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Impact of probiotic cell inclusion and metabolic activity on the rheological properties of low-methoxyl pectin solutions and gels

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ABSTRACT

The rheological behavior of low-methoxyl (LM) pectin solutions and gels was characterized to evaluate the impact of the inclusion of probiotic cells (*Lactobacillus rhamnosus* GG and *L. casei*) and its metabolites. Considering that probiotic cells metabolize macro and/or micro-nutrients in growth media and/or can modulate pectin-Ca^{2+} interactions occupying the free volume in the gel, two scenarios were tested: 1) A 2% pectin in free-carbohydrate basal medium (P-MRS-B) (control sample) was mixed with probiotic cells ($\sim 10^7$ cells/mL) and incubated at 37 °C for 48 h (fermented samples); 2) A probiotic suspension in P-MRS-B was prepared as described above and used immediately to measure rheological properties (*in situ* samples).

All gel-forming solutions presented Newtonian behavior and their corresponding gels showed an intermediate behavior between weak-gel and strong-gel. Mean viscosity (η) values ranged between 15.9 and 20.1 mPa s and no significant differences ($p = 0.643$) were observed between the samples. Mean elastic modulus at 1 rad/s (G'_1) values ranged between 447 and 499 Pa, with no significant differences ($p = 0.975$) between the samples. These results suggest that: 1) The volume fraction of probiotics was so low that its presence did not have a significant effect on the viscosity and on the elastic modulus, and neither on the gelation mechanism of the LM-pectin; 2) Metabolic products of LM-pectin fermentation did not have a significant effect on the viscosity of the solutions, nor on the elastic modulus of the gels and their gelation mechanism.

1. Introduction

Pectin has been widely used as gelling, thickening, stabilizing and emulsifying agent in the food industry, and has been typically applied in products such as jams, jellies, and marmalades, confectionary products and bakery fillings (Fraeye et al., 2010). It is supposed that pectin will have a fascinating trade market in the future compared to some other hydrocolloids because of its drastic benefits at low quantities rather than the cost (Ciriminna et al., 2016).

Pectin is a pectic polysaccharide-based biopolymer derived from the primary cell wall and the intracellular layer of higher plants (Padma Ishwarya et al., 2021; Van Buren, 1991). It is composed of a backbone of linearly connected α (1–4) D-galacturonic acid residues and their methyl esters, containing significant amounts of L-rhamnose (Rha), D-arabinose (Ara) and D-galactose (Gal), and 13 different monosaccharides along with these (Kaya et al., 2014).

Carboxyl groups of the galacturonic acid residues of pectin may be esterified with non-sugar components such as methyl or acetyl groups. Depending on the degree of methyl-esterification or methylation (DM), pectin is classified as either low-methoxyl (LMP; DM <50%) or high-methoxyl (HMP; DM >50%) type (Walkinshaw & Arnott, 1981).

The DM determines the mechanism of formation of pectin gels, and their conformational and rheological properties (Fishman et al., 1984; Grosso & Rao, 1998; Gigli et al., 2009). In HM pectin gels formation is governed by both hydrogen bonds and hydrophobic interactions, and requires conditions of low pH (~3) and water activity (typically, high sugar content ~ 65%) (Oakenfull, 1991). Oh the other hand, LM pectins form gels by reaction with calcium ions (Ca^{2+}, or other divalent metal ions) over a wide range of pH, with or without sugar. The "egg box" model have been widely accepted to describe this gelling mechanism (Grant et al., 1973) where carboxyl groups of different pectin chains are held together via ionic linkages with calcium ions, forming more or less

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extended junction zones (Axelos & Thibault, 1991; Fraeye et al., 2010).

Because of their ability to gel even without the presence of sugar, LM pectin gels have many applications in low calorie and dietetic foods (May, 1990). Moreover, encouraging findings on pectin's structure-function relationship in recent years has led to interesting food applications of pectin hydrogels such as fat replacer, texturizer, edible ink for 3D food printing, packaging film, and encapsulant or carrier material for the targeted delivery of nutrients/bioactives (Padma Ishwarya et al., 2021). In addition, pectin and its derivatives have potential to act as candidates for new-generation prebiotics (Hotchkiss et al., 2003). As dietary fiber, pectin escapes digestion in the small intestine and passes into large intestine, where it is metabolized by the gut microbiota, modulating its diversity and composition producing acetate, propionate and butyrate (Gullón et al., 2013; Reichembach & de Oliveira Petkowicz, 2021).

