, *Lactobacillus casei* LC01 and *Bifidobacterium animalis* subsp. *lactis* Bb12 inoculated in a commercial

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rice drink; attenuation did not affect the viability of probiotics or the sensory scores of the beverage. Moreover, some preliminary experiments performed by these authors showed that the probiotic traits were not affected by attenuation, but to the best of our knowledge, this is the only report on the effect of US-attenuation of the properties of probiotic bacteria. Thus, the main goal of this paper was to assess the effect of US on some selected probiotic properties (survival at low pH and in presence of bile salts, hydrophobicity, and adhesion to Caco-2 cells); as an additional goal, the growth under different conditions was assessed as a tool to study the effect of attenuation on the overall profile.

A wild strain of *L. plantarum*, isolated from an Italian sourdough, and *L. reuteri* DSM 20016 were used as targets, because of some promising results found in a previous publication or for their robustness (Bevilacqua et al., 2016; Corbo et al., 2014; Perricone et al., 2014b). The experiments were also done on two commercial strains of bifidobacteria (*B. longum* Bb46 and *B. infantis* Bb02).

2. Materials and methods

2.1. Strains

Four microorganisms were used in this research: i) *Lactobacillus plantarum* L12, isolated from a sourdough, and belonging to the Culture Collection of the Department of the Science of Agriculture, Food and Environment, University of Foggia (Corbo et al., 2014); ii) *Lactobacillus reuteri* DSM 20016, purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germania); iii) *Bifidobacterium longum* Bb46 and *Bifidobacterium infantis* Bb02, purchased from Chr. Hansen (Hørsholm, Denmark).

Lactobacilli were stored at −20 °C in MRS broth (Oxoid, Milan, Italy) added with 33% of sterile glycerol (J.T. Baker, Milan, Italy), while *B. longum* and *B. infantis* were stored in MRS broth supplemented with 0.5% cysteine (cMRS) (Sigma-Aldrich, Milan, Italy). Before each assay, the strains were grown under anaerobic conditions in MRS broth or cMRS broth, incubated at 37 °C for 24 h. The microorganisms were centrifuged at 1500g for 10 min; the supernatant was discarded and the pellet was suspended in sterile distilled water. The viable count of bacterial cultures was ca. 9 log cfu/ml.

2.2. US — treatment and acidification

Bacterial cultures (cells in distilled water, Section 2.1) were treated with ultrasound (US) through a VC Vibra Cell Ultrasound equipment; model VC 130 (Sonics and Materials Inc., Newtown, CT, USA). The main variables of the treatment were the net power (40, 60 and 80%) and the duration of the treatment (2, 4, and 6 min); pulse was set to 2 s. Thus, 9 different treatments were assayed and tested (Table 1).

US equipment worked at 20 kHz (frequency)–130 W (acoustic energy) and the mean efficiency of probe was ca. 70%; therefore, the net energy of treatment varied from 36.4 to 72.8 W. Before each treatment, the ultrasonic probe was washed with sterile distilled water; after sonication, the sample was cooled in ice. Immediately after the treatment, lactobacilli and bifidobacteria were respectively inoculated in MRS or cMRS broth; acidification was tested after 6 and 24 h at 37 °C. Untreated bacteria were used as controls to test acidification before sonication. The pH of the medium was evaluated through a pH-meter Crison (Crison Instruments, Barcelona, Spain). Data from pH were modeled as pH decrease. The experiments were performed in triplicate over three different batches.

2.3. Effect of US on the viable count

The microorganisms were US-treated at 60%/6 min/2 s, 80%/4 min/2 s and 80%/6 min/2 s (power/time/pulse). Viable count was determined before and after the sonication on MRS agar (lactobacilli; 37 °C for 48 h under anaerobic conditions) or cMRS agar (bifidobacteria; 37 °C for 48 h under anaerobiosis).

2.4. Effect of attenuation on some technological and functional properties

The microorganisms were US-treated (60%/6 min/2 s); immediately after the sonication, the following experiments were done: i) acidification throughout the storage at 4 and 15 °C; ii) growth at pH 4.0 and 9.0, in presence of NaCl or at 15, 37 and 45 °C; iii) survival at pH 2.0/2.5 or in presence of 0.3% bile salts; iv) hydrophobicity. Untreated bacteria were always used as controls.

