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Effect of different biopolymer-based structured systems on the survival of probiotic strains during storage and *in vitro* digestion

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Abstract

BACKGROUND: This study aimed to evaluate the protective effect of different biopolymer systems on the viability of two probiotics (*Lactobacillus rhamnosus* and *Streptococcus thermophilus*) during storage and *in vitro* digestion. Methylcellulose (MC), sodium alginate (SA), and whey protein (WP)-based structures were designed and characterized in terms of pH, rheological properties, and visual appearance.

RESULTS: The results highlighted that the WP-system ensured probiotic protection during both storage and *in vitro* digestion. This result was attributed to a combined effect of the physical barrier offered by the protein gel network and whey proteins as a nutrient for microbes. On the other hand, surprisingly, the viscous methylcellulose-based system was able to guarantee good microbial viability during storage. However, this was not confirmed during *in vitro* digestion. The opposite results were obtained for sodium alginate beads.

CONCLUSION: The results suggest that the capacity of a polymeric structure to protect probiotic bacteria is a combination of structural organization and system formulation.

Keywords: *Lactobacillus rhamnosus*; *Streptococcus thermophilus*; structured systems; viability protection; cold storage; *in vitro* digestion

INTRODUCTION

There is an increasing demand for functional foods containing probiotic bacteria. This is due to their demonstrated capacity to confer health benefits to the host,[1] such as immune stimulation, inhibition of pathogen growth, prevention of cancer, maintenance of healthy gut microflora, and the prevention, alleviation or cure of the adverse effects of constipation, inflammatory bowel disease, and food allergies.[2,3] However, to provide such benefits, the recommended minimum probiotic population should be in the range of 10^6–10^7 CFU g^{-1} in the final product.[4] For this reason, probiotics should remain viable and metabolically active from inoculation and throughout processing and storage, as well as in transit through the gastrointestinal tract during digestion.[5] Unfortunately, several challenges could limit their application in foods, such as their susceptibility to compositional and environmental effects during both food manufacturing and digestion.[6–8] In the latter case, the acidic condition of the stomach and the presence of enzymes and bile salts in the gastrointestinal tract may influence microbial viability.[9] Efforts have therefore been made in recent years to protect probiotics, increasing their resistance to adverse conditions.

The structuring capacity of several edible biopolymers, such as polysaccharides (alginate, starch, xanthan gum), proteins (casein, gelatin, whey proteins), or a combination of them,[10–15] have been exploited to increase probiotic survival in food and in the gut. It is well known that microbial tolerance to digestion-related stresses is strongly dependent not only on the microbial strain but also on the organic nature of the structured support as well as on its physico-chemical characteristics.[16,17] The literature is rich in studies of structure design for probiotic protection as well as on their possible applications in foods.[18,19] On the other hand, only a few reports, which are not comparable, are available on the efficacy of these protective approaches during gastrointestinal digestion. Although *in vivo* analyses are still considered the 'gold standard', *in vitro* methods are nowadays more commonly used because they are rapid, less costly, less laborious, and allow many samples to be analyzed in parallel without ethical restrictions. In recent decades, many *in vitro* protocols have been used, which, however, are characterized by different parameters impeding comparison of the results.[20] For this reason, a standardized *in vitro* digestion model has been proposed recently in the COST action INFOGEST.[21] This method has been used widely to investigate mainly the digestibility and bioaccessibility of nutrients and bioactive

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compounds.[22] Moreover, it has proved to be successful in determining the microbial protective effect of some strategies on *in vitro* digestion.[23,24] Nevertheless, only a few studies have evaluated the effects of probiotic viability on *in vitro* digestion in accordance with this protocol.[14,23–25] Other studies presented significant modifications to the standardized method in terms of pH values, enzyme concentration, or digestion fluid composition, or did not reproduce the entire digestion process.[26–29] It is therefore difficult to compare the ability of structured polymeric systems to protect probiotic bacteria. This information is essential to select the best performing strategy to preserve microbial viability in foods as well as during human digestion.

