BioTechnologia vol. 101 (2) • pp. 159–165 • 2020
Journal of Biotechnology, Computational Biology and Bionanotechnology RESEARCH PAPERS
http://doi.org/10.5114/bta.2020.94774

Ultrasound conditioning of *Streptococcus thermophilus* CNRZ 447: growth, biofilm formation, exopolysaccharide production, and cell membrane permeability

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Abstract

Sonication is one of the new and innovative approaches that is being increasingly used in food industry to control fermentation processes and to eradicate spoiling. Recently, this approach has seen new industrial applications such as enhancing microbial productivity. The present study aimed to assess the effects of ultrasound conditioning on the metabolism and extracellular matrix production of *Streptococcus thermophilus*. Bacterial suspensions were treated in ultrasonic bath (35 kHz, 240/60 W peak/nominal power, 1.8 l capacity) for different time periods (5, 10, 15, 20, 30, 45, and 65 min), and the growth improvement, adhesion ability, biofilm formation, and exopolysaccharide production of the bacterial strain were measured. The bacterial strain exhibited resistance to the treatment, and the conditioning improved the growth, adhesion, membrane permeability, biofilm formation, and exopolysaccharide production ability. An optimal treatment was obtained for 30 minutes of conditioning. An excellent yield of desirable exopolysaccharides (1788 mg glucose equivalent/l) was achieved. Ultrasound conditioning may be used as a potential approach to enhance certain biotechnological properties of industrial microorganisms.

Key words: *Streptococcus thermophilus*, ultrasound conditioning, bacterial adhesion, biofilm, exopolysaccharides

Introduction

Lactic acid bacteria (LABs) are a group of microorganisms that are generally recognized as safe (GRAS) and are highly used in biotech industry (such as cell reactors) and healthcare (such as probiotics). They are ranked second after yeasts as the most valued microbial bioresource for humans (Xiao et al., 2014). For instance, LABs were first used for fermentation in dairy industry, and their use is related to their ability to transform basic milk to high-grade products such as yogurt, cheese, ice cream, and kefir. The principal qualities of dairy products are preservation from spoiling, organoleptic properties, and texturing/rheological properties (Novel, 1993; Mozzi et al., 1996). In addition, contemporary consumers are increasingly anxious about the health-related properties of food and are raising concerns for safety and probiotic properties of dairy products. Because of their beneficial uses, LABs are widely considered in mainstream research, and many efforts have been made to improve their functional properties. The most investigated properties are their benefits to human health as probiotics and their industrial performance as cell reactors (Burgain et al., 2011).

Probiotic properties imply the ability of the microorganisms to tolerate digestive tract conditions, to nest in (at least for some time), to compete (through either production of antimicrobial or outgrowth against pathogens), and/or to improve digestion and nutrition (FAO/WHO, 2001; Boubakeur et al., 2018). Biofilm is a physiological state of microorganisms that exhibits

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specific traits, including improving adhesion ability and production of exopolysaccharides (EPS). Adhesion ability is an important characteristic that facilitates probiotic nesting in the mucous membranes of the digestive tract (FAO/WHO, 2001; O'Grady and Gibson, 2005). EPS are biomolecules belonging to the class of dietary fibers, which are known for their health effects as prebiotics and shield for the microorganisms against the adverse gastric environment (Caggianiello et al., 2016; London et al., 2016). In addition, these microbial metabolites exhibit antispoiling, rheological, and textural properties in milk products (Patel et al., 2012). However, only few dairy fermentative microorganisms can efficiently produce these multifunctional molecules, and the highest production yield currently reported is 3 g/l (Welman and Maddox, 2003). For instance, the most efficient dairy fermentative bacteria *Streptococcus thermophilus* (*S. thermophilus*) can yield only 1–2 g/l (Wu et al., 2014; Kanamarlapudi and Muddada, 2017).

