Metabolic attenuation of probiotics: a strategy for functional beverages development

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This PhD thesis dealt with the development of a strategy to counteract the deviation of probiotic food characteristics induced by probiotic metabolism. The strategy applied, namely metabolic attenuation, was based on a multiple physical approach, sonication and microencapsulation, to control the fermentative metabolism of the probiotic *Lacticaseibacillus casei* ATCC 393.

Attenuazione metabolica dei probiotici: una strategia per lo sviluppo di bevande funzionali

Questa tesi di dottorato ha riguardato lo sviluppo di una strategia per contrastare l’alterazione delle caratteristiche di un alimento probiotico indotte dal metabolismo del probiotico stesso. La strategia applicata, ovvero l'attenuazione metabolica, ha riguardato un approccio fisico multiplo, la sonicazione e la microincapsulazione, per controllare il metabolismo fermentativo del probiotico *Lacticaseibacillus casei* ATCC 393.

**Key words**: sonication, microencapsulation, flow cytometry, cultivability, acidification, surface properties.

# 1. Introduction

In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following three activities:

A1) sonication process design and optimization and characterization of its effects on *L. casei* ATCC 393;

A3) microencapsulation process design and optimization;

A4) sonication and microencapsulation assessment as multiple strategy.

# 2. Sonication

Ultrasound has gained much attention in the last decade as a mean to manipulate microbial cells. Mechanical and chemical events (temperature and pressure increase, high shear forces and free radical generation) occur upon the implosion of the cavitation bubbles. Thus, the physiology of the cell is altered. The microorganism response, stimulation, inactivation, or destruction, depends on the intensity of the phenomenon of cavitation (Zupanc *et al*., 2019). So far, very little is known about the modulation of probiotic’s activity and of the attenuation effect.

# 3. Microencapsulation

Microencapsulation is a well-known entrapment process used in the food and probiotic field. Microcapsule controls the rate of nutrient uptake and metabolite release, slowing them down enabling minimal cell-environment interactions (Sun *et al.*, 2023). From this point of view, microencapsulation can be considered an attenuation technology. To our knowledge, microencapsulation has never been used to modulate probiotic metabolism.

# 4. Experimental Procedure

In this PhD thesis the experimental procedure was set up by performing sonication experiments on the probiotic *L. casei* ATCC 393 followed the determination of its effects on several cell characteristics by applying conventional and multiparametric analysis. An independent microencapsulation experiment was then performed to choose the encapsulating agents and their concentrations. Finally, the two technologies were combined.

# 5. Materials and Methods

Two pulsed sonication treatments (6 or 8 min) with fixed power and frequency were carried out on water bacteria suspension. Attenuation was assessed as pH decrease of MRS broth after 6 and 24 h of incubation at 37 °C (Racioppo et al., 2017). Then, cultivability (spread plate count and growth index), auto-aggregation, hydrophobicity (cells affinity to iso-octane), membrane permeability and biofilm production were evaluated. Probiotic resuscitation through a growth curve was assessed. Light microscopy and light scattering angles were used for a morphological characterization. SYTO24TIM and cFDA (carboxyfluorescein diacetate) were combined with PI (propidium iodide) for viability and esterase activity evaluations through a flow cytometer. Alginate concentration (0.8, 1.0, 1.2, 1.5%) and chitosan coating (0.7%) were evaluated in the attenuation efficacy of microencapsulation. Microcapsules shape and size were also studied. Finally, sonicated cells were entrapped in 1.5% alginate and in chitosan-alginate microcapsules.

# 6. Results and Discussion

## 6.1 Sonication

## *6.1.1 Acidifying capabilities and cultivability*

Table 1 shows the results of ultrasound-induced attenuation and probiotic cultivability. The 6 min-treatment induced temporary attenuation while the 8 min-treatment induced complete attenuation. The findings imply that LC\_S6 can restore its metabolism and that increasing the sonication intensity results in a stronger attenuation effect. Hypothetically, free radicals, formed due to implosion of cavitation bubbles, can interact with enzymes involved in the sugar transport and metabolization system leading to their oxidation and, consequently, alteration of normal function.