Based on these considerations, three biopolymers (whey proteins, sodium alginate, and methylcellulose) able to structure in water at pH close to neutrality were considered in this study. Whey protein (WP) and sodium alginate (SA) gels were selected based on their well known capacity to deliver probiotic bacteria.[30–33] Both polymers are able to form cross-linked structures, which are considered to be the main protection mechanism for microbial cells during gastrointestinal digestion.[18] Finally, methylcellulose (MC) was included as reference viscous material, even though it was not reported to have probiotic protection capacity.

In this work, three biopolymer structured systems were enriched with *Lactobacillus rhamnosus* or *Streptococcus thermophilus*. Bacteria survival was studied during cold storage at 4 °C and during digestion under INFOGEST simulated conditions.

MATERIAL AND METHODS

Materials

Methylcellulose (MC) (1200–1800 mPa·s), sodium alginate from brown algae (SA), α-amylase from *Bacillus* sp. (EC 3.2.1.1), porcine pepsin (EC 3.4.23.1), porcine pancreatin (EC 232–468-9, 8 xUSP), porcine bile extract, phosphate buffer saline (PBS), citric acid, sodium dihydrogen phosphate dihydrate, calcium chloride, calcium chloride dihydrate, sodium carbonate, sodium bicarbonate, potassium chloride, sodium chloride, potassium dihydrogen phosphate, magnesium chloride hexahydrate, and ammonium carbonate were purchased from Sigma Aldrich (Milan, Italy). Hydrochloric acid and sodium hydroxide were purchased from J. T. Baker (Center Valley, USA). Whey protein isolate (WPI) (94.7% protein content; 74.6% β-lactoglobulin, 23.8% α-lactalbumin, 1.6% bovine serum albumin) was purchased from Davisco Food International Inc. (Le Sueur, MN, USA). Maximum Recovery Diluent (MRD), MRS agar, MRS broth, M17 agar and M17 broth were purchased from Oxoid (Milan, Italy). *Lactobacillus rhamnosus* (Lyofast LRB) was purchased from Sacco Srl (Cadorago, Como, Italy). *Streptococcus thermophilus* DSMZ 20617^T was obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). Deionized water (System advantage A10®, Millipore S.A.S, Molsheim, France) was used.

Culture preparation

Lactobacillus rhamnosus and *S. thermophilus* were stored at −80 °C as 30% (v/v) glycerol stock-cultures in MRS broth and M17 broth, respectively. Before each experiment, overnight cultures were prepared by sub-culturing 100 µL of stock-cultures in 100 mL of MRS broth or M17 broth at 37 °C for 18 h. *Lactobacillus rhamnosus* was grown in anaerobic conditions and *S. thermophilus* was grown in the presence of oxygen. Cells were then recovered by centrifugation at 13 000×*g* for 10 min at 4 °C, washed three times with PBS, and resuspended in PBS to a final viability of about 10^{10} CFU mL^{-1}.

Preparation of structured systems

Three different systems loaded with probiotics were prepared in two biological replicates. At the same time, controls (free cells) made of saline solution (NaCl 8.5% w/v), separately inoculated with the probiotics (final viable count about 10^8 CFU mL^{-1}) were prepared in duplicate.

Methylcellulose system

The methylcellulose system (S-MC) was prepared by a two-step method.[34] In the first step, 4 g of MC powder was gently mixed by a magnetic stirrer in 64 mL of water at 40 °C for 15 min and then vigorously stirred using a high-speed homogenizer (Ika-Werke, DI 25 basic, Staufen, Germany) at 800×*g* for 2 min. Then 1 mL of microbial suspension (*L. rhamnosus* or *S. thermophilus*) in PBS was added to 31 mL of cold water to reach a final concentration of about 10^8 CFU mL^{-1}, and the entire suspension was added to the MC stirred solution. The latter was gently mixed at 4 °C for 18 h to allow the maximum hydration of the MC.

Sodium alginate beads

Sodium alginate beads (S-SA) were prepared as previously described.[35] A 2% (w/v) sodium alginate solution was used to suspend *L. rhamnosus* or *S. thermophilus* to reach a final viability of about 10^8 CFU mL^{-1}. The suspension was inserted into a 5 mL sterile syringe fitted on a 27.5 G needle with a nominal inner diameter of 0.241 mm (Sol-Millenium Medical, Inc., USA). The distance from the needle tip to the calcium chloride solution was kept constant at 10 cm. The mixture of alginate-bacteria was manually extruded through the syringe into 100 mL of 0.1 M calcium chloride. The system was maintained under aseptic conditions. After 30 min, the beads were separated by decantation and rinsed with 0.1 M calcium chloride.

