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A low-power ultrasound attenuation improves the stability of biofilm and hydrophobicity of *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271 and *Acidipropionibacterium jensenii* DSM 20535

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ABSTRACT

The main topic of this paper was to study the effect of ultrasound-attenuation (US) on the surface properties of propionibacteria (*Acidipropionibacterium jensenii* DSM 20535 and *Propionibacterium freudenreichii* DSM 20271). A preliminary screening was done by using different power levels (40 and 60%) and treatment times (4, 6, and 8 min); immediately after sonication, acidification and viable count were tested. The best combinations to avoid post-acidification after 6 h were the following: *A. jensenii* DSM 20535: power, 40%; time, 8 min; *P. freudenreichii* subsp. *freudenreichii* DSM 20271: power, 60%; time, 4 min.

Moreover, the effect of US on the growth patterns, surface properties (biofilm formation and hydrophobicity), resistance to some selected antibiotics, and release of intracellular components was evaluated; the experiments were done immediately after the treatment. US-treatment improved the stability of biofilm after 5–7 days, caused an increase of hydrophobicity (from 15 to 27%) immediately after sonication, and determined an increase of cell permeability, as suggested by the release of intracellular components within 24 h and by the increased sensitivity to some antibiotics. This paper is the first report on US-attenuation on propionibacteria and could the background for future researches to modulate the surface properties of these microorganisms.

1. Introduction

A problem found in some foods after the addition of probiotics is the over-acidification or post-acidification, i.e. an uncontrolled decrease of pH during the storage. A way to address this challenge could be the modulation of the metabolism of probiotics through attenuation.

Attenuation is a method able to delay acidification without a negative effect on the viability (Bevilacqua et al., 2016; Racioppo et al., 2017); this effect can be achieved by a wide variety of methods (spray drying, freeze drying, fragilization using lysozyme or solvents, or the selection of lactose negative mutants' strains, and other physical approaches, like the high pressure of homogenization-HPH) (Di Cagno et al., 2012; Klein and Lortal, 1999; Lanciotti et al., 2007; Tabanelli et al., 2013). The first preparation of attenuated starters as a cheese additive was proposed by Petterson and Sjöström (1975) to accelerate the ripening of a semi-hard cheese; they used a mild thermal treatment (69 °C for 15 s) and achieved an accelerated release of microbial enzymes and a reduced ripening duration of cheese, due to a fast proteolysis.

Another possible way for attenuation is an ultrasound treatment (US); this method was used in the past to counteract the over-acidification exerted by lactobacilli in an organic rice beverage (Bevilacqua et al., 2016) or in a model system by *Lactobacillus reuteri* (Racioppo et al., 2017).

Erriu et al. (2014) reported that US could exert a dual effect on bacteria: inhibition/inactivation or growth enhancement, as a function of frequency and energy. These two effects are the results of two main physical phenomena: a stable cavitation (low intensity resulting in bubbles which do not collapse) and a transient or collapse cavitation (produced when the intensity is high enough so that the bubble radius is reduced to a very small value at the end of the contraction cycle) (Bigelow et al., 2009; Erriu et al., 2014; Guzman et al., 2003; Kodama et al., 2006; Pitt and Ross, 2003).

The effects at cell level are at least four: a) surface resonance; b) shear forces inside cells; c) free radicals in aqueous media; d) production of peroxides (Joyce et al., 2003). These four effects could result in bacteria inactivation or stimulation, increase of the growth rate, bacteria declumping, and increase of the membrane permeability (Erriu

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et al., 2014; Joyce et al., 2003; Pitt and Ross, 2003; Runyan et al., 2006).