Many strategies have been investigated to enhance microbial biosynthesis. These strategies include improvements of the culture media and conditions (i.e., temperature, pressure, pH, shear stress, and oxygen supply). The use of ultrasound is an emerging technology in biotech industry to improve microbial performance (Christi, 2003; Shikha et al., 2016). Ultrasounds are human-inaudible sound waves (frequencies superior or equal to 20 kHz) that can interfere mechanically and chemically with cell structure, metabolism, and physiology (Leighton, 2007). Few research studies have focused on investigating microbial conditioning by using this technology in terms of biofilm and EPS production capacities. In our previous study (Boubakeur et al., 2018), we reported interesting functional properties (prebiotic effects) of EPS of a local dairy starter isolate *S. thermophilus*. Therefore, with an interest in this microbial metabolite, the purpose of this study was to investigate the effects of ultrasound conditioning on biofilm formation, cell aggregation, EPS synthesis, and cell membrane permeability properties of the reference starter culture *S. thermophilus* CNRZ 447.

Materials and methods

Bacterial suspension preparation

The reference dairy starter culture *S. thermophilus* CNRZ 447 was obtained from INRA Rennes-France microbial collection. To assess the purity of the strain, it was grown aerobically on M17 agar (Pronadisa, Spain) at 42 °C. An overnight grown new culture was always prepared on M17 agar for analysis. The colonies were picked with a Pasteur pipette and suspended in saline physiological water to optical density (OD) of 0.11 (10^8 CFU/ml) at 578 nm wavelength (BIOCHROM Libra S6).

Ultrasound conditioning

A series of glass test tubes were prepared, each containing 9 ml of M17 broth (Pronadisa) and 1 ml of 10^8 CFU/ml *S. thermophilus*, and sonicated using the cavitation effect in Sonorex ultrasonic bath (35 kHz, 240/60 W peak/nominal powers, 1.8 l capacity) (SONOREX TK 52). Briefly, the test tubes were placed in a porous rack and put in the sonicator tank. The tank was filled to the mark with 5% Tickopur R 33 (Sigma-Aldrich), and the sonication was operated continuously. The time was measured with a chronometer, and duplicate tubes were withdrawn after 5, 10, 15, 30, 45, and 65 minutes. The experiment was performed at room temperature of 25 °C.

Survival and growth ability

The sonicated bacterial suspensions were incubated at 42 °C for 24 h. Bacterial growth after the sonication treatments was subjectively assessed through OD measurement at 578 nm wavelength (BIOCHROM Libra S6). The OD reading was then converted to CFU log10/ml. The tolerance of the strain to sonication was also verified on agar plates.

Biofilm quantification

Microplate containing M17 broth was inoculated with the sonicated inocula and then incubated at 37 °C for 24h and the biofilm was quantified using the crystal violet staining method as described by O'Toole (2011). The stained biofilm was read at the determined optimal wavelength of 492 nm (ELx800TM Biotek microplate reader).

Membrane permeability

The cell membrane permeability was determined using the quantification of the leaked protein and nucleic acid according to Dai et al. (2016). The culture supernatant was collected by centrifugation at 3000 *g* for 20 minutes and subsequently diluted (1 : 9 v/v supernatant: distilled water). The extracellular proteins and

nucleic acids were measured by absorbance (A) at 280 and 260, respectively. The membrane permeability (P%) was calculated using equation (1):

$$P\% = 100 \times (A_{ultrasound} - A_{without\ ultrasound}) / A_{ultrasound} \quad (1)$$

Autoaggregation ability

The autoaggregation kinetics were measured according to Kos et al. (2003). The bacterial suspension was incubated for 18 h at 42 °C and centrifuged at 5000 *g* for 15 minutes. The cell sediment was then washed twice with saline phosphate buffer (PBS) and dissolved again in PBS to achieve a cell concentration of 10^7 to 10^8 (OD_0 = 0.11 at 578 nm). Subsequently, a 10 ml aliquot was transferred into a glass test tube and isolated from shocks. The OD of the 1–2 cm upper layer of the suspension was monitored after 1, 2, 3 and 4 hours (OD_t). The autoaggregation percentage (A%) was calculated using equation (2):

$$A\% = 100 \times (1 - OD_t / OD_0) \quad (2)$$

EPS production

The ultrasonic-treated and nontreated bacterial suspensions in M17 broth were grown for 18 h at 42 °C. The EPS were released using a thermal shock in water bath (80 °C for 15 minutes) and ethanol precipitation as described by Ricciardi et al. (2002).