Whey protein system

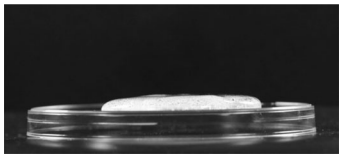
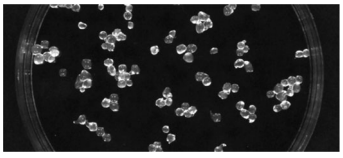
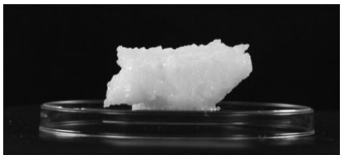
The whey protein system (S-WP) was prepared by heat denaturation followed by acidification, as previously described[36] with some modifications. Whey proteins (10% w/w) were suspended in water, stirred for 1 h, and then stored at 4 °C overnight to allow maximum hydration. The solution was then heated in a water bath at 78 °C for 30 min under continuous stirring, and cooled at room temperature. A citrate–phosphate buffer (pH 5.2), containing the microbial culture (*L. rhamnosus* or *S. thermophilus*; about 10^8 CFU mL^{-1}) was added in a 1:1 ratio to the whey protein suspension. The resulting mixture was then homogenized using a high-speed homogenizer at 1500×*g* for 20 s and stored at 4 °C for 24 h until use.

Physicochemical analysis

Image acquisition

Images were acquired using an image acquisition cabinet (Immagini & Computer, Bareggio, Italy) equipped with a digital camera (EOS 550D, Canon, Milan, Italy). The samples were placed on a black background and the digital camera was located on an adjustable stand positioned 45 cm above or in front of methylcellulose or whey protein-based systems and sodium alginate beads, respectively. The light was provided by 4100 W frosted photographic floodlights. Images were saved in *.jpeg* format, resulting in 5184 × 3456 pixels.

Table 1. Visual appearance and pH values of methylcellulose (S-MC) and whey protein (S-WP) based systems and sodium alginate beads (S-SA)

System	Visual appearance	pH
S-MC		6.3 ± 0.1
S-SA		6.5 ± 0.1
S-WP		5.9 ± 0.1

Determination of sodium alginate beads' diameter and volume

One hundred alginate beads were observed using optical microscopy (Leica DM2000, Leica Microsystems, Heerburg, Switzerland) connected with a digital camera (Leica EC3, Leica Microsystems). One bead was placed in the middle of a glass slide and analyzed at 400× magnification at room temperature. The diameter was obtained using the software Leica Suite LAS EZ (Leica Microsystems) and bead volume (mm^3) was calculated using Eqn (1):

$$V = \frac{4}{3}\pi r^3 \quad (1)$$

where V is the bead volume (mm^3) and r is the bead radius (mm).

Rheological testing

The structured systems were characterized from a rheological point of view at 20 °C using an RS6000 Rheometer (Thermo Scientific RheoStress, Haake, Germany) equipped with a Peltier cell. A parallel plate geometry was used, and the measuring gap was set at 2 mm. To determine the linear viscoelastic region for each sample, stress sweep tests were performed increasing stress from 0.1 to 100 Pa at 1 Hz frequency. Frequency sweep tests were performed, increasing frequency from 0.1 to 10 Hz using a fixed stress value included in the linear viscoelastic region. In the case of sodium alginate, the bead dimensions allowed them to be compacted into a monolayer that was carefully placed at the measuring plate in order to minimize the space between beads.[37]

pH measurement

The pH was measured on the samples at 25 °C using a standard pH meter (Hanna Instruments pH 301, Padua, Italy). For the sodium alginate beads, the pH was measured on the sodium alginate solution before adding $CaCl_2$. The calibration was performed using three different buffer solutions at pH 4, 7, and 9.