In particular, LM pectin hydrogels have proved to function as an effective probiotic delivery system. Low-methoxyl pectin and its combinations with other chemicals have been used to entrap probiotic bacteria by internal gelation mechanisms and acceptable results were observed in microbial viability under simulated gastrointestinal conditions and food matrix (Gebara et al., 2013; Ghibaudo et al., 2017; Khorasani & Shojaosadati, 2016; Raddatz et al., 2020; Tarifa et al., 2021). The capacity of pectin to act as a delivery vehicle for probiotics, its prebiotic nature and nutraceutical properties, can enable its utilization in promoting health.

As regards to the impact of the inclusion of probiotic cells on the rheological properties of pectin gels, it is often underestimated. Similarly, how the use of pectin by probiotic cells as a fermentable substrate affects these properties has not been studied. Although the pellet of living cells is incorporated at rather low concentrations in the gel-forming solution, the bacterial cells can interact with the conveying material in several ways including: (a) metabolizing macro (prebiotics)- or micro-nutrients into secondary metabolic products (postbiotics), e.g., organic acids, exopolysaccharides, amino acids and peptides, etc. (Zendeboodi et al., 2020), (b) interacting via short or long range forces with proteins or polysaccharides through their surface cellular elements, e.g., pili (Burgain et al., 2013), and (c) occupying the free volume between the biopolymers and thus, modulating the polymer-polymer interactions (Kanmani & Lim, 2013; Tapia et al., 2007).

Concerning to the rheological properties of gels, the impact (either positive or adverse) of probiotic cells have been related exclusively with the ability of the cells to be enclosed in the interspaces between entangled polymer chains, altering the porosity of the gel and the molecular mobility of the polymer chains (Tapia et al., 2007; Kanmani & Lim, 2013; Hellebois, 2020).

Considering the pectin gels as potential matrices to carry viable microorganisms, to our knowledge, no reports on the effect of metabolic activity of probiotic cells on the rheological properties of pectin gels have been published hereto.

In view of the above, the objective of this study was to evaluate the impact of two recognized probiotic microorganisms (*Lactobacillus casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 53103 (strain GG onwards *L. rhamnosus* 53103) in gels based on low methoxyl pectin solutions, and to characterize the rheological properties of the probiotic solutions and their corresponding gels.

2. Materials and methods

2.1. Chemical reagents and culture media

Low methoxyl pectin (Genu Pectin LM104 AS, CPKelco, Limeira, Brazil) used had an esterification degree of 27%, and 20% of the original carboxyl groups were replaced by amide groups. Calcium chloride (Anedra S.A., San Fernando, Buenos Aires, Argentina) used in this study as cross-linking agent was of analytical grade. D (+)-Glucose was purchased from Merck (Germany).

The culture media used for experiments were: Mann Rogosa Sharpe (MRS) broth and agar (Merck, Germany), Tryptic Soy Broth (TSB, Difco, Detroit, USA), Tryptic Soy Agar (TSA, Difco, Detroit, USA), MRS carbohydrate-free basal medium (MRS-B) which does not contain either glucose or meat extract (peptone 10.0 g/L, yeast extract 5.0 g/L, potassium phosphate 2 g/L, sodium acetate 5.0 g/L, magnesium sulphate 0.20 g/L, manganese sulphate 0.05 g/L, ammonium citrate 2.0 g/L, Tween 80 1.08 g/L, pH 6.5) and M9 minimal medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 1 mL/L $MgSO_4{\cdot}7H_20$ 1 M, 10 mL/L $CaCl_2$ anhydrous 0.01 M, pH 7.4). All chemicals used were of reagent grade.

2.2. Bacterial strains and culture conditions

The bacterial strains used in the study were *Lactobacillus casei* ATCC 393, *Lactobacillus rhamnosus* ATCC 53103 (strain GG onwards *L. rhamnosus* 53103) and *Escherichia coli* ATCC 25922 purchased from the American Type Culture Collection (ATCC, Rockville, MD). Stock cultures of the strains were stored at −70 °C in 20% (v/v) glycerol.

