Lactic acid bacteria microencapsulation in sodium alginate and other gelling hydrocolloids mixtures

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Summary
Lactic acid bacteria were microencapsulated with alginate and other gelling hydrocolloids (gellan gum and κ-carrageenan/locust bean gum). Sodium alginate microcapsules with gellan gum resulted in a harder and more resilient less viscous texture, with low particle size diameter, with enhanced viability of the microencapsulated bacteria. In contrast, sodium alginate microcapsules with κ-carrageenan/locust bean gum resulted in softer and less resilient (more viscous) texture. Although the particle size diameter was higher than alginate + gellan gum treatment, the microcapsules were smaller and with better viability than sodium alginate alone. Correlation analysis showed that microcapsules with lower surface area diameter were harder, more resilient and less viscous, enhancing cells viability and reducing acidification rates. Higher surface area affected negatively the growth and fermentative capability of encapsulated lactic acid bacteria. The incorporation of gellan or κ-carrageenan/locust bean gum into alginate beads resulted in a lower surface area that, on one hand, enhanced cell viability and, on the other hand, in moderate milk acidification in fermentation tests. The use of alginate with gellan gum represents a good alternative to protect lactic acid bacteria in order to be inoculated in functional processed foods.

Keywords
microencapsulation; lactic acid bacteria; functional foods; alginate; mixed hydrocolloid; microstructure

At the point of consumption, the viability of lactic acid bacteria in food products is an important parameter of their efficacy, as they have to survive during the processing and shelf life of food [1]. The viability and stability of lactic acid bacteria is both a marketing and technological challenge for industrial producers, since lactic acid bacteria should maintain a suitable level of viable cells during the product's shelf life, without increasing production costs [2]. Microencapsulation can be used to entrap or enclose microorganisms by segregating them from the external environment with a coating of hydrocolloids, which enhances microbial survival and activity during fermentation [3]. Use of encapsulation is a way to protect lactic acid bacteria or probiotics during food processing, preparation and consumption, and during the passage through the gastrointestinal tract. Lactic acid bacteria as probiotics should not undergo interfacial inactivation, stimulation of production and excretion of secondary metabolites, and continuous metabolism. Viability of the encapsulated cells may be affected by the physicochemical properties of the capsules. Type and concentration of the coating material, particle size, initial cell numbers and bacterial strains are some of the parameters to be considered [3].

Microencapsulation by emulsion involves combining a mixture of microorganisms and an encapsulating agent such as sodium alginate with vegetable oil, where the alginate beads are formed by slowly adding calcium chloride to the emulsion while stirring [4, 5]. In the emulsion technique, the droplet size can be controlled by the mixer since oil phase is emulsified until the desired droplet size to form water in oil emulsion into the aqueous

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phase containing biopolymer solution and microorganism [6]. There are many supporting materials used in the emulsion technique including mixture of κ-carrageenan and locust bean gum, cellulose acetate phthalate, alginate, chitosan and gelatin [7, 8]. Alginate and gellan gum are favoured above all other supporting materials for encapsulating probiotics because they are non-toxic, biocompatible and inexpensive [9, 10].

The use of different gelling hydrocolloids for microencapsulation will affect the cell viability and, hence, the beneficial properties of the microorganisms. The objective of this study was to evaluate the effect of incorporation of different gelling hydrocolloids, namely, gellan gum and κ-carrageenan/locust bean gum, on gel texture, cells viability and particle size, determining the correlation between the employed encapsulating materials (texture) and cells viability.

MATERIALS AND METHODS

Gelling hydrocolloids

In order to study the effect of hydrocolloid mixture on cells viability, three different mixtures of hydrocolloids were employed (commercial samples):

- Protanal SF 120 sodium alginate (1.0%; FMC Biopolymers, Philadelphia, Pennsylvania, USA), as control;
- Protanal SF 120 sodium alginate + Kelcogel LT 100 high acyl gellan gum (0.1%; CP Kelco, San Diego, California, USA);
- Protanal SF 120 sodium alginate + Gelcarin ME 913 κ-carrageenan (0.3%; FMC Biopolymers) and Viscogum FA locust bean gum (0.3%; Degussa Texturant Systems, Atlanta, Georgia, USA).

Mixtures of hydrocolloids were dissolved in distilled water.

Hydrocolloids gels texture

To each hydrocolloid mixture, 0.04 $mol \cdot l^{-1}$ $CaCO_3$ was added to form a gel after a quick addition of acetic acid (0.32 $mol \cdot l^{-1}$) into 20 mm diameter cellulose sausage casings (Fabpsa, Mexico City, Mexico). Gels were carefully removed from the casing and cut in 20 mm high cylinders. Only homogeneous gel parts were employed in testing. Textural profile analysis (TPA) was performed in a TAXT2i texture analyser (Texture Technologies, Scarsdale, New York, USA / Stable Micro Systems, Surrey, United Kingdom). Samples were compressed with a aluminium plunger (diameter, 5.08 mm) at 10 mm of the original height (50%) in a double cycle mode at a constant rate of 1.0 $mm \cdot s^{-1}$ and a wait period of 3.0 s between compressions. From the force-deformation curves, texture parameters were calculated and defined as: hardness (force necessary to attain a given deformation), cohesiveness (strength of the internal bonds making up the body of the product) and resilience (energy accumulated that allows the sample to recover its original shape after deformation) [11–13].

Rheological parameters of the gels, namely, viscosity index and apparent elasticity, were determined from force-deformation curves with equations of Hickson et al. [14]. To hydrocolloids mixtures, $CaCO_3$ (0.04 $mol \cdot l^{-1}$) was added and gel was formed after a quick addition of acetic acid (0.32 $mol \cdot l^{-1}$) into glass test tube (internal diameter, 1.6 mm). Gels were penetrated until rupture occurred with an 8 mm diameter acrylic probe at a constant rate of 1.0 $mm \cdot s^{-1}$ in same texture analyser.