Viability of probiotic bacteria during 4 °C storage

Aliquots of 1 g of each sample were suspended in 9 mL of MRD and homogenized in a LabBlender 400 (PBI International, Milan, Italy) for 2 min. Sodium alginate beads were preliminarily homogenized using a high-speed homogenizer (Ika-Werke, Staufen, Germany) at 290×*g* for 1 s. Decimal dilutions were then spread plated on MRS agar (for *L. rhamnosus*) or M17 agar (for *S. thermophilus*) and incubated at 37 °C for 48 h. Viable counts in the systems were compared to those of control samples stored at 4 °C. Structured systems and controls were analyzed for viable counts at 0, 7, and 14 days.

Viability of probiotic bacteria during *in vitro* digestion

In vitro digestion was carried out in accordance with the INFOGEST protocol.[21] The simulated salivary (SSF), gastric (SGF), and intestinal (SIF) fluids were prepared and stored at 4 °C. Before *in vitro* digestion, the fluids were heated at 37 °C; 2.5 g of each sample and 2.5 mL of each control were weighted in 50 mL Falcon tubes. The oral phase was performed by adding 13 µL of 0.3 M $CaCl_2$ $(H_2O)_2$, 488 µL of water and 2 mL of 6.55 mg mL^{-1} α-amylase solution in SSF (final activity 75 U mL^{-1}). The entire mixture was maintained at 37 °C under stirring at 13 rpm for 2 min. Subsequently, 3 µL of 0.3 M $CaCl_2$ $(H_2O)_2$, 347 µL of water, and 4.55 mL of a 0.07 mg mL^{-1} pepsin solution in SGF (2000 U mL^{-1} in the final mixture) were added. The pH was adjusted to 3 by adding 6 M HCl to start the gastric phase. The chime was maintained under stirring at 13 rpm at 37 °C for 2 h. Finally, the gastric chime was mixed with 20 µL of 0.3 M $CaCl_2$ $(H_2O)_2$, 655 µL of water, 1.25 mL of 160 mmol L^{-1} bile extract in SIF, and 8 mL of 22.15 mg mL^{-1} pancreatin solution in SIF (100 U mL^{-1} in the final mixture). The pH was adjusted to 7 by adding 1 M NaOH and the mixture was stirred at 13 rpm at 37 °C for 2 h. At the end of each phase, a sample was collected and put in an ice bath to stop the enzymatic reaction. *Lactobacillus rhamnosus* and *S. thermophilus* viabilities were evaluated as previously reported before digestion and at the end of each phase and compared to control samples.

Data analysis

All determinations were expressed as the mean ± standard deviation (SD) of at least two measurements from two experimental replications. Analysis of variance (ANOVA) was performed by using R v. 3.1.1 for Windows (The R foundation for statistical computing). A Tukey's *post-hoc* test was used to assess differences between means ($P < 0.05$).

RESULTS AND DISCUSSION

Characterization of polymer-based structured systems

Table 1 shows the visual appearance and the pH values of the methylcellulose-based system (S-MC), the sodium alginate gel (S-SA), and the whey protein-based system (S-WP). The S-MC was a clear, transparent, and viscous material, whereas the S-WP appeared as a white, self-standing material. The S-SA was formed into spherically shaped beads with an average diameter of 2.05 ± 0.12 mm and a mean volume of 4.55 ± 0.77 mm^3. The selected beads' dimensions are sufficiently small to be suitable for food applications from a sensory point of view as well as to perform

rheological analysis. In fact, the sensory perception of beads is strictly related to their size and volume,[38,39] affecting, for instance, the attributes of 'gritty', 'pasty', 'smooth', and 'melting'.[40,41] Moreover, bead size and volume could affect the functionality and viability of entrapped probiotics during refrigerated storage, freezing, and drying. It has been shown that large volumes provide greater protection to the cells during such stressful steps.[42–44] It has also been suggested that the size of alginate beads might be a pivotal factor in the metabolic activity of encapsulated probiotics.[45]

The pH of the samples ranged from 5.9 to 6.5. The probiotic strains used in this study belong to the group of lactic acid bacteria, which are known for having an optimal growth pH of about 5.5–6.0.[46] Thus, the pH differences among matrixes is expected to have negligible effects on bacterial viability during the experiments.