Rheological analysis of pectin gels and solutions with probiotics were initiated by growing *Lactobacillus* strains in MRS broth for 24 h at 37 °C. For prebiotic activity tests, *E. coli* was cultured in TSB for 24 h at 37 °C and probiotic cells as described above. The cells were harvested by centrifugation at 5000×*g* for 10 min and washed twice in sterile phosphate buffered saline (PBS, pH 7.2).

2.3. Prebiotic activity score (PAS)

Considering growth rates of *L. casei 393*, *L. rhamnosus* 53103 and *E. coli* ATCC 25922 attained using glucose and pectin as C source, PAS was determined using the equation reported by Huebner et.al., (2007) with modifications:

$$A_{preb} = \frac{(Log\ P_{24} - Log\ P_0)_{prebiotic}}{(Log\ P_{24} - Log\ P_0)_{glucose}} - \frac{(Log\ E_{24} - Log\ E_0)_{prebiotic}}{(Log\ E_{24} - Log\ E_0)_{glucose}}$$

where A_{preb} is the prebiotic activity score; Log P are the log of growth expressed as Colony Forming Units (CFU)/mL of the probiotic bacteria at 24 h (P_{24}) and 0 h (P_0) of culture on prebiotic and glucose; Log E are the log of growth (CFU/mL) of *E. coli* ATCC 25922 at 24 h (E_{24}) and 0 h (E_0) of culture on prebiotic and glucose.

Briefly, the assays were carried by adding stock solutions of filter-sterilized glucose or pectin (final concentrations of 1% and 2% (v/v)), to tubes with the corresponding autoclaved basic media: MRS-B for *L. casei* and *L. rhamnosus* and M9 minimal medium for *E. coli*. The basic media plus prebiotic and glucose (as positive control of growth) were inoculated with 1% (v/v) of the bacterial cultures prepared as described in 2.2. The assays included a negative control without carbohydrate.

After 24 h of incubation at 37 °C in aerobic conditions, samples were enumerated on TSA for *E. coli* and MRS agar for probiotic strains.

2.4. Preparation of the pectin solution with/without probiotics

The initial suspensions of *L. casei* and *L. rhamnosus* were prepared as described in 2.2 and adjusted by optical density (OD) at 600 nm to 0.250 (~10^8 cells/mL). The pectin (2% w/v) was dissolved in MRS-B (P-MRS-B) under vigorous agitation at 50 °C for 1 h. Then, to reduce microbial load, solution was heated under agitation until boiling for 5 min. The procedure resulted in clear suspensions with no evidence of undissolved material.

Two scenarios were tested to evaluate the impact of the inclusion of probiotic cells on rheological properties of pectin solutions and gels, considering that probiotic cells metabolize macro and/or micro-nutrients in growth media and/or can modulate pectin-Ca^{2+} interactions occupying the free volume in the gel: 1) P-MRS-B solution was mixed with probiotic suspension in a volume ratio of 45:5 (~10^7 cells/

mL) by magnetic stirring and incubated at 37 °C for 48 h (samples 53103 F and 393 F, where F stands for fermented); 2) A probiotic suspension in P-MRS-B was prepared as described above and used immediately to measure rheological properties (samples 53103 IS and 393 IS, where IS stands for *in situ*).

For Control sample, the pectin solution with none probiotic were prepared following a similar procedure as described above and with addition of sterile PBS buffer instead of bacterial suspension. Each solution sample was prepared at least three times.

2.5. Gel preparation

Pectin gels were prepared following the method used by Löfgren et al. (2002), with slight modifications. Basically, equal parts (~30 g) of the 2% pectin solution (with or without probiotics, obtained as described in Section 2.4) and a 0.5% $CaCl_2$ solution were heated under agitation until boiling. Then, the $CaCl_2$ solution was dripped into the pectin solution under vigorous agitation. Finally, 5 ml aliquots of the final solution (1% pectin, 0.25% $CaCl_2$) were poured into 4 Petri dishes (5 cm diameter), and stored for 24 h at 4 °C to achieve complete gelation before rheological measurements. Each gel sample was prepared at least three times.

2.6. Rheological properties

Rheological properties were determined in a Paar Physica MCR 301 rheometer (Anton Paar GmbH, Graz, Austria). Viscosity of the 2% pectin solutions (with or without probiotics, obtained as described in Section 2.4) was determined from flow curves (shear stress vs shear rate, in the range 0.1–100 s^{-1}), using a concentric cylinder geometry. Each fresh sample was measured in triplicate.