Cellular strains and microencapsulation

Four strains of lactic acid bacteria, namely, *Enterococcus faecium* UAM10a, *Lactobacillus plantarum* UAM15c, *Aerococcus viridans* UAM21 and *Pediococcus pentosaceus* UAM22 (Biotechnology Department, Universidad Autonoma Metropolitana Iztapalapa, Mexico City, Mexico) were employed. The strains were previously identified and described as having a probiotic potential by Ramirez-Chavarin et al. [15, 16]. Lactic acid bacteria were cultured in de Man, Rogosa and Sharpe (MRS) broth at 37 °C during 20–22 h. To harvest cells, broth was centrifuged (2000 ×*g* during 10 min) to obtain a cellular sediment, according to recommendation of Mandal et al. [17].

Alginate microcapsules were produced by emulsification/internal ionotropic gelation of sodium alginate. Microencapsulation of bacterial strains was carried out adapting the technique of Homayouni et al. [18]. Cellular sediment of each strain was resuspended in each hydrocolloid solution (100 ml) plus 200 ml of canola oil (Capullo vegetable oil; Unilever, Tultitlan, Mexico) and 2.5 ml of Tween 80 (Sigma-Aldrich, St. Louis, Missouri, USA) were mixed in a glass beaker with a magnetic stirring for 10 min at high speed. A mixture of 40 ml of canola oil with 2 ml of acetic acid was gently added to the emulsion during stirring in order to allow beads formation. After 20 min, microcapsules were removed from the aqueous phase and washed twice with phosphate buffer (pH 7.2). Microcapsules were incubated in hermetically sealed containers for 24 h at 4 °C to fully harden.

Cell viability

Live bacteria were enumerated using spread plating on MRS agar after 24 h incubation at 37 °C [19]. Tests were carried out in triplicate and reported as colony forming units per millilitre.

Fermentation test was carried out in 100 ml of sterilized skimmed reconstituted milk inoculated with microencapsulated cells (2%). Average initial inoculum was 9.56 ± 0.6, 9.23 ± 1.7, 7.50 ± 1.1 and 10.46 ± 0.7 for *A. viridans*, *E. faecium*, *L. plantarum* and *P. pentosaceus*, respectively. The milk was tempered to 42 °C, inoculated with different microcapsules of each treatment and with non-encapsulated cultures as control, and incubated in a thermostatically controlled water bath during 24 h. The pH value was monitored every 30 min during 24 h, employing non-encapsulated cultures as control. Fermentations for each lactic acid strain microencapsulated in each hydrocolloid mixture were carried out in triplicate. Kinetics parameters calculated were: V_{max} as maximum acidification rate (change of pH value per minute); time to reach V_{max} (in hours); pH at maximum acidification rate; and final pH (after 24 h of fermentation) [20].

L13152 LIVE/DEAD *BacLight* Bacterial viability kit (Molecular Probes, Eugene, Oregon, USA) was used to determine viability of free and encapsulated cells by fluorescence microscopy according to PIMENTEL-GONZALEZ et al. [21]. The bacterial viability kit contained two dyes: SYTO 9, which stains intact cells in green, while propidium iodide stains damaged cells in red. Both dyes were mixed in 1 : 1 ratio. A volume of 100 μl of the dye mixture was added to 100 μl of the sample and maintained out of the light for 45 min. After this time, samples were observed in a Olympus BX41 fluorescence microscope (Olympus, Shinjuku, Japan) with light intensity LED indicator built-in filter and camera Evolution VF Color Cooled (Media Cybernetics, Rockville, Maryland, USA).

Particle size and scanning electron microscopy

The particle size of the microcapsules was determined using a Malvern Mastersizer 2000 instrument (Malvern Instruments, Worcestershire, United Kingdom). The surface area mean diameter ($D_{[3,2]}$) and the volume median diameter $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ were calculated for each microencapsulation treatment (10%, 50% and 90% of the total volume is made up with particle with diameter lower than the value, respectively).

Microstructure of the encapsulated lactic acid bacteria were determined employing scanning electron microscopy, adapting the methodology of MUTHUKUMARASAMY et al. [22]. Samples were fixed with glutaraldehyde at 5% (v/v) in 0.1 mol·l^{-1} phosphate buffer (pH 7.0) solution followed by osmium tetroxide for further fixation. Fixed samples were washed with a series of 20% (v/v) to 100% (v/v) ethanol. Samples were then transferred to microporous specimen capsules, dried to critical point in a Samdri 795 semi-automatic dryer (Tousimis, Rockville, Maryland, USA), and gold covered Denton Vacuum Desk III (Denton Vacuum, Moorestown, New Jersey, USA). Samples were observed in a JEOL JSM-5900LV scanning electron microscope (JEOL, Tokyo, Japan) operated at 13 kV accelerating voltage to examine the external appearance of the microcapsules.

Statistical analysis

The effect of the different mixtures of hydrocolloids on the gel texture, cell viability and acidification kinetics parameters, as well as particle size characteristics, were analysed by PROC ANOVA procedure in SAS Statistical Software v. 8.0 (SAS Institute, Cary, North Carolina, USA). Significant ($p < 0.05$) differences between means were determined by Duncan's mean test included in the software. In order to determine the relations between textural properties of hydrocolloids mixtures (hardness, cohesiveness, resilience, apparent elasticity and viscosity index), lactic acid bacteria viability (colony forming units, acidification kinetic parameters), and particle size characteristics (surface area mean diameter $D_{[3,2]}$ and $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ volume median diameters), a correlation analysis and principal component analysis (PCA) were performed, irrespectively of the microencapsulated strain. Pearson's correlation coefficients were determined with the PROC CORR procedure. Principal component analysis was performed employing the PROC FACTOR with orthogonal rotation (ROTATE = VARIMAX) to derive non-correlated factors.