Dairy propionibacteria are beneficial microorganisms used in the food industry for the production of vitamins, for cheese ripening, and for their probiotic properties (Cousin et al., 2010). They could exert a probiotic effect, like the modulation of intestinal inflammation (Foligné et al., 2010, 2013; Mitsuyama et al., 2007), the production of beneficial metabolites such as short-chain fatty acids (Cousin et al., 2012; Jan et al., 2002; Lan et al., 2007), vitamins and bifidogenic compounds (DHNA) (Bouglé et al., 1999; Kaneko, 1999; Hojo et al., 2002; Mitsuyama et al., 2007; Ouwehand et al., 2002; Seki et al., 2004). Moreover, some strains were shown to adhere to mice intestinal epithelial cells both *ex vivo* and *in vivo* as well as to cultured human intestinal cell lines *in vitro* (Huang and Adams, 2003; Moussavi and Adams, 2010; Campaniello et al., 2015), and produce biofilms (Campaniello et al., 2018).

In a previous research, US was tested on *L. reuteri*; apart from the delay of the acidification, another interesting result was the increase of bacterial cell hydrophobicity by 20% and a 10-fold enhancement of adhesion to CaCo-2 cells (Racioppo et al., 2017). However, the research was done on a single strain of *Lactobacillus* spp., whereas no data are available on propionibacteria; the effects of US are strongly strain-dependent and the combinations for attenuation could be different depending on the kind of microorganisms.

The aim of this study was to evaluate the effects of US-attenuation on two beneficial propionibacteria (*Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271; *P. jensenii* DSM 20535, now *Acidipropionibacterium jensenii*; Scholz and Kilian, 2016) with interesting functional traits (Campaniello et al., 2015, 2018), to assess if attenuation could be successfully proposed to avoid post-acidification. In addition, the effect of US on some surface properties (hydrophobicity, biofilm formation), on membrane permeability (release of cell components) and growth patterns was studied.

2. Materials and methods

2.1. Strains

This study focused on *P. freudenreichii* subsp. *freudenreichii* DSM 20271 and *A. jensenii* DSM 20535 (previously known as *P. jensenii*); the strains were purchased from the German Collection of Microorganisms (DSMZ). The microorganisms were stored at −20 °C in MRS broth (Oxoid, Milan, Italy) supplemented with 33% sterile glycerol (J.T. Baker, Milan, Italy). The microorganisms were grown in MRS broth, incubated at 37 °C for 24 h in anaerobiosis. Then, the cultures were centrifuged at 3000 × *g* for 10 min at 4 °C; the supernatant was discarded and the pellet suspended in distilled water.

2.2. US – treatment

The strains were treated with ultrasound (US) through a VC Vibra Cell Ultrasound equipment; model VC 130 (Sonics and Materials Inc., Newtown, CT, USA: net power, 130W). The main variables of the treatment were the net power (40, and 60%) and the duration of the treatment (4–8 min); the pulse was set to 2 s. Before each treatment, the ultrasonic probe was washed with sterile distilled water; immediately after processing, the sample was cooled in ice. The viable count and acidification were measured before and after each treatment.

The viable count was determined on MRS agar (37 °C for 48 h under anaerobic conditions). For acidification, aliquots of MRS broth were individually inoculated with each strain to 7 log cfu/mL and incubated at 37 °C; the pH of the medium was evaluated after 6 h through a pH-meter Crison (Crison Instruments, Barcelona, Spain). Data from pH were modelled as pH decrease.

2.3. Growth patterns

The bacteria were inoculated in MRS broth at 6 log cfu/mL. The media were adjusted to pH 4-5-8 or 9 (through HCl or NaOH 1.0 N), supplemented with NaCl (2–4%), or incubated at different temperatures (37–45 °C). Growth was evaluated after 24 and 48 h as absorbance at 600 nm using a spectrophotometer UV–Vis DU 640 Beckman (Fullerton, CA, USA).

The results were modelled as Growth Index (Bevilacqua et al., 2009; modified by Racioppo et al., 2017):

$$GI = Abs_s/abs_c*100$$

Where: Abs_s is the absorbance of US-treated microorganisms and Abs_c is the absorbance of the reference strains (untreated bacteria).