Viscoelastic properties of the gels were determined by small deformation dynamic oscillatory measurements, using a parallel-plate geometry (diameter = 50 mm). The gels prepared in Section 2.5 (~50 mm diameter, ~2 mm thick) were carefully removed from the Petri dishes and placed in the rheometer plate (previously set at 20 °C), covering all of its surface.

Then, the upper plate was lowered to ~2 mm gap. The exact final gap was adjusted by gradually lowering the upper plate (0.1 mm steps) until reaching a normal force of ~1 N, which assured full contact between the upper plate and the gel, without squeezing it. Finally, storage (*G'*) and loss (*G"*) moduli of each sample were measured at 20 °C, as a function of angular frequency (ω) from 100 to 0.1 rad/s, and at 0.5% strain (γ), which was within the linear viscoelastic range (LVR), as determined in preliminary experiments. Each fresh sample was measured four times.

2.7. Scanning electron microscopy analysis

SEM was utilized to examine the morphology of LAB cells in pectin solutions. The cell pellet obtained by centrifugation was first washed with sterile saline solution (0.85% (w/v) NaCl solution) and then resuspended in 2.5% (w/v) glutaraldehyde. The cell suspension was kept at 4 °C for 2 h to fix the cells. After that, LAB cells were collected by centrifugation, followed by washing with sterile deionized water three times.

The resulting cell pellet was dehydrated with a grade series of ethanol solution, namely 30%, 50%, 70%, 80%, 90%, and 100% (v/v), for 10 min at each grade. The dehydration was ended with repeated treatments in 100% ethanol for another three times, 15 min each time. The dehydrated cells were placed in a vacuum oven at 40 °C for 2 days to allow the ethanol to evaporate. Then, the LAB cells were carefully mounted to an aluminum stub using conducting carbon tape.

2.8. Statistical analysis

For prebiotic activity assays, all analyses were performed in triplicate under identical conditions in two independent trials and the results expressed as mean and standard deviation (mean ± SD). Data were analyzed by one-way ANOVA, followed by Tukey test at a significant level of 5% ($p < 0.05$) with GraphPad InStat (Software, Inc. 2003).

For rheological assays, each sample was prepared in at least three independent experiments, and per each experiment at least three rheological measurements were performed, averaged and statistically analyzed through Analysis of Variance (ANOVA) tests and Fisher's least significant difference (LSD) comparisons with a significance level of $\alpha = 0.05$ (InfoStat v 2014 software).

3. Results

3.1. Prebiotic activity score

PAS indicates the ability of a given substrate to promote the growth of probiotic strains relative to enteric bacteria such as *E. coli*, and relative to growth on non-prebiotic substrates, as glucose.

Therefore, carbohydrates have a positive PAS if they are fermented as well as glucose by probiotic strains and they are selectively fermented by probiotics but not by other intestinal bacteria. The viable counts of *L. casei* and *L. rhamnosus* after incubation for 24 h with 2% of pectin and glucose (included as positive control) are presented in Table 1. No growth was observed for any of the tested strains in the culture controls without carbohydrate source (data not shown).

Overall, pectin significantly enhanced the growth of the probiotics compared to positive control, with scores of 1.31 and 1.52 for *L. casei* and *L. rhamnosus*, respectively, at 2% pectin concentration, and scores of 0.79 and 1.08 for *L. casei* 393 and *L. rhamnosus* 53103, respectively, at 1%. The PA values obtained in this study were indicative of a selective use of pectin sample by *Lactobacillus strains* with respect to pathogenic strain.

3.2. Rheological properties

All gel solutions presented Newtonian behavior (not shown). The viscosity (η) of each sample measurement was obtained by fitting shear stress (τ) vs shear rate ($\dot{\gamma}$) data with Newton's Law ($R^2 > 0.999$):

$$\tau = \eta\dot{\gamma} \quad (1)$$

Mean viscosity values ranged between 15.9 and 20.1 mPa s (Table 2), and no significant differences ($p = 0.643$) were observed between the samples.

All gel samples showed an intermediate behavior between weak-gel and strong-gel (Lopes da Silva & Rao, 2007), with storage modulus (*G'*) higher than loss modulus (*G"*) values (Fig. 1), and slightly dependent on the angular frequency (ω), as has been observed before in LM pectin gels (see for example Gigli et al., 2009). *G'* vs ω data of each sample were fitted with a power law function ($R^2 > 0.990$):

$$G' = G'_1\omega^n \quad (2)$$

where the pre-exponential factor G'_1 predicts the G' value at $\omega = 1$ rad/s, and the exponent n represents the slope of the $logG'$ vs $log\omega$ curves.