F1 Figure 1 shows the rheological behavior of the systems, which confirmed the differences observed from their visual appearance. The S-MC was characterized by G″ values higher than G′ in the entire frequency domain that was considered, which indicated, as expected, a liquid-like behavior. As is well known, in these conditions, methylcellulose forms a viscous material due to the capability of the polymer to structure into a soft network through the formation of a three-dimensional crosslinked structure.[47]

In agreement with the literature, S-SA resulted in a strong gel because both moduli were independent of the frequency applied, with G′ higher than G″[48] and tan δ with a mean value of 0.2.[49] It is well known that when sodium alginate is introduced in a $CaCl_2$ solution, gelation occurs rapidly. Ca^{2+} ions bind to guluronate units of sodium alginate giving the so-called *egg-box* structure formed by junction zones involving two chains and chelated ions.[50] However, the gelling kinetic and mechanical properties of sodium alginate are influenced by different parameters, such as molecular weight, concentration, and composition. These factors, in turn, can affect the pore size distribution and then the ability of this matrix to ensure the diffusion of molecules or probiotics entrapped within.[48,51]

Finally, in S-WP the elastic modulus (G′) dominated across the measured frequency range and both moduli showed very limited frequency dependence. The tan δ was 0.2 in the entire frequency domain indicating a solid-like behavior. As extensively reported in the literature, whey proteins are very adaptable, and gels form not only by heating but also at room temperature. The latter process, called cold-set gelation, consists of two steps: the heat-induced denaturation and gelation at low temperatures. During heating above 70 °C, whey proteins unfold. This condition combined with a pH sufficiently far from the isoelectric point (pH 4.6–5.2) guarantees that they do not immediately aggregate but form a filamentous structure.[52,53] After cooling, controlled acidification or increasing of ionic strength causes a reduction of electrostatic repulsions between protein filamentous aggregates and subsequently gelation, forming a three-dimensional protein network.[36,52]

In conclusion, considering the restrictions in structuring ability of the selected biopolymers, SA and WP were structured at pH close to 6 and with a compared ratio between G″ and G′ (tan δ). The methylcellulose (MC)-based system was included as reference viscous material in addition to the unstructured water solution control sample.

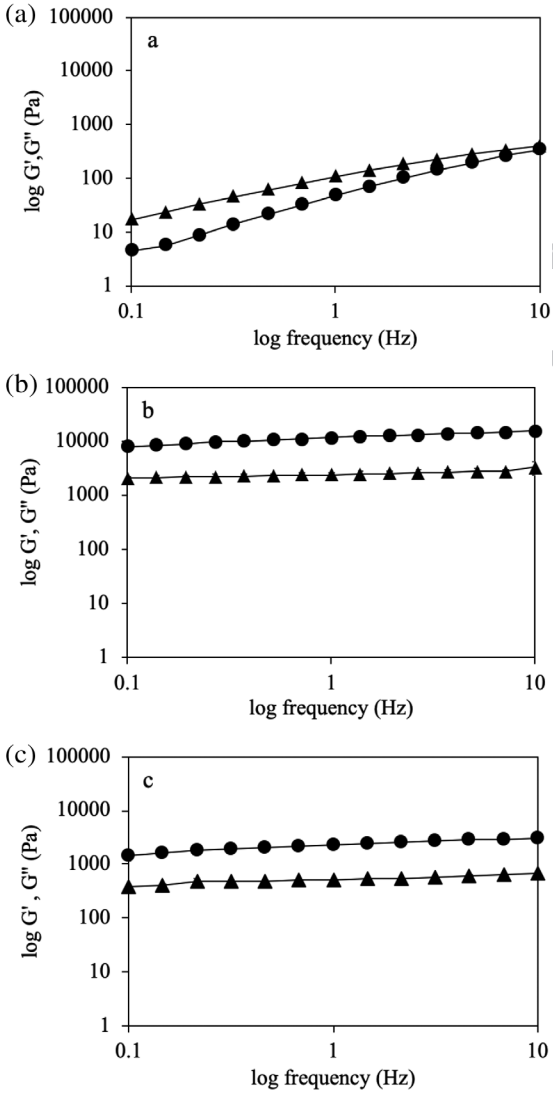


Figure 1. Storage (G′) and loss (G″) moduli versus frequency at 20 °C for methylcellulose-based system (S-MC) (a), sodium alginate beads (S-SA) (b) and whey protein gel (S-WP) (c). G′; G″.