RESULTS AND DISCUSSION

Texture of hydrocolloid gels

The results for the textural profile analysis of the gelled hydrocolloids mixtures are shown in Tab. 1. The incorporation of other hydrocolloids to alginate during gel formation modified textural parameters. Hydrocolloids mixtures had significantly ($p < 0.05$) different gel hardness. Gels containing gellan presented a harder texture than alginate gels. When κ-carrageenan/locust bean gum were mixed with alginate, a softer texture was detected. Cohesiveness of gels formulated with the different hydrocolloids mixtures were not signifi-

Tab. 1. Textural properties of the gels formed with different hydrocolloids mixtures.

Textural parameter	Sodium alginate	Sodium alginate + gellan gum	Sodium alginate + κ-carrageenan/locust bean gum
Hardness [N]	3.91 ± 0.89 b	4.78 ± 1.01 a	3.50 ± 0.32 c
Cohesiveness	0.3335 ± 0.013 a	0.3530 ± 0.053 a	0.3304 ± 0.010 a
Resilience	0.2913 ± 0.014 b	0.3148 ± 0.023 a	0.1940 ± 0.004 c
Apparent elasticity [N·cm^{-2}]	1.93 ± 0.37 b	3.45 ± 1.31 a	4.22 ± 1.87 a
Viscosity index (Poise)	2.76 ± 0.31 b	2.21 ± 0.36 c	3.28 ± 0.51 a

Means with same lowercase letter in superscript in same row are not significantly different ($p > 0.05$) for individual treatments.

cantly ($p > 0.05$) different. Resilience of the gels with κ-carrageenan/locust bean gum was significantly ($p < 0.05$) lower than alginate and alginate-gellan gum gels.

For the rheological properties, gels with κ-carrageenan/locust bean gum or with gellan gum presented significantly ($p < 0.05$) higher apparent elasticity values than alginate gels. The viscosity index was significantly ($p < 0.05$) highest for gels with κ–carrageenan/locust bean gum; the lowest viscosity index values were detected for gels containing gellan gum (Tab. 1).

In TPA, a harder and more resilient texture was found when sodium alginate was mixed with gellan gum, and more elastic but less viscous texture was observed in penetration test. Less hard and resilient but more viscous gels were obtained when alginate was mixed with κ–carrageenan/locust bean gum. In the ionotropic gelation of alginate, gelation modes and types of molecular associations responsible for gelation depend on Ca^{2+} dose and sequential parameters of the polysaccharide [23]. When sodium alginate was mixed with other hydrocolloids, different structures arose. This probably can be explained on the basis of different gelation mechanisms of the hydrocolloids mixtures components. Gellan gum was found to provide a synergistic effect in terms of gel strength and stability to form a complex structure when was added into sodium caseinate solution [24]. Incorporation of low concentrations of gellan increased the breaking stress of alginate gels, but since the gels breaking strain remained unaffected, probably the brittle gellan network was distributed as dispersed particles embedded in the calcium alginate matrix [25]. Possibly coacervation governed the interaction between alginate and gellan since, at low pH (due to acetic acid employed during gel formation), both polymers were negatively charged [26]. In this view, the sodium alginate with gellan gum gels seem to present associative interaction since mixed gels had higher values of hardness, resilience and apparent elasticity than alginate or alginate with κ-carrageenan/locust bean gum gels. In contrast, in alginate + κ-carrageenan gels, the alginate cross-linking reactions are responsible for the gel structure, with no carrageenan effect [27]. Besides that, the strong synergetic effect reported among κ-carrageenan and locust bean gum [28, 29] improved κ-carrageenan gelation forming strong, elastic gels with low syneresis [30, 31]. The combination of κ-carrageenan with locust bean gum was recently used to encapsulate lactic acid bacteria [8]. Due to the strong interaction between these two hydrocolloids, the resulting structure was softer and rubbery, and less resilient than sodium alginate gels.

Viability of the encapsulated bacteria

Microencapsulation of lactic acid bacteria in the different hydrocolloids mixtures resulted in a significant ($p < 0.05$) decrease in cells viability. The incorporation of other gelling hydrocolloids resulted in higher viability, as compared to microencapsulation with sodium alginate only. Regarding differences between strains, *P. pentosaceus* retained significantly ($p < 0.05$) higher viability, followed by *A. viridans* (Tab. 2).

Regarding kinetic parameters of acidification (Tab. 2), cultures obtained from non-encapsulated cells had significantly ($p < 0.05$) highest V_{max} values of fermentation, followed by microcapsules formed with alginate + gellan gum, and the lowest acidification rate was detected in fermentations with only alginate-microencapsulated samples. In this parameter, *A. viridans* microencapsulated samples resulted in significantly ($p < 0.05$) highest V_{max} values, followed by *L. plantarum* samples. The time to reach V_{max} was significantly ($p < 0.05$) lowest in non-encapsulated culture fermentations, followed by the samples of alginate mixed with other gelling hydrocolloids fermentations. Longest times to reach V_{max} were detected for the sodium alginate microencapsulated samples.