Mean G'_1 values ranged between 447 and 499 Pa (Table 2), with no

Table 1
Viable cell count (Log CFU/mL) of probiotic strains and *E. coli* ATCC 25922 after 24 h incubation with pectin and glucose.

Strain	Supplement	
	Pectin 2%	Glucose 2%
L. casei 393	11.30 ± 0.15 ***	10.23 ± 0.13
L. rhamnosus 53103	11.48 ± 0.10***	10.00 ± 0.07
E. coli 25922	7.78 ± 0.08 ***	10.48 ± 0.10

Results are given as mean ± SD, ***$p < 0.001$.

Table 2
Viscosity (η) of pectin solutions and viscoelastic properties (G'_1 and n from Eq. (2)) of pectin gels, without and with probiotics.

Sample	η [mPa.s]	G'_1 [Pa]	n [−]
Control	18.2 ± 4.8 [a]	499 ± 81 [a]	0.0705 ± 0.0244 [a]
53103 IS	16.5 ± 3.0 [a]	473 ± 85 [a]	0.0718 ± 0.0170 [a]
393 IS	15.9 ± 3.7 [a]	447 ± 101 [a]	0.0648 ± 0.0016 [a]
53103 F	17.7 ± 2.4 [a]	474 ± 113 [a]	0.0825 ± 0.0245 [a]
393 F	20.1 ± 2.9 [a]	488 ± 122 [a]	0.0677 ± 0.0384 [a]

Means with a common letter are not significantly different ($p > 0.05$).

significant differences ($p = 0.975$) between the samples. Mean n values ranged between 0.0648 and 0.0824, and no significant differences ($p = 0.917$) between the samples were observed either.

It should be noted that Fisher's LSD test is the most sensitive to detect differences between treatments, so if it doesn't find them means that there is more certainty that there are no differences between the samples, as observed in this work. This was attributed to the fact that there was a relatively high variability within samples, with values of up to 26% for viscosity, 25% for G'_1, and 57% for n, even though several outliers were eliminated for statistical analysis. On the other hand, the effect of the treatments was negligible compared to this variability, as discussed in the next section.

4. Discussion

As evidenced by the results, pectin acted as good growth prebiotic substrate for *L. rhamnosus* ATCC 53103 and *L. casei* 393 with significant differences ($p < 0.001$) compared with control. The higher the activity score, the higher the relative growth of the probiotic and/or the lower the relative growth of the pathogen, which indicates a higher and more selective use of prebiotic in relation to glucose by the probiotic microorganism and/or a limited use of prebiotic in relation to glucose by the pathogenic microorganism.

The degradation of pectin is facilitated by different bacteria-derived enzymes such as pectinases, methylesterases, acetylsterases, and lyases, generating different pectic oligosaccharides that will vary depending on microbial strain and pectin structure (Blanco-Pérez et al., 2021). In order to ferment pectin, lactic acid bacteria must possess 2-keto-3-deoxy-6-phosphogluconate aldolase, an enzyme that catalyses galacturonate metabolism (Biz et al., 2014). However, metabolic pathways for D-galacturonate metabolism and the resulting product profile in Lactobacilli have not been previously studied in detail (Valk et al., 2020).

Amount and type of compounds produced by LAB during pectin fermentation process will depend on LAB strains, culture medium composition and growth conditions (Ammor et al., 2006). Gómez et al. (2019) reported that the main organic acids derived from pectin and pectic oligosaccharides fermentation by *L. rhamnosus* GG in MRS carbohydrate-free basal medium were acetic, lactic and formic acids. To the best of our knowledge, no data on metabolic products derived from pectin fermentation by *L. casei* 393 are available in the literature.