EPS HPLC- profiling

The EPS of the isolate were HPLC-profiled using the Agilent LC 1260 - Refractive index detector system. The EPS were dissolved in 1 ml of HPLC grade water (Sigma-Aldrich). Then, 100 µl of the extract suspension was mixed with 200 µl of 2M HCl and sonicated for 45 min a 45 °C bath and continuously vortexed. The acid-hydrolyzed EPS volume was made up to 1 ml with the eluent 80% acetonitrile and 20% water (HPLC grade, Sigma-Aldrich). A volume of 300 µl of crude extract was also mixed with the eluent. The extracts were filtered (0.22 µm), and a volume of 10 µl was injected. The crude and hydrolyzed extracts were analyzed on an Agilent Zorbax Carbohydrate analysis column (4.6 × 150 mm, 5 µm). The flow rate was 1.3 ml/minute, and the operation temperature was 30 °C.

EPS quantification

Crude EPS extracts were quantified as glucose equivalent according to the phenol-sulfuric acid method (Dubois et al., 1956).

Statistical analysis

All the analyses were performed in duplicate and expressed as mean ± standard deviation. ANOVA one-way and Tukey's HSD were performed with the significance level of 0.05 by using Statistical Package for Social Sciences (SPSS) 21.0.

Results and discussion

Effect of ultrasound conditioning on growth

The sonication effects on the growth ability of the model strain *S. thermophilus* CNRZ 447 is shown in Figure 1. The species tolerated the sonication treatment for the duration below 30 minutes. For longer treatments (45 and 65 minutes), the sonication significantly affected the bacterial growth ability (7.76 log10 vs 8.55 log10 and 7.66 log 10 vs 8.55 log10, respectively) ($P < 0.05$), thus suggesting the destructive effect of the ultrasound treatment. In contrast, Nguyen et al. (2009) indicated that ultrasounds (20 kHz) can have dual effects on bifidobacteria: a negative effect because of the reduction in the number of viable cells and a positive one because of the fact that broken or destroyed cells can provide essential nutrients and signaling molecules that can promote culture growth. In agreement with our results, low intensity ultrasounds and short exposition times (less than 30 minutes) were demonstrated to improve the growth ability of *Lactobacillus helveticus* PTCC 1332, *Lactobacillus acidophilus* PTCC 1643, *Bifidobacterium longum* FTDC 8943, *B. longum* FTDC 2113, and *Lactobacillus casei* ATCC 393 (Yeo et al., 2011; Hashemi et al., 2018). It could be interpreted that ultrasounds can interfere with bacterial metabolisms and conditions important for physiological effects (Christi, 2003; Leighton, 2007; Shikha et al., 2016). However, depending on the application conditions (time, viscosity of the media, potency, and frequency of the wave) of the sonication and the characteristics of the bacterial species (cellular wall structures), ultrasounds can have different consequences that are either deleterious or beneficial for the bacteria (Tabatabaie and Mortasavi, 2008; Kobayashi et al., 2009). Tabatabaie and Mortasavi (2008) showed that selected LABs could tolerate sonication at different intensities. According to Garcin et al. (2015), LABs are more resistant to high-power ultrasonic treatment than yeast cells and suggested that this is due to the differences in cell sizes—sensitivity to sonication is greater when

the cell is larger. Even among bacteria, cell sizes seem to have an important contribution to resistance to sonication treatment, as observed with our laboratory experience on resistance of *Lactobacillus bulgaricus* and *S. thermophilus* strains (data not published).