2.4. Biofilm formation

Glass slides (25.4 mm × 76.2 mm) were used as surfaces to get the biofilm attached. All slides were cleaned with acetone before soaking in 3.5% sodium hypochlorite (V/V) at 75 °C for 5 min, rinsed and transferred into 7.0 g/L phosphoric acid solution for 5 min. Then, the slides were rinsed in distilled water, air dried and autoclaved at 121 °C for 15 min (Arizcun et al., 1998). The samples were prepared pouring 45 mL of MRS broth into sterile tubes and vertically dipping sterile slides in; the inoculum with 10^7 cfu/mL was performed in each of them and the samples were incubated at 37 °C for 7 days (static conditions). The populations in planktonic and sessile states were periodically determined by a standard plate count on MRS Agar. To determine the viable sessile cells, the slides were aseptically removed from the medium, rinsed with sterile distilled water to remove the unattached cells, and placed into a test-tube containing 45 mL of sterile saline solution (0.9% NaCl) and sonicated at 20% power "Vibra Cell" for 3 min (Speranza et al., 2011).

2.5. Hydrophobicity

The hydrophobicity of the bacterial cell surface before and after sonication was assessed by measuring the microbial adhesion to xylene (Bautista-Gallego et al., 2013). US-treated and untreated bacteria were centrifuged (1500 × *g* for 10 min); the pellet was washed twice in PBS (Phosphate Buffer saline, Sigma-Aldrich), suspended in 10 mL of 0.1 M KNO_3 (C. Erba, Milan, Italy), and the absorbance at 600 nm was measured (A_0). Then, 3 mL of xylene were added to form a two-phase system and after 10-min pre-incubation at room temperature, the samples were mixed for 20 s and stored under static conditions at room temperature for 20 min, 1, 2 or 3 h. At each sampling point, the aqueous phase was carefully removed and the absorbance at 600 nm was assessed (A_1). The hydrophobicity (H%) was calculated using the following formula:

$$H\% = (1 - A_1/A_0)*100$$

2.6. Release of intracellular components

Untreated and treated bacteria (8 log cfu/mL) were centrifuged at 6000 × *g* for 10 min. The UV absorbance of the supernatant was measured at 260 nm and at 280 nm with a spectrophotometer UV-VIS DU 640 Beckman (Fullerton, CA) (Virto et al., 2005).

The results were modelled using the following formula:

$((Abs_s-Abs_c)/Abs_c)*100$Where: Abs_s is the absorbance of US-treated microorganisms and Abs_c is the absorbance of untreated bacteria.

2.7. Antibiotic-resistance

Antibiotic-resistance of treated and untreated bacteria was carried out through an E-test (Liofilchem, Roseto degli Abruzzi, Italy). The following compounds were used: Ampicillin (0.016–256 μg/mL), Ciprofloxacin (0.002–32 μg/mL), Clarithromycin (0.016–256 μg/mL), Chloramphenicol (0.016–256 μg/mL), Erythromycin (0.016–256 μg/mL), Gentamicin (0.064–1024 μg/mL), Tetracycline (0.016–256 μg/mL), Trimethoprim (0.002–32 μg/mL), Vancomycin (0.016–256 μg/mL). The strains were plated onto the surface of MRS agar; thereafter, the strip containing the antibiotic was placed onto the plates. The plates were incubated at 37 °C for 24 h in anaerobiosis.

At the end of incubation, the Minimal Inhibitory Concentration (MIC) was evaluated; it was read as the value on the strip where the edge of the inhibition ellipse intersected the strip itself.

2.8. Statistical analysis

The analyses were done on three independent batches; the results were analysed through a t-student's test (paired comparison, $P < 0.05$) or one-way ANOVA using the Tukey's test as the *post-hoc* test (multiple comparison) ($P < 0.05$, corrected for multiple comparisons). The data from hydrophobicity and biofilm formation were also analysed through a two way ANOVA ($P < 0.05$), by using the treatment (control or attenuated strains) and the time of sampling as categorical predictors. The statistic was performed through the software Statistica for Windows (Statsoft, Tulsa, and Okhla.).