E. faecium and *P. pentosaceus* presented significantly ($p < 0.05$) shortest times to reach V_{max}. Value of pH at V_{max} was significantly ($p < 0.05$) lowest for non-encapsulated cultures and highest pH values at this point were detected for alginate microencapsulation treatments. *E. faecium* and *P. pentosaceus* reached significantly ($p < 0.05$) lower pH values at V_{max} than *A. viridans* and *L. plantarum*. The final pH reached after 24 h of fermentation was significantly ($p < 0.05$) lowest for the non-microencapsulated cultures. Highest pH values at the end of milk fermentation (24 h) were found for alginate microencapsulated samples. Final pH reached for *E. faecium* and *P. pentosaceus* was significantly ($p < 0.05$) lower than for *A. viridans* and *L. plantarum*. As expected, compared to non-encapsulated cultures, the viable plate counts, acidification rate and time to reach maximum acidification rate decreased as a consequence of microencapsulation. From the above results, *E. faecium* and *P. pentosaceus* seem to have a better viability after microencapsulation with the different hydrocolloids mixtures. Qualitative viability of these lactic acid bacteria, determined by live/dead staining, is shown in Fig. 1. For *P. pentosaceus*, microencapsulation with sodium alginate and gellan gum or sodium alginate and κ-carrageenan/locust bean gum seem to enhance viability, since more live cells can be observed compared to *E. viridans*.

The inclusion of other gelling hydrocolloids (as gellan or κ-carrageenan/locust bean gum) changed

Tab. 2. Acidification kinetic parameters for lactic acid bacteria as non-encapsulated or encapsulated with different hydrocolloids mixtures.

Strain	Control (non-encapsulated)	Sodium alginate	Sodium alginate + gellan gum	Sodium alginate + κ-carrageenan/locust bean gum
Viable counts [log CFU·ml^{-1}]				
A. viridans	11.16 ± 0.22^{aB}	6.67 ± 1.41^{cB}	11.00 ± 0.04^{bB}	11.01 ± 0.36^{bB}
E. faecium	11.17 ± 0.59^{aC}	6.70 ± 2.19^{cC}	11.01 ± 0.94^{bC}	9.98 ± 2.07^{bC}
L. plantarum	11.20 ± 0.72^{aC}	4.63 ± 0.49^{cC}	8.56 ± 1.25^{bC}	9.31 ± 1.50^{bC}
P. pentosaceus	11.54 ± 0.76^{aA}	9.77 ± 1.07^{cA}	10.41 ± 0.46^{bA}	11.21 ± 0.66^{bA}
Maximum acidification rate V_{max} [min^{-1}]				
A. viridans	3.18 ± 0.01^{aA}	2.60 ± 0.01^{cA}	2.88 ± 0.01^{bA}	1.86 ± 0.01^{dA}
E. faecium	2.80 ± 0.01^{aC}	2.18 ± 0.01^{cC}	2.48 ± 0.01^{bC}	1.68 ± 0.01^{dC}
L. plantarum	3.10 ± 0.01^{aB}	2.26 ± 0.01^{cB}	2.68 ± 0.01^{bB}	1.80 ± 0.01^{dB}
P. pentosaceus	2.60 ± 0.01^{aD}	2.08 ± 0.01^{cD}	2.18 ± 0.01^{bD}	1.58 ± 0.01^{dD}
Time to reach V_{max} [h]				
A. viridans	13.0 ± 0.06^{cA}	24.0 ± 0.09^{aA}	14.5 ± 0.05^{bA}	22.5 ± 0.0^{aA}
E. faecium	12.0 ± 0.05^{cC}	14.0 ± 0.08^{aC}	13.5 ± 0.05^{bC}	14.1 ± 0.0^{aC}
L. plantarum	12.9 ± 0.01^{cB}	14.7 ± 0.07^{aB}	14.0 ± 0.02^{bB}	16.9 ± 0.0^{aB}
P. pentosaceus	12.0 ± 0.07^{cC}	14.5 ± 0.05^{aC}	13.2 ± 0.01^{bC}	14.3 ± 0.0^{aC}
pH at V_{max}				
A. viridans	6.08 ± 0.01^{dB}	6.25 ± 0.01^{bB}	6.20 ± 0.01^{cB}	6.34 ± 0.01^{aB}
E. faecium	5.67 ± 0.01^{dD}	5.98 ± 0.01^{bD}	5.87 ± 0.01^{cD}	6.12 ± 0.01^{aD}
L. plantarum	6.14 ± 0.01^{dA}	6.25 ± 0.01^{bA}	6.20 ± 0.01^{cA}	6.34 ± 0.01^{aA}
P. pentosaceus	5.98 ± 0.01^{dC}	6.13 ± 0.01^{bC}	6.10 ± 0.01^{cC}	6.27 ± 0.01^{aC}
Final pH (after 24 h)				
A. viridans	5.86 ± 0.01^{bA}	5.80 ± 0.01^{cA}	5.76 ± 0.01^{dA}	6.09 ± 0.01^{aA}
E. faecium	5.71 ± 0.01^{bC}	5.49 ± 0.01^{cC}	5.31 ± 0.01^{dC}	6.01 ± 0.01^{aC}
L. plantarum	5.85 ± 0.01^{bA}	5.79 ± 0.01^{cA}	5.74 ± 0.01^{dA}	6.09 ± 0.01^{aA}
P. pentosaceus	5.76 ± 0.01^{bB}	5.67 ± 0.01^{cB}	5.49 ± 0.01^{dB}	6.06 ± 0.01^{aB}

Means with same lowercase letter in superscript in same row are not significantly different ($p > 0.05$) for individual hydrocolloids mixtures. Means with same uppercase letter in superscript in same column are not significantly different ($p > 0.05$) for individual lactic acid bacterial strains.