Effect of ultrasound conditioning on biofilm propensity

The results of the biofilm propensity of the strain *S. thermophilus* CNRZ 447 are presented in Figure 2. Ultrasound conditioning improved the biofilm production ability of the strain for the treatment durations of 15, 30, 45, and 65 minutes as compared to the control without sonication (OD 0.20, 0.33, 0.22, and 0.25, respectively vs 0.1 for control). The biofilm production declined significantly to approximately 24–33% after 45 and 65 minutes of sonication as compared to the conditioning time of 30 minutes. This observation could be associated with the destruction of the bacterial cells with prolonged sonication treatments (Fig. 1). Generally, ultrasounds are being used as a cleaning technology to eradicate unwanted microorganisms and biofilms causing contamination. Nonetheless, the treatment can be gauged to trigger a stimulatory effect. As shown in Figure 1 and Figure 2, ultrasounds can destroy bacterial cells, but they can also be considered as a stressing or an interfering agent that can stimulate biofilm formation (a sophisticated protection mechanism of bacteria). We identified an optimal ultrasound conditioning time (30 minutes) to stimulate biofilm formation by *S. thermophilus* CNRZ 477. To the best of our knowledge, there is no study on the biofilm stimulation effect of ultrasound on LABs. Because of the long domestication of the LABs, they have lost, in contrast to the pathogens, the ability to produce consistent biofilms (Couvigny et al., 2015). Here, we showed that the ultrasound technology can be used to stimulate biofilm formation, which can be valuable for industrial microorganisms such as LABs. The biofilm production capacity can enhance several performances of the microorganisms, including resistance to antimicrobial agents and some industrial operations steps (shears and pH), the biosynthesis processes (extracellular compounds), and the probiotic properties (adhesion, colonization, and immunomodulatory effect) (Czaczyk and Myszka, 2011; Couvigny et al., 2015; Caggianiello et al., 2016).

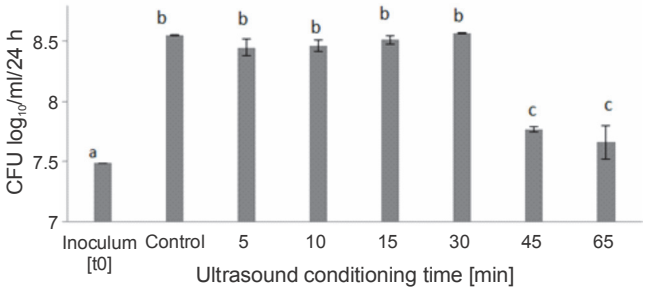

Fig. 1. Tolerance and growth ability of *S. thermophilus* CNRZ 447 as affected by ultrasound conditioning (values with different superscript letters (a, b, and c) are significantly different, $P < 0.05$)

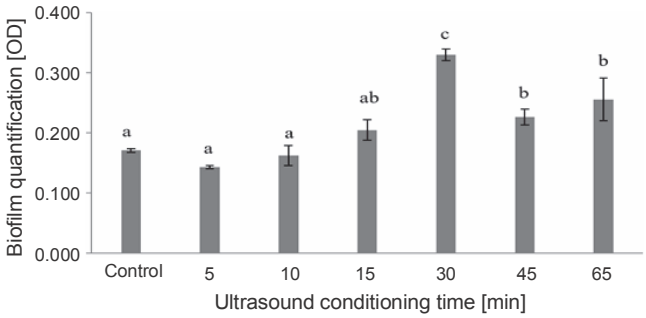

Fig. 2. Biofilm propensity of *S. thermophilus* CNRZ 447 as affected by ultrasound conditioning (values with different superscript letters (a, b, and c) are significantly different, $P < 0.05$)

Effects of ultrasound conditioning on membrane permeability

The precise mechanisms associated with biological activation with ultrasounds are still highly speculated. The most suggested mechanism is that the ultrasonic waves can induce microdiffusion, which can generate strong convection and improve substrate transfer (Christi, 2003; Leighton, 2007). Therefore, to understand the mechanochemical interferences of the ultrasounds that may have affected the strain's physiology, the protein and nucleic acid diffusion was assessed. The increase in the content of extracellular proteins and nucleic acids was $55 \pm 7.07\%$ and $1.55 \pm 0.7\%$, respectively. These results suggest that the ultrasounds had a low effect on nucleic acid diffusion but induced a high excretion of proteins, probably associated with the stimulation of protein synthesis and/or membrane permeability. The ultrasounds can effectively improve cell membrane permeability, facilitate the flow of nutrients and metabolites, and activate the metabolic processes