The size and morphology of cells in pectin solutions was evidenced by SEM (Fig. 2A and B). Comparing the sample without probiotics (Control) and the samples with probiotics incorporated just before measurement (53103 IS and 393 IS), it was found that the sole presence of the microorganisms did not have a significant effect on the viscosity of the pectin solutions. However, the presence of microscopic particles is expected to increase the viscosity of a suspension, in a magnitude that depends on their volume fraction (i.e. their volume relative to the total volume of the dispersion) and inter-particle colloidal forces (Genovese, 2012). Pectins are anionic in nature due the dominance of carboxyl groups in polygalacturonan backbone, which, thereby, may be involved in electrostatic interactions, while the surface charge of lactobacilli is negative at physiological pH. Larsen et al. (2018) proposed that due to electrostatic interactions, LM pectins will probably have a stronger tendency to bind to the oppositely charged groups on bacterial surfaces, improving bacterial resistance.

In order to theoretically predict the effect of probiotics on the viscosity of the suspension, and for the sake of simplicity, we are going to neglect those inter-particle forces as a first approach. Under these conditions, Einstein's equation for dilute microscopic suspensions predicts the increase in viscosity produced by hard-spheres (i.e. rigid, non-interacting spherical particles):

$$\eta_r = 1 + [\eta]\varphi \tag{3}$$

where η_r is the relative viscosity, i.e. the ratio between the viscosity of the suspension and that of the solvent, and $[\eta]$ and φ are the intrinsic viscosity and the volume fraction of particles. Both probiotic cultures studied in this work (*L. casei* 393 and *L. rhamnosus* 53103) are rod-shape cells with 1–2 $*10^{-6}$ m length and 0.3–0.7 $*10^{-6}$ m diameter (Gobbetti

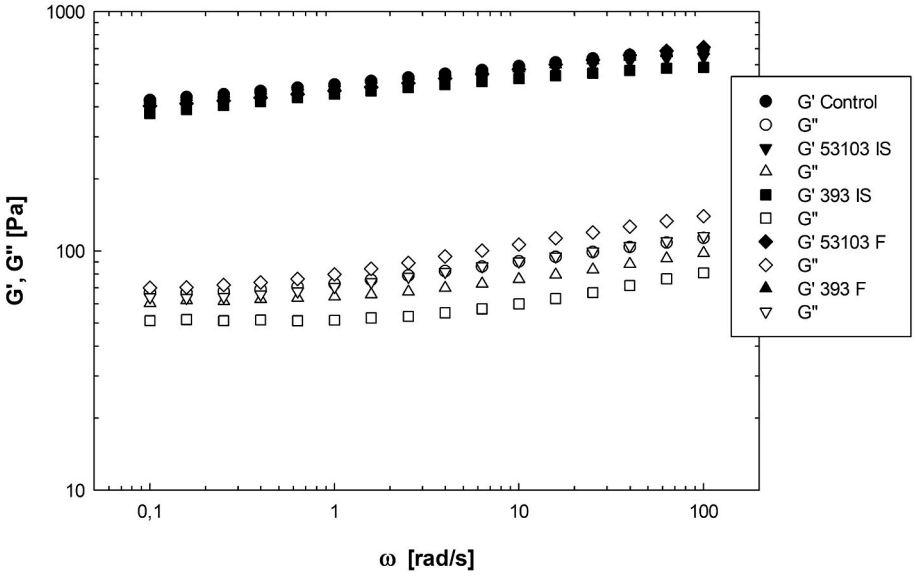

Fig. 1. Elastic modulus (G', solid symbols) and viscous modulus (G'', empty symbols) vs angular frequency (ω) of pectin gels without probiotics (Control Sample ● ○), with probiotics added and measured immediately (*in situ* samples 53103 IS ▼△ and 393 IS ■ □), and with probiotics added and measured after 48 h incubation (fermented samples 53103 F ◆ ⋄ and 393 F ▲▽).

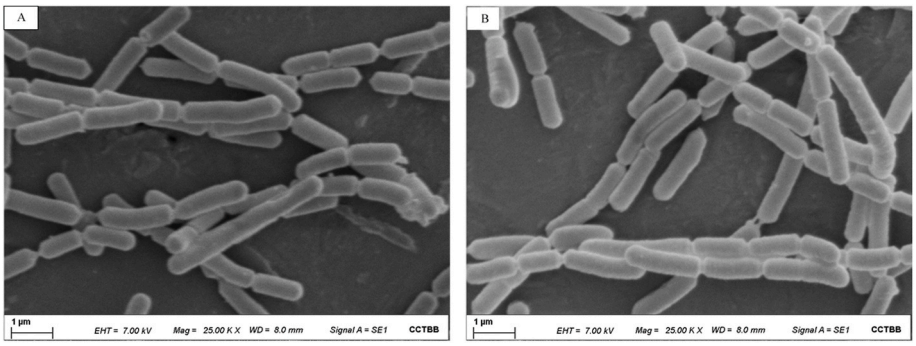

Fig. 2. SEM images of *Lactobacillus rhamnosus* ATCC 53103 (A) and *L. casei* 393 (B) in pectin solutions.