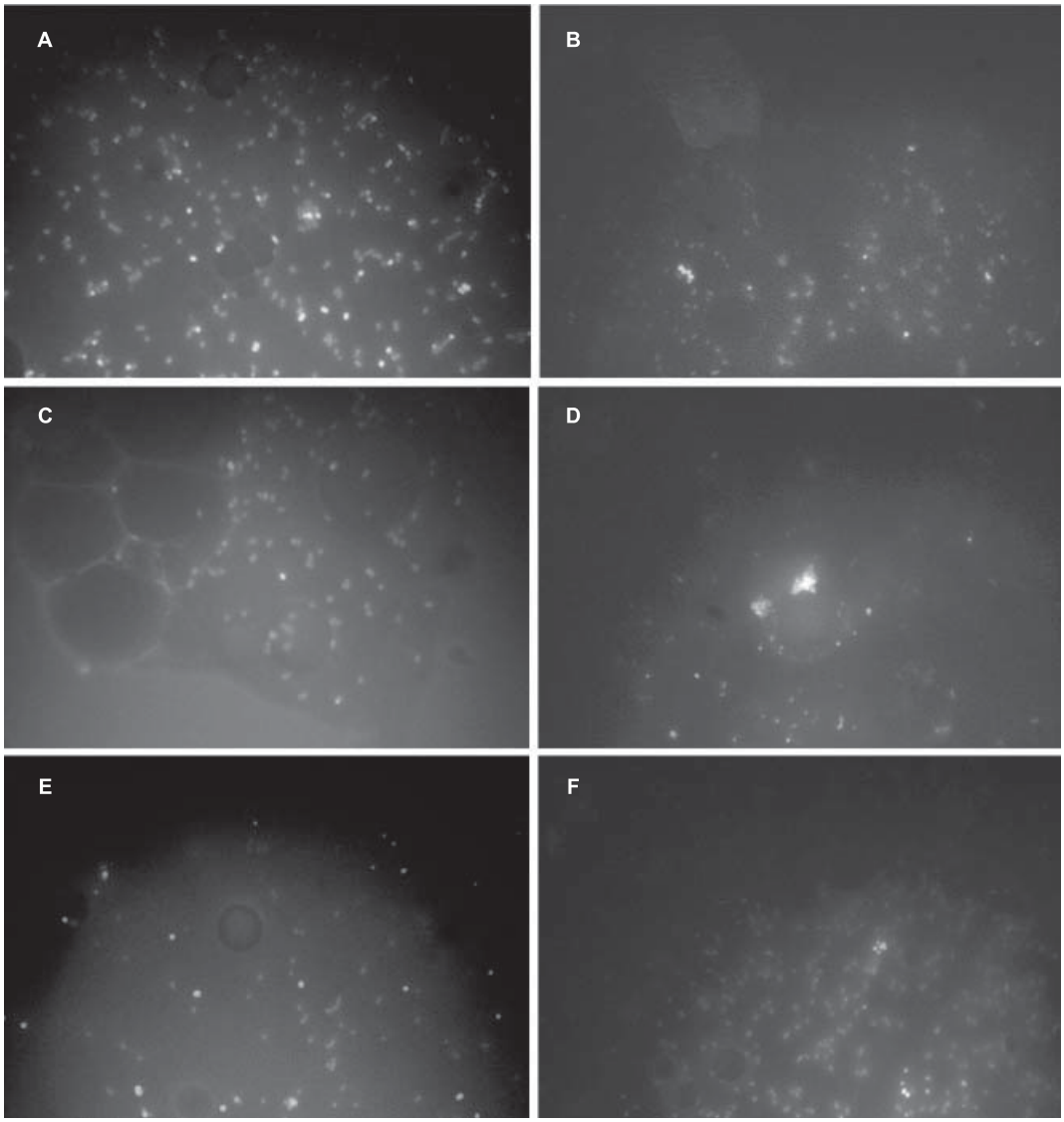

Fig. 1. Fluorescence micrograph (100x) for lactic acid bacteria after microencapsulation stained with the LIVE/DEAD BacLight viability stain.

Live cells seem brighter than damaged cells.
A – *E. faecium* in alginate, B – *P. pentosaceus* in alginate, C – *E. faecium* in alginate + gellan, D – *P. pentosaceus* in alginate + gellan, E – *E. faecium* in alginate + κ-carrageenan/locust bean gum, F – *P. pentosaceus* in alginate + κ-carrageenan/locust bean gum.

alginate gels texture in a way that positively influenced viability of the encapsulated microorganisms, resulting in acceptable fermentation ability. Advantages of alginate as encapsulating agent are the non-toxicity, formation of soft matrices and ability to trap sensitive materials, such as lactic acid bacteria. Although it was reported [1, 9, 32] that cells viability was not affected and alginate gels could be solubilized to release the encapsulated-immobilized microorganisms, some disadvantages of alginate beads, such as acid environment susceptibility and integrity deterioration by chelating agents that reduce barrier properties, were also reported [7, 33]. Blending of alginate with other hydrocolloids efficiently compensated these structural-textural disadvantages.

Particle size and scanning electron microscopy

The average particle size and volume mean diameters of the microcapsules formed with the different hydrocolloids mixtures with alginate for each lactic acid bacteria strain are listed in Tab. 3.

For the surface area mean diameter or average particle size $D_{[3,2]}$, the use of incorporation of gellan in alginate microencapsulation resulted in significantly ($p < 0.05$) lowest values, followed by alginate + κ-carrageenan/locust bean gum samples.

For the volume median diameter $d_{0.1}$, when other gelling hydrocolloid was employed, a significantly ($p < 0.05$) lowest diameter was obtained with alginate + gellan gum treatment (10% of the microcapsules were below 47.06 mm ± 11.75 mm average diameter), followed by the alginate + κ-carrageenan/locust bean gum containing samples (10% of the microcapsules were below 84.86 mm ± 17.50 mm average diameter). On average for alginate microcapsules, regardless lactic acid bacterial strain, 10% of the microcapsules were below 162.51 mm ± 26.50 mm in diameter.

For the volume median diameter $d_{0.5}$, alginate microencapsulation, with 50% of the microcapsules having the diameter below 136.26 mm ± 21.50 mm, treatment resulted in significantly ($p < 0.05$) higher particle size diameter. On average, 50% of microcapsules had diameter size below 33.46 mm ± 12.50 mm and 68.82 mm ± 13.50 mm for alginate + gellan gum and alginate + κ-carrageenan/locust bean gum, respectively, regardless the lactic acid bacterial strain.