Viability of *L. rhamnosus* and *S. thermophilus* during storage

Lactobacillus rhamnosus and *S. thermophilus* were added directly to the aqueous phase of each biopolymer-based structured system to evaluate the protective capability of the matrix on microbial viability during 4 °C storage for up to 14 days. The microbial viabilities in methylcellulose-, sodium alginate- and whey protein-based systems, as well as those in the control samples (free cells suspended in saline solution) are reported in Table 2. T2

At the beginning of the storage time, the structured systems and controls presented a microbial viability of around 10^8 CFU g^{-1} for both microorganisms. This result indicated that the process conditions, and the mechanical stress adopted during preparation, did not have an impact on cell survival. During storage, the control samples showed a viability loss of about 2 log for both microorganisms after 14 days storage. This reduction could be due to the absence of nutrients and the storage temperature, which is about 37 °C lower than the optimal growth temperature for both microorganisms.[54] Interestingly, the viability of the selected bacteria was higher after seven days of storage in the S-MC than in the control. Almost 2.5 log CFU mL^{-1} viability

Table 2. Viability (log CFU g^{-1} in structured systems or log CFU mL^{-1} in control samples) of *L. rhamnosus* and *S. thermophilus* in a methylcellulose-based system (S-MC), sodium alginate beads (S-SA), and a whey protein-based system (S-WP) and relative controls during 4 °C storage

		Time (days)		
Microorganism	System	0	7	14
L. rhamnosus	Control	$8.12 \pm 0.07^{a, A}$	$6.11 \pm 0.41^{b, B}$	$5.37 \pm 0.30^{b, B}$
	S-MC	$8.11 \pm 0.04^{a, A}$	$8.42 \pm 0.39^{a, A}$	$6.01 \pm 0.43^{b, AB}$
	S-SA	$8.42 \pm 0.47^{a, A}$	$7.26 \pm 0.63^{a, AB}$	$3.77 \pm 0.35^{b, C}$
	S-WP	$8.44 \pm 0.78^{a, A}$	$7.50 \pm 0.07^{a, AB}$	$7.17 \pm 0.26^{a, A}$
S. thermophilus	Control	$8.25 \pm 0.05^{a, A}$	$5.76 \pm 0.45^{b, B}$	$5.35 \pm 0.36^{b, B}$
	S-MC	$8.11 \pm 0.02^{a, A}$	$8.23 \pm 0.56^{a, A}$	$5.20 \pm 0.03^{b, B}$
	S-SA	$8.43 \pm 0.41^{a, A}$	$7.12 \pm 0.22^{a, AB}$	$4.03 \pm 0.30^{b, B}$
	S-WP	$8.38 \pm 0.25^{a, A}$	$7.89 \pm 0.07^{a, A}$	$7.60 \pm 0.56^{a, A}$

[a–b] In the same row, means indicated by different letters are significantly different ($P < 0.05$).
[A–C] In the same column, means indicated by different letters are significantly different ($P < 0.05$).

reduction was observed after seven days of storage in the control, whereas a similar viability reduction in structured samples was seen after 14 days. This result highlighted a protective effect of the network formed by methylcellulose. As far as we are aware, no studies were conducted on the probiotic protection capacity of systems composed exclusively of methylcellulose or cellulose derivatives. On the other hand, cellulose derivatives have been used in combination with other structuring molecules in edible films or emulsions to reinforce their structuring ability. The resulting denser and structured matrix was shown to protect probiotics better from environmental stresses such as acid pH, refrigerated storage, or intense heat.

It could be inferred that these results could be associated with higher viscosity of the MC-based system than the control sample.

With regard to S-SA, the structured system was not able to protect the microbial cells during storage. In the entire period of storage, the viability loss was almost 4 log CFU g^{-1} for both microorganisms. It is conceivable that the strong gel nature of alginate beads, despite their porosity, hinders migration to the outside of acid metabolites, which accumulate inside the beads causing a self-intoxication of probiotic cells in the long term.[55]

In S-WP no viability loss was detected during storage for *L. rhamnosus* and *S. thermophilus*. In fact, significant differences for both microorganisms can be observed by comparing control and structured system at 14 days of storage. In this case, it could be hypothesized that in the structured system the combination of formulation and structure had a protective effect on cell viability. The presence of whey proteins could provide nutrients to probiotic bacteria, especially essential amino acids, able to guarantee a metabolic intake necessary for cells' survival preserving their viability at levels higher than 10^6–10^7 CFU g^{-1} during the entire period of storage.[54] At the same time, the gel structure ensures a physical barrier against environmental stresses such as oxygen, water, and cold temperature.