3. Results and discussion

The basic requirement of attenuation is to avoid the acidification due to probiotics without affecting their viability; therefore, the first experiment was aimed at assessing the retention of viability after US-treatment and the delay of acidification (Table 1). Attenuation (i.e. acidification delay after 6 h) was found in the following conditions: 40% of power for 8 min for *A. jensenii*, and 60% for 4–8 min for *P. freudenreichii* subsp. *freudenreichii*. The second basic requisite was on the viability: *A. jensenii* was never affected at 40% of power, whereas *P. freudenreichii* was not affected at 60% for 4 or 6 min and underwent a significant reduction of the viable count (6.20 log cfu/mL) after 8 min. Therefore, the optimal combinations for attenuation were set as follows: 40% for 8 min for *A. jensenii* and 60% for 4 min for *P. freudenreichii*.

US-treated bacteria were also studied in relation to their ability to grow at different pH, temperature, and with salt added in order to test the effect of US on growth profiles of the target strains. The results were

Table 1
Viable count and acidification after 6 h (decrease of pH) of US-treated propionibacteria, compared to the control (untreated microorganism) of *A. jensenii* DSM 20535 and *P. freudenreichii* subsp. *freudenreichii* DSM 20271. Mean values ± standard deviation.

Power/duration	*A. jensenii*		*P. freudenreichii*	
	Acidification	*Viable count (log cfu/mL)*	*Acidification*	*Viable count (log cfu/mL)*
Control	0.83 ± 0.04^{A}	8.93 ± 0.11^{A}	0.80 ± 0.01^{A}	8.68 ± 0.12^{A}
40%/4min	0.84 ± 0.01^{A}	8.99 ± 0.23^{A}	0.88 ± 0.00^{A}	8.77 ± 0.35^{A}
40%/6min	0.88 ± 0.01^{A}	8.99 ± 0.25^{A}	0.85 ± 0.01^{A}	8.49 ± 0.25^{A}
40%/8min	0.23 ± 0.00^{B}	8.66 ± 0.09^{A}	0.82 ± 0.01^{A}	8.58 ± 0.32^{A}
60%/4min	0.28 ± 0.02^{B}	7.09 ± 0.33^{B}	0.23 ± 0.01^{B}	8.83 ± 0.13^{A}
60%/6min	0.26 ± 0.03^{B}	6.87 ± 0.11^{B}	0.20 ± 0.03^{B}	8.49 ± 0.21^{A}
60%/8min	0.18 ± 0.02^{C}	5.25 ± 0.13^{C}	0.14 ± 0.01^{D}	6.20 ± 0.12^{B}

^{A,B,C}Different letters within each column mean significant differences (one-way ANOVA and Tukey's test, $P < 0.05$).

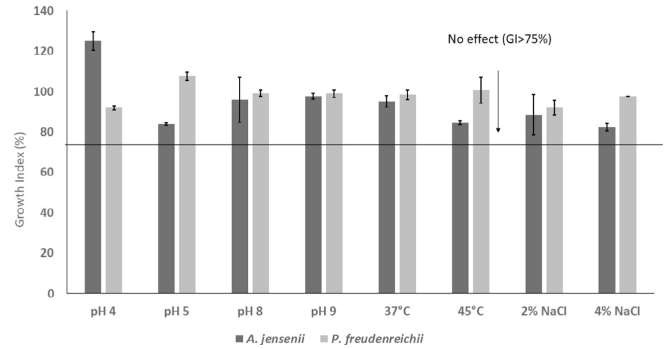

Fig. 1. Growth Index (%) of attenuated strains in MRS broth after 48 h. Mean values ± standard deviation.