***Table 1*** *Attenuation and cultivability alteration induced by ultrasound. LC\_S0:* L. caseiATCC 393 *non sonicated (control); LC\_S6:* L. caseiATCC 393 *sonicated for 6 min; LC\_S8:* L. caseiATCC 393 *sonicated for 8 min.\**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Sample** | **Acidification (ΔpH)** | | **Log CFU/ml** | **Growth index** | | |
| **t6** | **t24** | **GI > 75 %**  **No growth inhibition** | **25 < GI < 75 %**  **Partial growth inhibition** | **GI < 25 %**  **Complete growth inhibition** |
| LC\_S0 | 0.38 ± 0.05A | 2.16 ± 0.18A | 9.30 ± 0.02A | - | - | - |
| LC\_S6 | 0.06 ± 0.02B | 1.91 ± 0.12A | 8.58 ± 0.12B | + | - | - |
| LC\_S8 | 0.03 ± 0.02B | 0.97 ± 0.14B | 6.43 ± 0.04C | - | - | + |

Sonication reduced the plate count by approximately 1- and 3-Log for the 6 and 8 min-treatment, respectively. The growth index was calculated as follows:

(1)

where AUS is the absorbance of sonicated samples, and AC is the absorbance of the control.

Collected data show that there was no growth inhibition for sample LC\_S6 and complete inhibition in the case of LC\_S8. Therefore, probiotic cultivability was affected by sonication. In stress conditions, bacteria can enter in a viable but non-culturable (VBNC) state. However, further analysis is required to proper define the ultrasound-induced VBNC status. Results obtained from the growth index analysis suggest that LC\_S6 resuscitate. Although the more intense treatment may have caused LC\_S8 inactivation, Brandão et al. (2021) demonstrated that ultrasound inactivation does not impair the health benefits of probiotics.

## *6.1.2 Cell surface characterization*

Cell surface properties of *L. casei* ATCC 393 and of the sonicated probiotic are summarized in Table 2.

Membrane damage is given by:

(2)

Auto-aggregation and hydrophobicity were calculated as follows:

(3)

where A0 is the absorbance of samples at time 0, and At is the absorbance after incubation.

Biofilm production was quantified by establishing a low cut-off (ODc) and comparing OD570 of the samples with it. Sonicated *L. casei* ATCC 393 resulted in increased membrane permeability due its damage and weakening. Upon sonication, a negative correlation was found between sonication treatment and probiotic auto-aggregation, while hydrophobicity increased, and biofilm production improved. Our results suggest that ultrasound alters the surface structure of *L. casei* ATCC 393, thus affecting its normal function and physiological activities. This could explain the different adhesive properties of the sonicated strains. Furthermore, comparing our data with those in the literature, it is evident that the response to ultrasound treatment is strain- and species-specific.

***Table 2*** *Adhesive properties and membrane structure evaluation before (LC\_S0) and after sonication (LC\_S6: 6-min treatment; LC\_S8: 8-min treatment).\**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sample** | **Auto-aggregation (%)** | **Hydrophobicity (%)** | **Biofilm production** | **Membrane permeability (%)** | |
| **A260** | **A280** |
| LC\_S0 | 23.98 ± 1.32A | 6.29 ± 0.68A | Weak (OD ≤ ODc) | - | - |
| LC\_S6 | 3.39 ± 0.78B | 11.68 ± 2.65B | Strong (4ODc < OD) | 216 ± 8.16A | 140 ± 10.00A |
| LC\_S8 | 0.65 ± 0.26C | 15.01 ± 1.59B | Strong (4ODc < OD) | 256 ± 10.69B | 165 ± 9.64B |

## *6.1.3 Cell morphology*

Microscope images (Figure 1) show the ultrasound-induced morphology variation. Sonicated bacteria presented a single cell morphology, resulted in a smaller rod cell compared to the *Streptobacillus* morphology of the control. FCM analysis also confirmed these changes. Forward Scatter (FSC) is related to the cell size and surface area while the Side Scatter (SSC) is related to the granularity or internal complexity. Both parameters were reduced in sonicated samples (Table 3) proving that the high shear forces and shock waves impair the membrane structure, thus changing the cell morphology, and also cause the leakage of intracellular components, thus reducing internal complexity.