For the volume median diameter $d_{0.9}$, alginate microencapsulation resulted in significantly ($p < 0.05$) highest diameter (90% of the microcapsules were below 111.14 mm ± 20.75 mm in diameter), with lowest particle size diameters in alginate + gellan treatments (90% the average diameter size were below 54.99 mm ± 8.75 mm and 67.00 mm ± 14.10 mm for alginate + gellan gum and alginate + κ-carrageenan/locust bean gum, respectively; Tab. 3).

Regarding differences between bacterial strains, the surface area mean diameter $D_{[3,2]}$ was significantly lowest for microcapsules with *P. pen-*

Tab. 3. Surface area mean diameter and volume median diameters for lactic acid bacteria as non-encapsulated or encapsulated with different hydrocolloids mixtures.

Strain	Sodium alginate	Sodium alginate + gellan gum	Sodium alginate + κ-carrageenan/locust bean gum
Surface area mean diameter $D_{[3,2]}$ [μm]			
A. viridans	226.9 ± 30.5 ^{aA}	41.6 ± 14.2 ^{cA}	98.0 ± 12.1 ^{bA}
E. faecium	198.0 ± 40.3 ^{aC}	36.1 ± 11.0 ^{cC}	59.1 ± 11.2 ^{bC}
L. plantarum	207.0 ± 56.1 ^{aB}	40.2 ± 12.5 ^{cB}	78.2 ± 14.3 ^{bB}
P. pentosaceus	195.0 ± 10.4 ^{aD}	39.3 ± 10.4 ^{cD}	55.7 ± 13.5 ^{bD}
Volume median diameter $d_{0.1}$ [μm]			
A. viridans	165.31 ± 15.61 ^{aAD}	49.45 ± 13.54 ^{cAD}	89.73 ± 19.11 ^{bAD}
E. faecium	162.40 ± 35.44 ^{aBD}	47.37 ± 10.94 ^{cBD}	84.13 ± 16.71 ^{bBD}
L. plantarum	162.00 ± 30.63 ^{aBD}	47.18 ± 11.43 ^{cBD}	85.81 ± 17.00 ^{bBD}
P. pentosaceus	160.33 ± 26.51 ^{aCD}	44.25 ± 13.32 ^{cCD}	79.75 ± 18.42 ^{bCD}
Volume median diameter $d_{0.5}$ [μm]			
A. viridans	140.71 ± 23.50 ^{aAE}	38.13 ± 15.25 ^{cAE}	72.53 ± 14.15 ^{bAE}
E. faecium	134.32 ± 15.68 ^{aBE}	31.44 ± 14.63 ^{cBE}	70.93 ± 15.35 ^{bBE}
L. plantarum	139.30 ± 25.63 ^{aBE}	34.00 ± 10.92 ^{cBE}	68.23 ± 11.23 ^{bBE}
P. pentosaceus	130.71 ± 23.92 ^{aCE}	30.26 ± 11.45 ^{cCE}	63.60 ± 14.50 ^{bCE}
Volume median diameter $d_{0.9}$ [μm]			
A. viridans	119.01 ± 13.21 ^{aAF}	59.43 ± 9.56 ^{cAF}	70.25 ± 16.11 ^{bAF}
E. faecium	117.12 ± 28.44 ^{aBF}	54.95 ± 8.72 ^{cBF}	68.19 ± 15.63 ^{bBF}
L. plantarum	113.16 ± 27.33 ^{aBF}	54.52 ± 9.37 ^{cBF}	69.72 ± 11.24 ^{bBF}
P. pentosaceus	95.25 ± 15.10 ^{aCF}	51.06 ± 9.11 ^{cCF}	59.82 ± 14.02 ^{bCF}

Means with same lowercase letter in superscript in same row are not significantly different ($p > 0.05$) for individual hydrocolloids mixtures.

A, B, C – Means with same uppercase letter in superscript in same column are not significantly different ($p > 0.05$) for individual lactic acid bacterial strains.

D, E, F – Means with same uppercase letter in superscript in same column are not significantly different ($p > 0.05$) for the given volume median diameter.

tosaceus. The volume median diameters ($d_{0.1}$, $d_{0.5}$ and $d_{0.9}$) followed the same tendency, where microencapsulation with *P. pentosaceus* resulted in significantly ($p < 0.05$) lowest values. Highest microcapsules size was observed for *A. viridans* samples (Tab. 3).

In general, microencapsulation of the employed lactic acid bacteria strains at the actual experimental conditions incorporating other gelling hydrocolloids resulted in smaller particle size. Particle median diameter size was significantly ($p < 0.05$) lowest for $d_{0.9}$ and highest for $d_{0.1}$, meaning that most of the microcapsules (90%) had smaller particle size, and only a small portion (10%) had large particle size. The mixture of alginate with other gelling hydrocolloids as gellan gum or κ-carrageenan/locust bean gum modified the textural characteristics of the emulsion-formed microcapsules. This textural modification enhanced the cells viability since the highest viable counts and fastest milk acidification were detected in mixed alginate treatments. These results seem to correlate with particle size (determined as surface area mean diameter and volume mean diameter), since the samples with other hydrocolloids were smaller than alginate microcapsules. The microcapsule diameter influenced the fermentation rate since smaller beads are known to have higher