modelled with the equation of Growth Index (GI) by Bevilacqua et al. (2009), modified for attenuation (ratio treated *vs* untreated microorganisms) (Racioppo et al., 2017). A GI > 75% suggests that the treatment does not affect the growth kinetic of the target strains; on the other hand a GI < 25% or in the range 25–75% pinpoints a strong or a partial inhibition, respectively. US-treatment did not affect the growth of both *Propionibacterium*, and after 48 h the strains showed a GI of at least 90% (no inhibition) (Fig. 1). The impact of this assay is different from the viable count: in fact, the viable count showed that US did not affect viability, thus the strains did not die. On the other hand, GI evaluated the ability of the target to grow: as a function of a treatment, a microorganism could not lose its viability, but the growth could be affected, delayed or inhibited. This effect could be found in the case of sub-lethal injuries: a sub-lethal injured microorganism has suffered some form of stress but it has the potential to regain viability and to grow (Wu, 2008). Therefore, GI evaluation focused on a delay of growth as a function of US treatment and suggested that the treatment had no effect on this trait.

Apart from the technological effect of US on the delay of acidification for 6 h, in the second part of this research the target trait was biofilm formation, as Erriu et al. (2014) stressed the effects of US on the ability of bacteria to produce a layer on different surfaces. Two way ANOVA suggested that sonication, both as a single hurdle or in interaction with time, could significantly affect the formation of biofilm, with a $p < 0.01$. The results for *A. jensenii* are in Fig. 2.

After 3 days, the levels of sessile cells was 4.86 log cfu/cm^2 for the control cells and 5.39 log cfu/cm^2 for US-treated bacteria; the effect of sonication was not significant. On the other hand, the effect was significant after 5 and 7 days, as the level of sessile cells of US-treated *A. jensenii* was higher than the control microorganism (4.72 vs 3.20 log

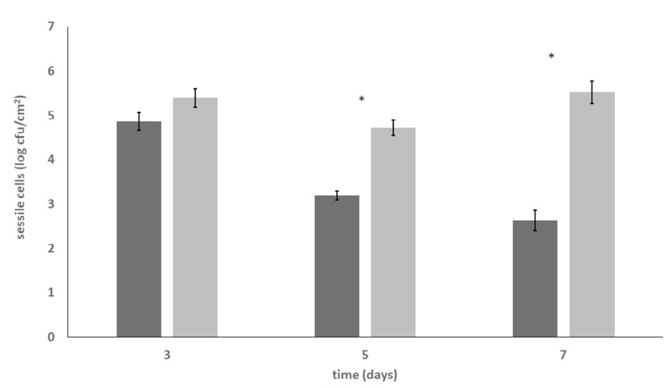

Fig. 2. Level of sessile cells of *A. jensenii* DSM 20535 after 3, 5 and 7 days of incubation. Mean values ± standard deviation. CNT, reference strain; TR, attenuated strain.
The symbol "*" indicates a significant difference between the reference and the attenuated strain.

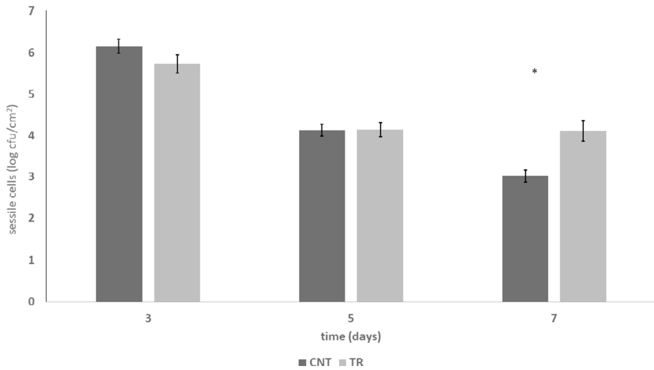

Fig. 3. Level of sessile cells of *P. freudenreichii* DSM 20271 after 3, 5 and 7 days of incubation. Mean values ± standard deviation. CNT, reference strain; TR, attenuated strain.
The symbol "*" indicates a significant difference between the reference and the attenuated strain.