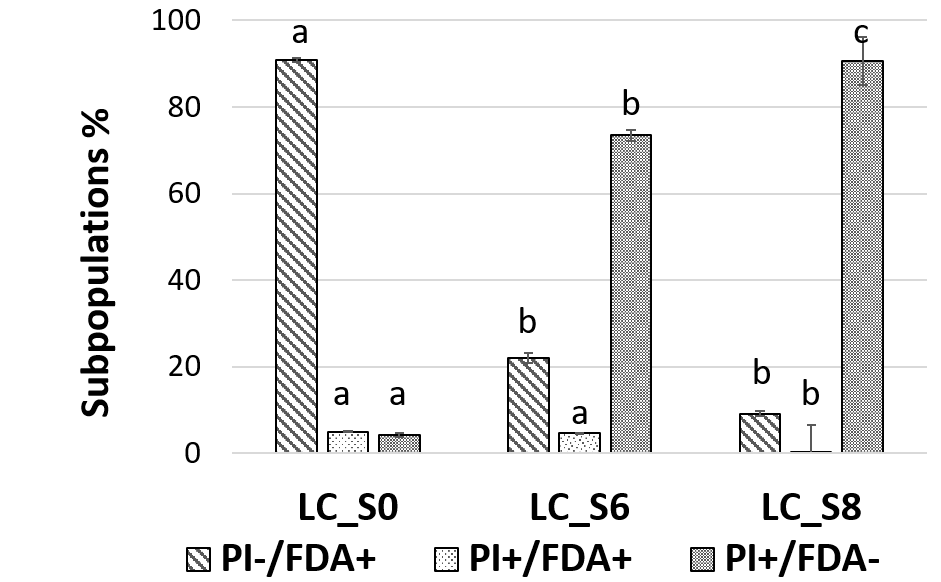
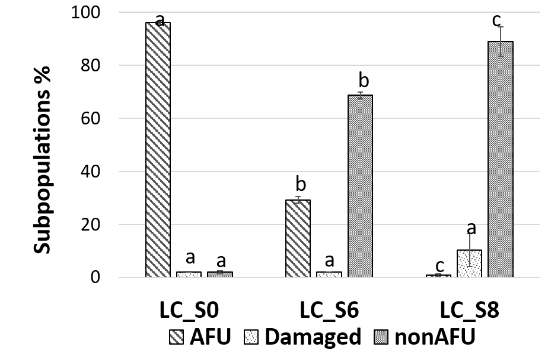
**Figure 1** *Microscope images (400x magnification) of* Lacticaseibacillus casei *ATCC 393 (a);* L. casei *sonicated for 6 (b) and 8 min (c).*

***Table 3*** *Values and percentage decrease of* Lacticaseibacillus casei *ATCC 393 Forward and Side Scatter angles. LC\_S0: L. casei non-sonicated (control); LC\_S6: L. casei sonicated for 6 min; LC\_S8: L. casei sonicated for 8 min.\**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample** | **FSC** | **ΔFSC-H %** | **SSC** | **ΔSSC-H %** |
| LC\_S0 | 48,202 ± 268A | - | 30,817 ± 972A | - |
| LC\_S6 | 17,373 ± 177B | -63.96 ± 0.57A | 10,247 ± 146B | -66.74 ± 0.57A |
| LC\_S8 | 17,443 ± 443B | -63.81 ± 1.12A | 9,840 ± 404B | -68.07 ± 0.31A |

## *6.1.4 Membrane integrity and esterase activity*

Stressful treatments generate subpopulations in different physiological states that can be detected by multiparametric flow cytometry analysis. As shown in Figure 2, the double staining with the cell permeant and impermeant nucleic acid dyes, SYTO 24(TIM) and PI, revealed a depletion of the viable population in both sonicated treatments in favour of dead subpopulation. In addition, sublethal injured cells were not detected. Our results suggested that the membrane of *L. casei* is impaired due to the violent events generated during ultrasound propagation and that ultrasound induced an “all-or-nothing” phenomenon. Figure 3 shows the distribution of metabolic active subpopulations in the suspension. The percentage of the cells able to metabolize the non-fluorescent cFDA in the fluorescent cF was reduced upon sonication. Thus, ultrasound affects the esterase activity of the probiotic.