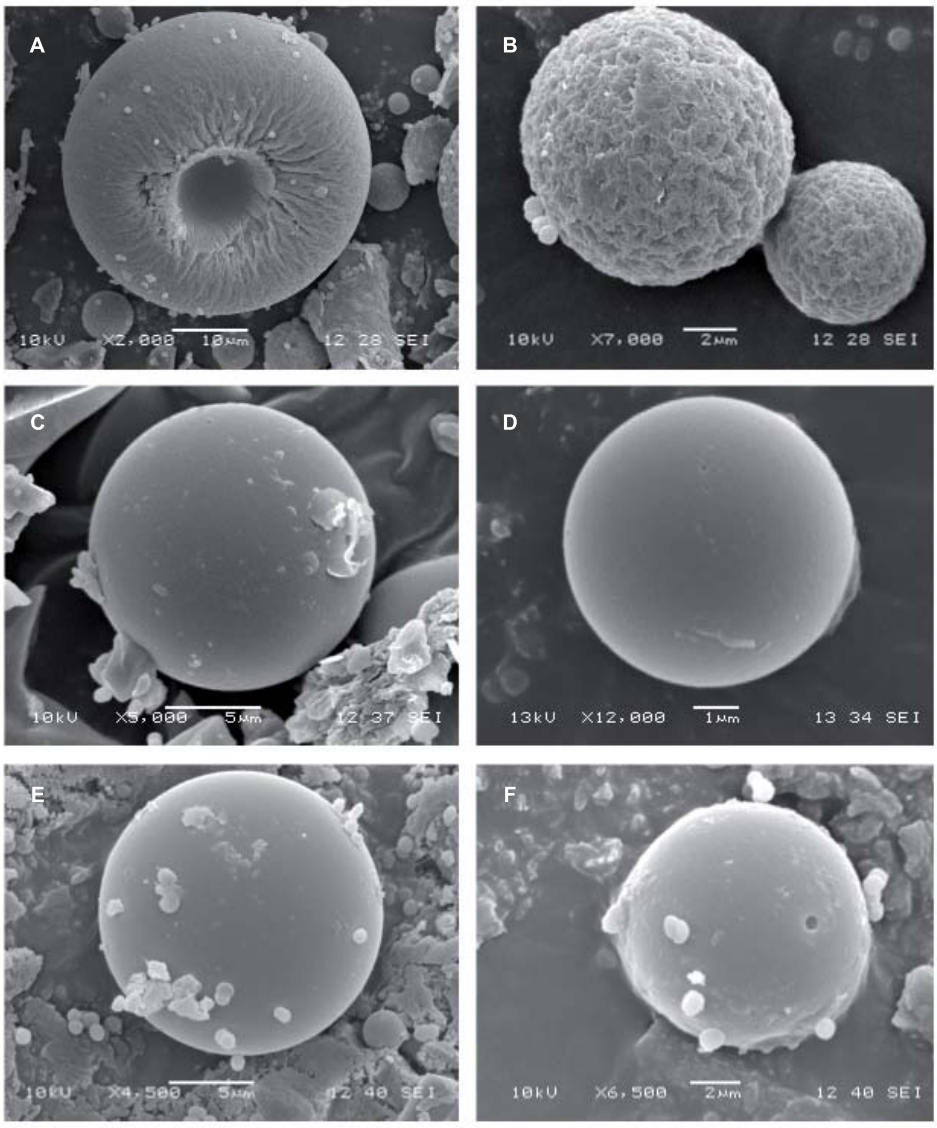

Fig. 2. Scanning electron micrographs of microencapsulated *E. faecium*.

A, B – bacteria microencapsulated in alginate (at 2000× and 7000×, respectively); C, D – alginate + gellan gum (at 5000× and 12000×, respectively); E, F – alginate + κ-carrageenan/locust bean gum (at 4500× and 6500×, respectively).

cell release rates, lactose utilization and acid production [34]. In addition, the method and the type of material employed for microencapsulation is known to have an effect on the shape of microcapsules [22, 35], and the bacterial cells may be considered as particles that may influence the particle size distribution [4].

Microcapsules morphology is illustrated on scanning electron micrographs for *E. faecium* (Fig. 2) and *P. pentosaceus* (Fig. 3). For both strains, sodium alginate microcapsules presented a wrinkled surface, in contrast to the smooth, corrugated or crumpled surface in alginate with gellan gum or alginate with k-carrageenan/locust bean gum mixture. This rough texture in alginate microcapsules had been observed by YOUNG et al. [36], SONG et al. [37] and ZANJINI et al. [38]. CHEN et al. [39] reported that the beads made of sodium alginate alone were softer than those containing gellan gum and had irregular shapes. Alginate microparticles usually had a core due to the heterogeneous gelation mechanism. Since polysaccharide ionotropic gels formed by diffusion of calcium ions into solutions of alginate exhibited various degrees of heterogeneity, the polymer concentration was much higher on the surface than in the centre of the gels, which resulted in uneven surfaces [40]. The incorporation of other gelling hydrocolloids