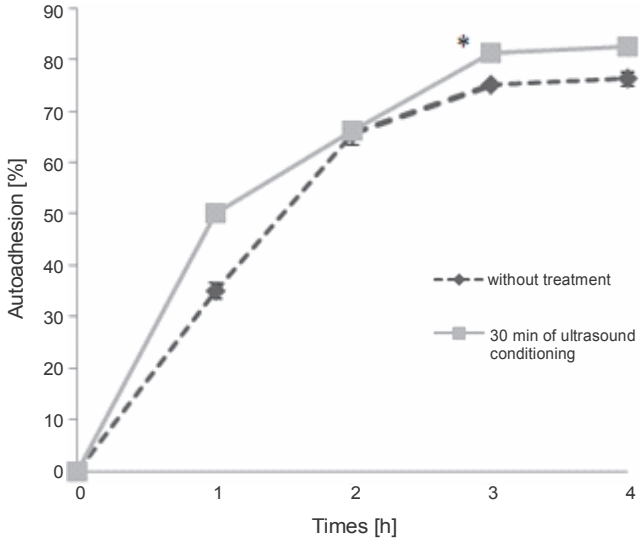

Fig. 3. Autoadhesion of *S. thermophilus* CNRZ 447 as affected by ultrasound conditioning (* indicates that the value is significantly different compared to the control without treatment (0.05))

(Pitt and Ross, 2003; Yang et al., 2010). The cell membrane permeability is associated with passive diffusion of the quorum-sensing protein signaling molecules that can explain the stimulation of biofilm formation (Kamaraju et al., 2011; Fitzgerald et al., 2018).

Effects of ultrasound conditioning on autoaggregation

Adhesion ability is a fundamental step for probiotics to exert their health effect on the host. Several molecules may be involved in this process, such as cell wall teichoic and lipoteichoic acids, peptides and proteins, peptidoglycans, and EPS (Sanchez et al., 2007). The autoaggregation kinetics of the ultrasound-conditioned cells were compared to those of the control cells, as shown in Figure 3. *S. thermophilus* CNRZ 447 exhibited strong autoaggregation ability (above 70% after 3 hours), which is consistent with previous observations suggesting that *S. thermophilus* species are known to have strong adhesion ability (above 50%) (Leighton, 2007; Khalil, 2010; Tuncer and Tuncer, 2014). Ultrasound-conditioning (30 minutes treatment) significantly improved the adhesion capacity of the strain (to approximately 82%) ($P < 0.05$). We did not find any study that has evaluated the effect of ultrasounds on the aggregation capacity of this strain to better understand the nature of the interactions between ultrasounds and the adhesive properties of the strain. However, it could be inferred that the ultrasound can erode the surface structure of the cells and expose the adhesion molecules. Tabatabaie and Mortasavi (2008) showed that after an ultrasonic treatment (20 kHz) with increasing duration, some proteins of the cell wall were activated and promoted good adhesion of the bacteria to surfaces.