2.4.1. Combination of attenuation and refrigeration

US-treated and untreated bacteria were inoculated in MRS or cMRS broth at level of 6 log cfu/ml; the samples were stored at 4 and 15 °C and acidification and viable count were evaluated after 2, 7 and 14 days.

2.4.2. Growth assays

The bacteria were inoculated in MRS broth or cMRS at level of 6 log cfu/ml. The media were adjusted to pH 4 or 9 (through HCl or NaOH 1.0 N), supplemented with NaCl (7%) or incubated at different temperatures (15, 37 and 45 °C). Microbial growth was evaluated after 24 h or 7 days (at 15 °C) as absorbance at 600 nm using a spectrophotometer UV–Vis DU 640 Beckman (Fullerton, CA, USA). Data were modeled as growth index, as reported by Bevilacqua et al. (2009):

$$GI = \frac{Abs_s}{Abs_c} * 100$$

where:

Abs_s is the absorbance of US-treated microorganisms and Abs_c is the absorbance of the controls (untreated bacteria).

Table 1
Decrease of pH (mean values ± standard deviation) in MRS broth or cMRS inoculated with lactobacilli and bifidobacteria; the measurements were done after 6 and 24 h at 37 °C. The letters indicate the significant differences for each sampling point and each microorganism (one-way ANOVA and Tukey's test, $P < 0.05$).

	L. reuteri		*L. plantarum*		*B. infantis*		*B. longum*	
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h
Untreated bacteria	0.81 ± 0.02A	2.00 ± 0.01A	0.97 ± 0.01A	2.49 ± 0.00A	1.21 ± 0.04A	2.15 ± 0.00A	1.34 ± 0.03A	2.01 ± 0.01A
US-treated bacteria								
40% 2 min	0.83 ± 0.02A	1.98 ± 0.00A	0.88 ± 0.03B	2.50 ± 0.01A	1.26 ± 0.01A	2.14 ± 0.00A	1.26 ± 0.03A	2.02 ± 0.01A
60% 2 min	0.74 ± 0.01B	1.98 ± 0.01A	0.88 ± 0.01B	2.50 ± 0.01A	1.23 ± 0.03A	2.11 ± 0.01A	1.21 ± 0.02A	2.01 ± 0.01A
80% 2 min	0.68 ± 0.00B	1.97 ± 0.02A	0.64 ± 0.05C	2.49 ± 0.01A	1.07 ± 0.02B	2.05 ± 0.01A	0.95 ± 0.02B	2.00 ± 0.02A
40% 4 min	0.68 ± 0.02B	1.96 ± 0.01A	0.94 ± 0.02A	2.50 ± 0.01A	1.39 ± 0.00A	2.11 ± 0.01A	1.23 ± 0.01A	2.01 ± 0.01A
60% 4 min	0.66 ± 0.03B	1.94 ± 0.02A	0.75 ± 0.06B,C	2.48 ± 0.01A	0.98 ± 0.06B	2.06 ± 0.01A	1.06 ± 0.04B	1.96 ± 0.01A
80% 4 min	0.18 ± 0.06C	1.88 ± 0.04A	0.07 ± 0.04D	2.43 ± 0.02A	0.12 ± 0.07C	2.01 ± 0.03A	0.05 ± 0.03C	1.97 ± 0.01A
40% 6 min	0.69 ± 0.01B	1.98 ± 0.01A	0.76 ± 0.01B,C	2.49 ± 0.01A	1.13 ± 0.14B	2.05 ± 0.02A	1.21 ± 0.03A	1.97 ± 0.05A
60% 6 min	0.09 ± 0.04C	1.85 ± 0.01A	0.02 ± 0.01D	2.39 ± 0.01A	0.10 ± 0.02C	2.02 ± 0.01A	0.04 ± 0.01C	1.96 ± 0.01A
80% 6 min	0.01 ± 0.02C	1.70 ± 0.13A	0.00 ± 0.00D	2.36 ± 0.02A	0.02 ± 0.01C	1.97 ± 0.03A	0.02 ± 0.00C	1.86 ± 0.03B

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