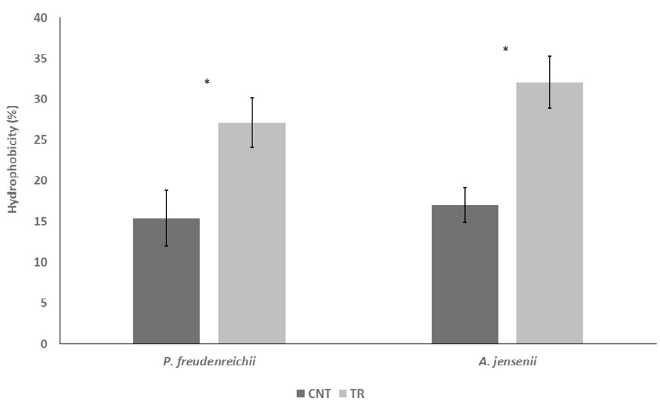

Fig. 4. Mean hydrophobicity (%) of *P. freudenreichii* DSM 20271 and *A. jensenii* DSM 20535. Mean values ± standard deviation. CNT, reference strain; TR, attenuated strain.
The symbol "*" indicates a significant difference between the reference and the attenuated strain.

cfu/cm^2 after 5 days and 5.53 vs 2.62 log cfu/cm^2 after 7 days). US also affected the stability of the biofilm of *P. freudenreichii* (Fig. 3); for this microorganism, the population of sessile cells experienced a decreasing trend for both the untreated and the US-treated microorganism. However, after 7 days the level of sessile cells in US-treated population was significantly higher than in untreated population (4.11 log cfu/cm^2 vs 3.02 log cfu/cm^2).

In the gastrointestinal tract, bacteria may be free-living or attached to mucus, mucosa surface, food particles or digestive residues. The attached bacteria produce micro-colonies, leading to the development of biofilms, which initially may be composed of only one bacterial species, but frequently develop into a complex community composed of different bacterial species. The formation of a biofilm on the mucus is important for some probiotic activities exerted by several genera and species, like the modification of some signaling pathways within the host cells (Yadav et al., 2017).

Bacterial attachment could be strongly affected by US, both with negative (detachment) or positive impacts (enhancement of adhesion) (Erriu et al., 2014). Monsen et al. (2009) and Pitt and Ross (2003) found that US stimulated bacterial metabolism and increased the ability to adhere to inert surfaces by enhancing the mechanisms related to biofilm formation. This positive effect could be the result of an increase of nutrient transportation in the deeper layers of biofilm (Peterson and Pitt, 2000; Qian et al., 1996). The effect of nutrient transportation could lead to a higher stability of biofilm and could offer an idea on why the level of sessile cells of US-treated *A. jensenii* and *P. freudereichii* were