Viability of *L. rhamnosus* and *S. thermophilus* during *in vitro* digestion

To evaluate the protective effect of the different polymeric networks against the stressful conditions during gastrointestinal transit, the structured systems containing *L. rhamnosus* and *S. thermophilus* were subjected to the INFOGEST *in vitro* digestion protocol, which consists of oral, gastric, and intestinal phases mimicking *in vivo* human conditions.[21] The viability before the digestion process (pre digestion) and after oral, gastric, and intestinal phases was measured by considering both controls (free cells suspended in saline solution) and structured systems. Figure 2 shows the viability of *L. rhamnosus* (a) and *S. thermophilus*

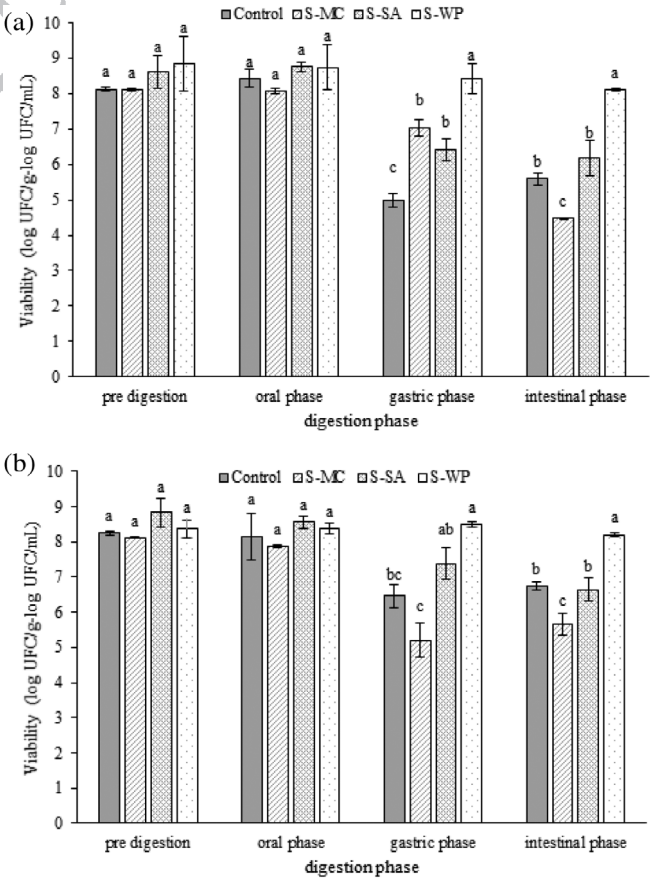


Figure 2. Viability of *L. rhamnosus* a and *S. thermophilus* b (log CFU g^{-1} or log CFU mL^{-1}) as a function of digestion phase in methylcellulose-based system (S-MC), sodium alginate beads (S-SA) and whey protein gel (S-WP) and controls. Means indicated by different letters are significantly different ($P < 0.05$) within the same digestion phase.

(b) entrapped in all the systems considered and in the control at the end of each digestion step.

At the end of the oral phase, no probiotic viability loss was observed in any sample. This result agrees with the literature which reported the same behavior regardless of the encapsulation material.[14] Nevertheless, the exposure to strong acid conditions, typical of the gastric environment, is known to cause damage to the cell membrane, DNA, and proteins.[56,57] Instead, in the small intestine, the presence of bile salts could induce membrane damage and protein misfolding, causing DNA injury by oxidative shock and low intracellular pH.[56,58] These factors usually lead to a strong cell viability reduction during digestion.