& Minervini, 2014). Taking the upper limits of both parameters (to predict the maximum effect), gives a volume of $\sim 7.7*10^{-19}$ m^3 per each cell. Considering that there were 10^8 cells per mL of suspension, this gives a maximum volume fraction of probiotics of $\varphi \approx 7.7*10^{-5}$. On the other hand, $[\eta]$ depends on particle shape, being 2.5 for spheres. For rod-shape particles as our probiotic cells, $[\eta] = 0.7*q^{5/3}$, where q is the axial ratio of the ellipsoid (Barnes, 1981). In this case, q = L/d ~2.9, giving $[\eta] \sim 4.0$. Finally, using Eq. (3) the predicted increase in viscosity produced by the presence of probiotics is ~0.03%. If inter-particle forces are to be considered, this value might be higher to some extent. However, experimental evidence suggests that the volume fraction of probiotics was so low that their mere presence did not have a significant effect on the viscosity. In agreement with our results, it has been reported that the addition of probiotic bacteria to film-forming dispersions of kefiran (Piermaria, Diosma, Aquino, Garrote, & Abraham, 2015) and cassava starch and inulin (Orozco-Parra et al., 2020) did not have a significant effect on their rheological properties (consistency index, flow behavior index and apparent viscosity).

The second conclusion when comparing samples Control vs 53103 IS and 393 IS, is that the presence of the probiotics did not have a significant effect on the elastic modulus of the pectin gels, and presumably neither on the gelation mechanism of the LM pectin. The presence of particles (called fillers) in gels (called filler-matrix composite gels) is expected to affect the viscoelastic properties of the gel (mainly the elastic modulus) in a magnitude that depends on the volume fraction of the filler, its rigidity compared to the matrix gel, and the interaction or affinity between the filler and the matrix (Genovese, 2012). So this result suggests that either the probiotics had a similar rigidity than the gel, and/or the interaction between the probiotics and the gel was neutral, and/or their volume fraction was too low to produce a significant effect on the strength of the gels.

In order to theoretically estimate the maximum effect of the presence of the probiotic cells on the elastic modulus of the pectin gel, it will be assumed that there is a positive interaction between the cells and the gel, and that the cells are rigid particles. The reinforcement effect of particles on composite gels has been defined as the relative elastic modulus, $G'_r = G'_c/G'_m$, where G'_c and G'_m are the elastic moduli of the composite (the gel with particles) and the matrix (the gel without particles), respectively (Genovese, 2012). One of the most recognized models to predict G'_r is the one of Lewis and Nielsen (1970):

$$G'_r = \frac{1 + 1.5 \bullet B \bullet \varphi}{1 - B \bullet \left[1 - exp\left(-\frac{\varphi}{1-\varphi/\varphi_m}\right)\right]}$$

$$B = \frac{M - 1}{M + 1.5}$$

where $\varphi_m \approx 0.64$ is the maximum packing fraction of particles, and $M = G'_p/G'_m$ is the relative rigidity of the particles, that is the ratio of the elastic modulus of the particles to that of the matrix. In the gels, the volume fraction of probiotics is half of that in the solutions (as in order to prepare each gel the pectin + probiotic solution is mixed with equal parts of $CaCl_2$ solution), so $\varphi \approx 3.85*10^{-5}$). Assuming the probiotic cells as rigid particles ($B \approx 1$), this model predicts that the presence of probiotic cells would increase the elastic modulus of the gel about 0.01%. As previously mentioned, this is the maximum expected effect. To the best of our knowledge, the rigidity of individual probiotic cells has not been quantified, but it is surely less than infinite, which would give an even lower contribution to the elastic modulus of the gel. This is in agreement with our experimental results.