**Figure 2** *Subpopulations detected with SYTO 24TIM/PI double staining of* L. casei *before sonication* *(LC\_S0)* *and* *after sonication (LC\_S6: 6-min treatment; LC\_S8: 8-min treatment)*. *AFU (Active Fluorescent Unit): SYTO+/PI-; Damaged: SYTO+/PI+; nonAFU (non-Active Fluorescent Unit): SYTO-/PI+.*

**Figure 3** *Subpopulations detected with cFDA/PI double staining of* L. casei *before sonication* *(LC\_S0)* *and* *after sonication (LC\_S6: 6-min treatment; LC\_S8: 8-min treatment)*. *PI-/FDA+: metabolic active cells; PI+/FDA+: damaged cells; PI+/FDA-: non metabolic active cells.*

## *6.1.5 Comparison between cultivable, viable and metabolic active population*

Multiple comparisons between the samples and between subpopulations of the same sample allows the discrimination of the ultrasound target on *L. casei*. Probiotic cultivability (Log CFU/ml), viability (Log AFU/ml) and metabolic activity (Log (PI-/FDA+)/ml) are summarized in Table 4. The 6-min treatment impaired the probiotic cultivability and metabolic activity, but not its viability. In addition, the three populations are not different. The higher intensity, instead, reduced the viability and cultivability while did not further impair the esterase activity. Furthermore, in LC\_S8 the viable population is no different from the cultivable and metabolically active ones, while the cultivable population is lower than the metabolically active one. Therefore, *L. casei* retains its esterase activity upon each treatment, whereas it does not retain cultivability and viability with the severe one. Moreover, The FCM data negate the previous hypothesis of VBNC induction.

***Table 4*** *Comparison of viable, cultivable and metabolically active (Log) populations of the non-sonicated (LC\_S0) and sonicated samples for 6 (LC\_S6) and 8 min (LC\_S8).\**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Cultivability** | **Viability** | **Metabolic activity** |
| LC\_S0 | 9.15 ± 0.03Ab | 8.89 ± 0.03Aa | 8.86 ± 0.07Aab |
| LC\_S6 | 8.52 ± 0.08Ba | 8.60 ± 0.12Aa | 8.57 ± 0.06Ba |
| LC\_S8 | 6.37 ± 0.05Cab | 7.20 ± 0.23Ba | 8.36 ± 0.02Bac |

## *6.1.6 Restoring of normal physiology*

Beyond the instantaneous effect of ultrasound on cultivability and viability, it could also affect the growth kinetics. The growth curve of the three samples is reported in Figure 4. Growth curves comparison revealed again a time-dependent effect of ultrasound on *L. casei* ATCC 393. A significant delay in the probiotic growth was found upon sonication. As postulated by Ojha et al., (2017), random and a high level of sonoporation lead to an uncontrolled efflux of cells components and to a delay of cell metabolism. These results also confirmed the growth index values.

## 6.2 Microencapsulation

**Figure 4** *Growth curve of* Lacticaseibacillus casei *ATCC 393 during 48 h of incubation at 37 °C. LC\_S0: L. casei non sonicated (control) (■); LC\_S6: L. casei 6-min sonicated (♦); LC\_S8: L. casei 8-min sonicated (▲).*

## *6.2.1 Microcapsules shape and size*

The bead shape affects the mechanical and chemical stability of the microcapsules while the bead size affects the efflux rate of nutrients and metabolites. Taking this into account, these parameters were evaluated. As shown in Figure 5, by increasing the alginate concentration an improvement of beads shape is obtained, reaching a perfectly spherical shape and smooth surface with the 1.5% solution (MC). On the other hand, chitosan-alginate microcapsules (CMC) appear with a wrinkled and rougher surface (Figure 6). Moreover, MC are characterized by a diameter of approximately 240 µm, while a slight contraction is observed in CMC.

**Figure 6** *Light microscope images (400x) of 1.5% alginate microcapsules (MC, a) and chitosan-alginate microcapsules (CMC, b).*

**Figure 5** *Light microscope images (400x) of microcapsules with different alginate concentration. a: 0.8%; b: 1.0%; c: 1.2%; d: 1.5%.*

## *6.2.2 Attenuation efficacy of microencapsulation*

Data of *L. casei* acidification abilities in microcapsules are reported in Table 5. Regardless the polymer concentration, the high-water content and high porosity of the alginate beads facilities the diffusion of molecules. Considering these results and the morphological evaluations, a chitosan coating was added on the 1.5% alginate microcapsules. Only, CMC were able to control the broth acidification. Alginate-chitosan high electrostatics interactions confer to the microcapsule new permeability properties, a more homogeneous surface and reduced porosity. Thus, the CMC capsule efficiently control the probiotic acidification abilities.