As for *L. rhamnosus*, although a viability loss of 1.03 ± 0.29 log CFU g^{-1} was observed during the gastric phase, S-MC was shown to be able to protect the probiotic compared with the control. Nevertheless, the intestinal phase had a considerable impact on cell viability. Indeed, at the end of the *in vitro* digestion process it reached a value of 4.47 ± 0.02 log CFU g^{-1}, which was lower than the control, as well as the minimum level needed to guarantee health benefits. Compared to *L. rhamnosus*, the MC-based system containing *S. thermophilus* was more susceptible to the gastric phase causing a viability reduction of 2.65 ± 0.45 log CFU g^{-1} while no further modifications were observed after the intestinal phase. This means that the network formed by methylcellulose in water did not improve the tolerance of either microbial strain during digestion. In fact, the only increase of viscosity of a food matrix was not able to protect, even partially, the microbial cell to the stressful digestive conditions. The same behavior was also observed for other matrices, such as chitosan, locust bean gum, and guar gum, which cannot by themselves guarantee enough probiotic protection. The strategy is therefore to combine them with other supporting components or to use them as coat / shell materials.[33,59]

Different results were obtained when considering S-SA, in which the viability of both microorganisms remained higher than 10^6 CFU g^{-1} during the entire *in vitro* digestion process. Once again, no significant loss of viability in either microorganism was shown after the oral phase. On the other hand, a slight reduction was noted after the gastric and intestinal phase. The strict conditions of the gastric phase probably had a great impact on viability, indicating that the beads alone were unable to protect the cells from the acid environment, while the intestinal phase did not have a pronounced effect. These results agree with other authors who report a viability reduction of *Lactobacillus acidophilus*, *L. plantarum* and *Bifidobacterium* encapsulated in sodium alginate beads during the gastric pass.[35,60] In fact, sodium alginate particles are very porous and, for this reason, moisture and digestion fluids can easily diffuse through the walls of the beads.[61] To minimize this behavior, possible approaches have been proposed, such as the application of an external coating or a combination of sodium alginate with other gelling agents to create a physical barrier able to protect cells from acid damage and ensure, at the same time, cell release at the target site.[35,62,63]

Finally, S-WP was able to ensure an unchanged *L. rhamnosus* and *S. thermophilus* viability upon the entire *in vitro* digestion. It is interesting to note that the WP-containing system showed the highest bacteria protection capacity despite the possible destructuring activity of proteolytic enzymes during gastric and intestinal phase. Other authors reported an efficient protective effect of whey proteins on *S. thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *L. rhamnosus* and *Bifidobacterium* viability.[13,17,64] The results indicated that the composition of the system played a key role in preserving the viability of probiotics due to the buffering capacity exerted by whey proteins.[36] Moreover, the heat treatment carried out on whey protein solution may cause the exposure of hydrophobic patches that are then buried in the interior of aggregate formed during the subsequent acidification. The resulting structure may safeguard probiotics, avoiding the release of cells from the protein network and reducing the acidity effect of the gastrointestinal tract.[36,65] In the intestinal phase, where bile salts are responsible for protein and DNA damage and for the emulsification of fats and bacterial lipid membrane, whey proteins could exert a barrier role in reducing bile damage to lipid membranes and in facilitating protein repair.[64]

CONCLUSIONS

In this study the protective performance of three biopolymers was compared during cold storage and during *in vitro* simulated digestion with the standardized INFOGEST protocol. It should be stressed that the application of a standardized digestion protocol is strongly recommended to compare the efficacy of different delivery systems to preserve probiotic viability at a level able to guarantee health benefits.

Moreover, even if more research is needed, especially on the destructuring behavior of polymers during digestion, the results suggest that the capacity of a polymeric structure to protect probiotic bacteria is a combination of structure organization and system formulation. The whey protein-based system was the best performing one. In fact, no viability reductions were noted during refrigerated storage and *in vitro* digestion trials. This result was attributed to a combined effect of the physical barrier offered by the protein gel network and whey proteins as a nutrient for microbes. On the other hand, surprisingly, the viscous methylcellulose-based system was able to guarantee good microbial viability during storage. However, this was not confirmed during *in vitro* digestion. The opposite results were obtained for sodium alginate beads. Thus, storage trials cannot be used to predict the gastrointestinal behavior of structured systems in protecting bacteria.

Only the combination of storage stability tests with digestion simulation trials could provide detailed information fundamental to the design of probiotic protection systems able to guarantee the adequate probiotic viability both in food and in the human body.

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