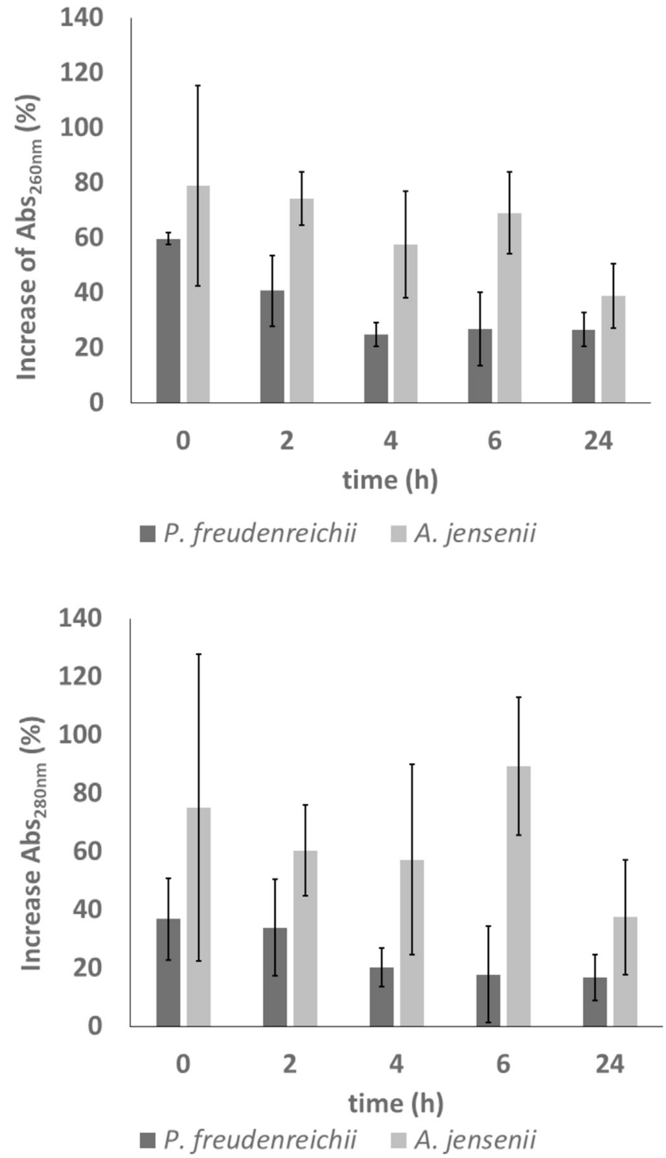

Fig. 5. Release of nucleic acids (increase of Abs at 260 nm) and proteins (increase of Abs at 280 nm) from propionibacteria (20535, *A. jensenii*; 20271, *P. freudenreichii* spp. *freudenreichii*) (0, immediately after sonication). Mean values ± standard deviation.

higher than the levels of untreated bacteria (reference strains) after 7 days.

Another possible effect of US on biofilm could be due to a clumping effect (increase of auto-aggregation), found by Tabatabaie and Mortazavi (2008) in *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactococcus lactis* subsp. *cremoris* after US-exposure.

Biofilm formation is affected by hydrophobicity (Donlan, 2002). ANOVA highlighted a significant effect of sonication on this parameter, too ($P < 0.01$): the results are in Fig. 4. Because of US-treatment, the mean hydrophobicity increased from 15.45 to 27.12% for *P. freudenreichii* and from 17.03 to 32.11% for *A. jensenii*.

The results on biofilm confirmed that the higher stability could be the result of the increase of hydrophobicity; further investigations are required to assess if other factors play a significant role. The results on hydrophobicity also have other interesting implications; in fact, the first line of contact of probiotics into the gut is the mucus. It acts as a protective and hydrophobic layer, thus the hydrophobicity of bacterial surface could favour the contact with host and promote the second stage of adhesion by specific cell wall components (de Wouters et al.,

Table 2
Minimal Inhibitory Concentration (μg/mL) of antibiotics toward propionibacteria. CNT, control sample; TR, US-attenuated strain (*A. jensenii* DSM 20535: power, 40%; time, 8 min; pulse; 2 s; *P. freudenreichii* spp. *freudenreichii* DSM 20271: power 60%; time 4 min; pulse 2 s). Mean values ± standard deviation.

	A. jensenii		*P. freudenreichii*	
	CNT	TR	CNT	TR
Chloramphenicol	6.00 ± 0.00	7.00 ± 1.04	2.00 ± 0.00	2.00 ± 0.00
Gentamycin	95.00 ± 1.41*	48.00 ± 0.00*	96.00 ± 0.00*	52.50 ± .0.70*
Clarithromycin	0.86 ± 0.18	0.57 ± 0.26	5.00 ± 1.40	3.00 ± 0.00
Trimethoprim	-**	–	–	–
Erythromycin	2.50 ± 0.70	2.50 ± 0.70	12.00 ± 0.00*	8.00 ± 0.00*
Ampicillin	1.13 ± 0.50	1.25 ± 0.40	0.38 ± 0.00	0.38 ± 0.00
Ciprofloxacin	–	–	–	–
Vancomycin	–	–	–	–
Tetracyclines	48.00 ± 0.00	48.00 ± 0.00	12.00 ± 0.00	12.00 ± 0.00

*For each microorganism and antibiotic, the difference between control and treated cells is significant (t-student, $P < 0.05$).
**The microorganism was resistant (no MIC detected).