## 6.8 Sonication and microencapsulation as multiple attenuation strategy

Table 5 also shows the results of the efficacy of sonication and microencapsulation as an attenuation strategy. Collected data suggested that sonication-induced attenuation is significantly improved when a chitosan-alginate microcapsule is built around the cells. Interesting, chitosan-alginate microcapsules showed different preperformances from free cells and alginate microcapsules for all the samples.

***Table 5*** *pH decrease\* of MRS broth after* Lacticaseibacillus casei *ATCC 393 inoculum in free form, in 1.5% alginate microcapsules (MC) and in chitosan-alginate microcapsules (CMC), not sonicated (LC\_S0), 6-min sonicated (LC\_S6), 8-min sonicated (LC\_S8).*

|  |  |  |  |
| --- | --- | --- | --- |
| **Samples** | **Free form** | **MC** | **CMC** |
| **6 h of incubation** | | |
| **LC\_S0** | 0.48 ± 0.03Aa | 0.26 ± 0.01Ab | 0.22 ± 0.05Ab |
| **LC\_S6** | 0.14 ± 0.02Ba | 0.16 ± 0.01Ba | 0.16 ± 0.01Ba |
| **LC\_S8** | 0.11 ± 0.03Ba | 0.15 ± 0.01Ba | 0.15 ± 0.01Ba |
|  | **24 h of incubation** | | |
| **LC\_S0** | 2.17 ± 0.04Aa | 2.04 ± 0.05Aa | 1.54 ± 0.09Ab |
| **LC\_S6** | 2.10 ± 0.02Aa | 1.83 ± 0.07Aa | 1.49 ± 0.04Ab |
| **LC\_S8** | 1.04 ± 0.03Ba | 0.77 ± 0.01Bb | 0.31 ± 0.03Bc |

*\*Data are reported as means value* ± *standard deviation* *(n = 3). Statistical analysis (One-way ANOVA, unpaired and paired t-Student tests) were performed by SPSS software (p < 0.05). Different capital letters in the same column and different lower-case letters in the same row indicate that the differences are significant.*

# 7. Conclusion and Future Perspectives

The concept of healthy food for healthy life leads food companies and scientists towards the formulation of new probiotic foods that can meet the demand of the most (or all) consumers. Food probiotication need an in dept knowledge of the probiotic-matrix interaction. Research efforts have been focused on developing systems capable of preserving cell viability rather than preserving the sensory characteristics of the product. Therefore, the PhD thesis was focused on the development of an attenuation strategy to modulate or to control the metabolism of probiotic in food.

Our results showed that the attenuation effect of sonication depends on the intensity of the treatment. Ultrasound was proved to be a suitable technology to modulate the *L. casei* ATCC 393 acidification abilities. Although it caused some side effects such as a major loss of membrane integrity, reduction of cultivability and auto-aggregation, it also enhanced the surface hydrophobicity and biofilm production thus improving the probiotic adhesion abilities. This underline that ultrasound has a broad spectrum of action on bacteria. FCM data also proved that not all the metabolic activities of the cells are impaired. Therefore, although further analyses are needed, we can assume that not all cellular functions, such as probiotic activities, are impaired. Although modulation of probiotic metabolism occurs at different levels in the cell, the loss of viability does not allow to test more intense sonication treatments. Microencapsulation overcomes the limits of sonication in probiotic activity modulation. The findings of the study highlight that microcapsules with adequate barrier properties are the key factor to develop an efficient attenuation system. Besides the physical modulation, microencapsulation could modulate the bacteria Quorum Sensing (QS) activity (Li et al., 2023).

The results obtained in laboratory need to be confirmed in the more complex environment of a food matrix. The operating parameters for both sonication and microencapsulation can be changed and used in several combination. This opens up the possibility to build unlimited systems with specific attenuation abilities and to introduce a new kind of probiotic food on the market. Moreover, sonication and microencapsulation applications on bacteria are not limited on the metabolism attenuation. A study on transcriptome and on QS could lead to a deeper comprehension of the phenomena that occur inside the cells upon sonication and inside the microcapsule, thus allowing a conscious manipulation of both technologies to achieve many different goals.

# 8. References

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