Chen et al. (2020) determined the viscoelastic properties of LM pectin gels containing micro-fibrillated soybean cellulose and lactic acid bacteria. However, they did not analyze the effect of the bacteria on the rheological properties of the gels. On the contrary, Yan et al. (2021) reported that the incorporation of *Lb. Paracasei LS14* in mixed gels of soy protein isolate and sugar beet pectin produced a decrease in gel elasticity (G') and hardness, by reducing physical interactions or numbers of covalent crosslinks between molecules, which probably affected the network connectivity. It should be noted that they used a probiotic concentration 100 times higher than ours.

Comparing the previous samples (Control, 53103 IS and 393 IS) and the samples with probiotics incorporated 48 h before measurement (53103 F and 393 F), it was found that the metabolic products of the microorganisms during this fermentation period did not have a significant effect on the viscosity of the pectin solutions, nor on the elastic modulus of the pectin gels and their gelation mechanism. However, they did have an effect on the visual aspect of the gels.

Fig. 3 shows representative images of the different pectin gel samples. Pectin gels without probiotics (Control sample, Fig. 3A) were translucent. The addition of probiotics *in situ* provided a slight turbidity to the gels (Fig. 3B and C), while probiotics fermentation increased the turbidity, particularly in the case of *L. casei* (Fig. 3E). It has been reported that incorporation of microorganisms in films resulted in a lower degree of transparency or higher opacity, since the presence of micrometer-scale cells could obstruct or disperse the passage of light, and consequently films and coatings appearance may be affected by the presence of microorganisms (Guimarães et al., 2018; Orozco-Parra et al., 2020). As observed in this work, the metabolic products derived from pectin fermentation by lactic acid bacteria (particularly *L. casei*) further increased the turbidity of the samples.

5. Conclusions

Based on the rheological stability of low-methoxyl pectin solutions and gels when *L. rhamnosus* and *L. casei* cells interact with and/or metabolize them, overall, the results of this investigation suggest that they would be effective matrices for the formulation of films, edible coatings, microparticles and other carriers for the delivery of probiotic cells.

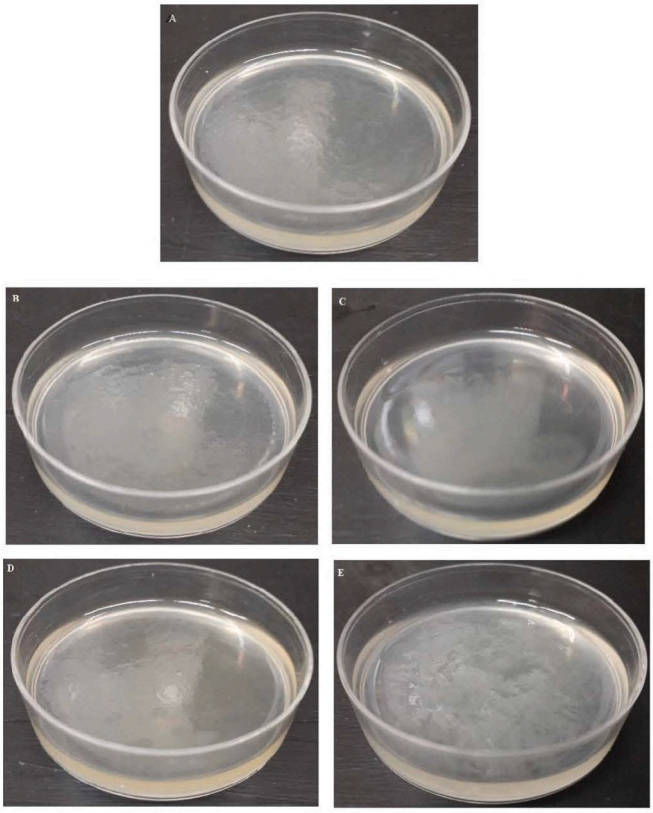

Fig. 3. Pectin gels without probiotics A) Control Sample, with probiotics added and measured immediately (*in situ* samples) B) 53103 IS and C) 393 IS, and with probiotics added and measured after 48 h incubation (fermented samples) D) 53103 F and E) 393 F.

CRediT authorship contribution statement

Lorena I. Brugnoni: Conceptualization, Formal analysis, interpretation of results, Writing – original draft, manuscript preparation, (microbiological assays), Both authors reviewed the results and approved the final version of the manuscript. **Diego B. Genovese:** Conceptualization, Formal analysis, interpretation of results, Writing – original draft, manuscript preparation, (rheological properties), Both authors reviewed the results and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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