Effects of ultrasound conditioning on EPS production

HPLC-profiling (Fig. 4) revealed that *S. thermophilus* CNRZ 447 EPS was mainly composed of two triholosides constituted mostly of glucose. The exact monosaccharide composition of the EPS was not adequately determined. Nonetheless, *S. thermophilus* species has been reported to produce heteropolysaccharides (Ricciardi et al., 2002; Kanamarlapudi and Muddada, 2017); however, the structural characterization of EPS of the *S. thermophilus* strains still needs further characterization. The present study showed that the *S. thermophilus* strain has important potential to produce EPS (Table 1). Ultrasound conditioning enhanced the metabolic activity of the strain and it outperformed EPS production (1745 mg glucose equivalent/l vs 176 ± 4 mg glucose equivalent/l) for an optimal time of 30 minutes of sonication. Importantly, EPS production increased after 15 minutes of sonication but declined after 65 minutes (541 ± 0.1 mg glucose equivalent/l and 83 ± 3 mg glucose equivalent/l, respectively). The decrease in EPS production after the prolonged sonication time might be explained by the deleterious effects of the treatment. Adequate time conditioning is thus essential for the use of ultrasound to improve the properties and performance of the beneficial microorganisms. Moncada and Aryana (2012) observed that low-potency sonication and controlled duration of exposure improve the desired properties of *S. thermophilus*, such as resistance to hostile gastrointestinal tract conditions (bile and pH) and secretion of bioactive metabolites. The performance achieved was comparable to the maximum EPS yield of *S. thermophilus* species (1–2 g/l) (Wu et al., 2014; Kanamarlapudi and Muddada, 2017). In agreement with our previous study (Boubakeur et al., 2018), it was possible to improve EPS yield (from 200 to 826 mg glucose/l, $P < 0.05$) by conditioning *S. thermophilus* with polyphenol extract and to demonstrate their properties as prebiotics.

Conclusions

The present study reported the potential of sonication in improving bacterial metabolism, conditioning biofilm physiological traits, and enhancing EPS produc-

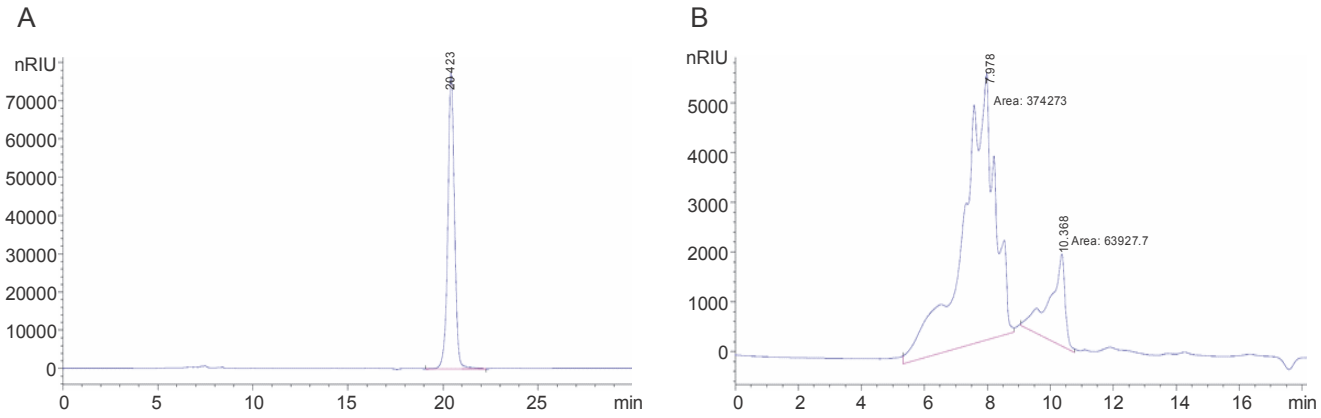

Fig. 4. HPLC-profiling of EPS of *S. thermophilus* CNRZ 447: A) A unique characteristic peak was observed in the EPS hydrolysis at 20.4 minute; it was identified as glucitol, which suggests that, B) the EPS is composed of at least two distinct triholosides (between 7 and 10 minutes) that are constituted of mainly glucose residues

Table 1. EPS productivity of *S. thermophilus* CNRZ 447 as affected by ultrasound conditioning

Time [min]	Control	15 min	30 min	65 min
EPS [mg glucose/l]	176 ± 4^{a}	541 ± 0.1^{b}	1745 ± 12^{c}	83 ± 3^{d}

Values with different superscript letters (a, b, c, and d) are significantly different ($P < 0.05$)

tion of a potential dairy starter and probiotic strain. There is increasing interest in the health-promoting properties of probiotics and their EPS. Physical conditioning techniques such as ultrasound should be further explored and used to enhance the properties of beneficial microorganisms.

Acknowledgments

The authors express their gratitude to Algeria Ministry of Higher Education and Scientific Research and to the General Directorate for Scientific Research and Technological Development for the PhD grant.

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