2015; Haddaji et al., 2015).

An open question is the time-dependency of US-treatment. The results of this paper suggested that some effects could be retained over the time, as the higher stability of biofilm was found after 5–7 days. In addition, some preliminary experiments on *L. plantarum* (Bevilacqua, unpublished results) highlighted that US-treated cells maintained some properties for 48 h. However, the stability of US-treated propionibacteria could be a challenge and should be further studied and investigated.

In the last step, the effect of US-attenuation on the permeability of the membrane was studied by means of the release of intracellular components (absorbance reading at 260 and 280 nm). US-treatment increased Abs at 260 nm and 280 nm (Fig. 5A and B), with some differences between the two strains, or due to the time of sampling (immediately after the treatment-0, or after an incubation for 2, 4, 6 and 24 h).

The most important phenomenon of sonication is the cavitation, along with bubble formation and implosion; these two events are related to the formation of injuries on the membranes. This effect was indirectly demonstrated by focusing on the release of intracellular components (absorbance at 260 and 280 nm-nucleic acids and proteins); injured cells often lose amino acids, nucleic acids, and proteins) through leakage into their surroundings, since the cell membrane appears to be the component most commonly affected.

As reported by Wu (2008), after thermal or not thermal technologies there may be one population of microbes which are dead, another population that are viable, and a third population that are sub-lethally injured. The combination of the results on viability, GI and absorbance reading at 260 and 280 nm (release of intracellular components) suggested that US treatment on propionibacteria at medium-to-low power impaired and increased the permeability of cell membrane (a kind of sub-lethal injury), but this effect was not so strong to exert a significant effect on the ability to grow and on the viability.

The effect of US-treatment on cell permeability was further assessed by means of antibiotic resistance. The antibiotics were selected on the basis of their mode of action in order to understand which was the goal and the target of US-treatment on cells. Gentamycin, tetracyclines, and clarithromycin inhibit the protein synthesis and act on the 30S; trimethoprim and ciprofloxacin have other targets (the metabolism of folic acid and DNA, respectively) (Alighardashi et al., 2009; Weinstein, 1967). All these antibiotic should enter the cell to exert their bioactivity, thus an increase of permeability could be related to a decreased MIC. On the other hand, ampicillin and vancomycin act on the cell wall and an increased permeability would not affect their effectiveness. The strains were resistant to vancomycin, ciprofloxacin, trimethoprim, and US did not modify these traits. On the other hand, US-attenuation caused a decrease of the Minimal Inhibitory Concentration (MIC) of gentamycin towards both propionibacteria, and erythromycin for only *P. freudenreichii* subsp. *freudenreichii* (Table 2). Thus, the decreased bioactivity of gentamycin and erythromycin confirmed the increased permeability of the membrane, although other mechanisms are probably involved, because for other antibiotics the decrease of MIC was not found.

In conclusion, the results of this research confirm that sonication could be used to control and/or modulate post-acidification of propionibacteria. The best combinations were the following: 40%/8 min for *A. jensenii;* 60%/4min for *P. freudenreichii* subsp. *freudenreichii*, as they could delay acidification for at least 6 h, without compromising viability or growth patterns.

Concerning some selected probiotic traits, this paper could be the background for future researches and to confirm the results *in vivo*. US-treatment improved the stability of biofilm, and this result could be linked to the increase in hydrophobicity found after sonication; finally, the treatment caused an increase of cell permeability, as suggested by the release of nucleic acids, and proteins and by the increased sensitivity to some antibiotics.

Further investigations are required to validate attenuation of propionibacteria in foods, as well as to assess the mechanisms beyond the higher stability of biofilm and the increase of hydrophobicity, along with the impacts of these results on the probiotic effect of propionibacteria. Moreover, the effect of US on the adhesion of propionibacteria to intestinal cell lines should be tested to confirm the positive effect on adhesion to mucus: adhesion, in fact, is a complex phenomenon and the formation of biofilm could be also affected by the kind of surface.

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