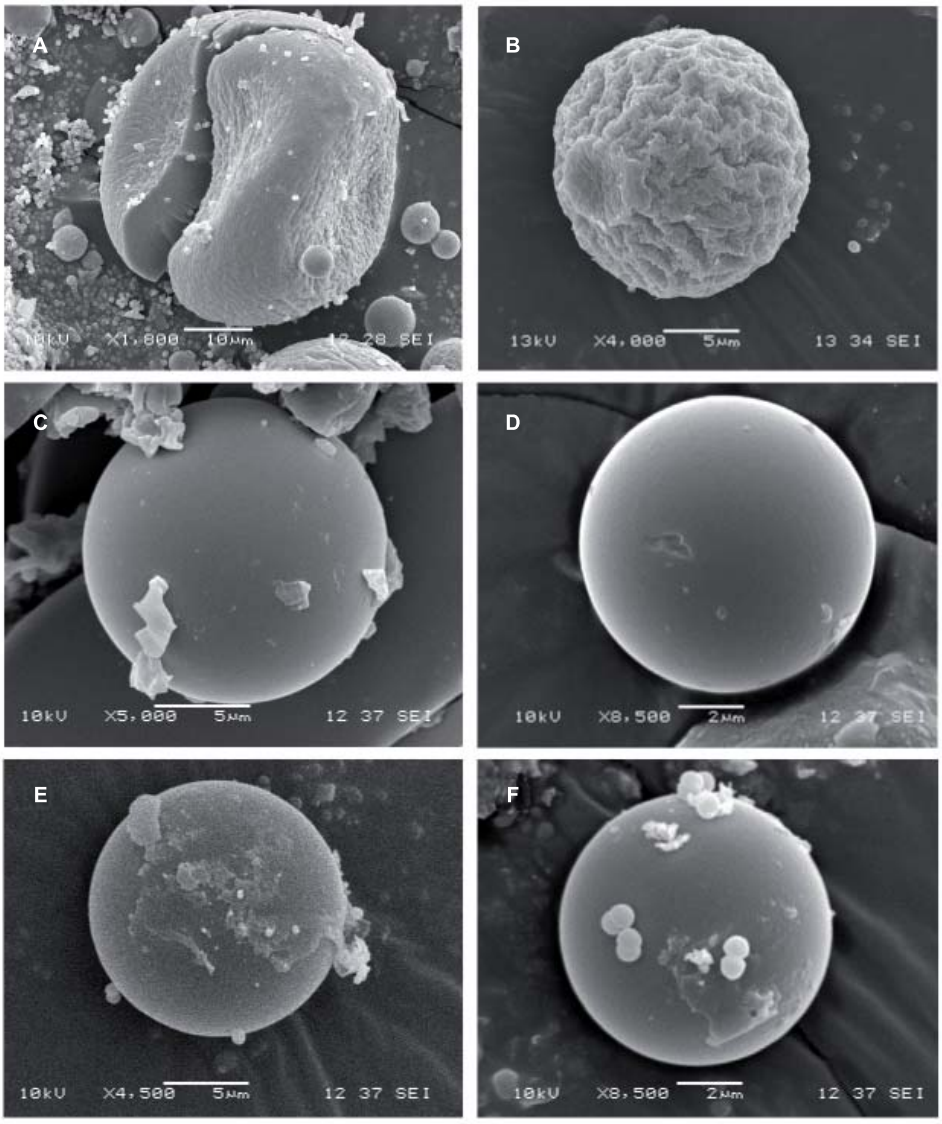

Fig. 3. Scanning electron micrographs of microencapsulated *P. pentosaceus*.

A, B – bacteria microencapsulated in alginate (at 1800× and 4000×, respectively); C, D – alginate + gellan gum (at 5000× and 8500×, respectively); E, F – alginate+κ-carrageenan/locust bean gum (at 4500× and 8500×, respectively).

Tab. 4. Pearson's correlation coefficients between textural properties, bacteria viability and particle size of the microcapsules containing lactic acid bacteria encapsulated using different hydrocolloids mixtures.

Variables	Hardness	Cohesiveness	Resilience	Apparent elasticity	Viscosity index	Viable count	V_{max}	Time to V_{max}	pH at V_{max}	Final pH	$D_{[3,2]}$	$d_{0.1}$	$d_{0.5}$	$d_{0.9}$
Textural properties														
Hardness	1.0000	0.9661**	0.8944**	0.3093	–0.1409	0.4558	0.5482	0.0793	–0.5081	–0.5236	–0.7409*	–0.3698	0.5945	0.2659
Cohesiveness		1.0000	0.8025**	0.5183	–0.0207	0.5882	0.3936	0.2321	–0.4815	–0.3706	–0.3631	–0.1893	0.5854	0.5488
Resilience			1.0000	–0.0608	–0.4715	0.8274**	0.8560**	–0.1814	–0.7325*	–0.8421*	–0.0554	0.1115	0.4588	0.7864*
Apparent elasticity				1.0000	0.7982*	0.2248	–0.4711	0.6206	0.0267	0.4885	0.2406	0.6212	0.6228	–0.5430
Viscosity index					1.0000	0.1074	0.7092	–0.0129	–0.8049**	0.7405*	0.7285*	0.5735	0.5016	–0.1991
Bacteria viability														
Viable count						1.0000	–0.3139	0.8567**	–0.3740	0.3049	–0.8381**	0.5595	0.3588	–0.7001
V_{max}							1.0000	–0.4690	–0.5831	–0.9972**	0.2104	–0.9527**	0.0414	–0.9524**
Time to V_{max}								1.0000	–0.4287	0.4268	0.9618**	0.7099	–0.0249	0.9168**
pH at V_{max}									1.0000	0.6282	0.7582*	0.3272	0.1281	0.0898
Final pH										1.0000	–0.1632	0.6392	0.0113	–0.7132
Particle size														
$D_{[3,2]}$											1.00000	0.7997*	0.7003	–0.6277
$d_{0.1}$												1.0000	0.0176	0.1927
$d_{0.5}$													1.0000	0.6041
$d_{0.9}$														1.00000

** – highly significant ($p < 0.01$), * – significant ($p < 0.05$). V_{max} – maximum acidification rate.

resulted in a smooth and regular surface, besides a smaller particle size that enhanced the encapsulated lactic acid bacteria viability in the experimental conditions employed in our study.

Correlation coefficients and principal components analysis

Pearson's correlation coefficients of the analysed variables are listed in Tab. 4. There was an inherent correlation amid the different microcapsules characteristics. For example, the texture profile analysis parameters (hardness, cohesiveness and resilience) presented a highly significant ($p < 0.01$) correlation among them, whereas apparent elasticity was significantly ($p < 0.05$) correlated with viscosity index. Regarding the cells viability, a highly significant ($p < 0.01$) correlation between viable counts (as colony forming units) and the time to reach the maximum acidification rate (Time to V_{max}) was found. This means that more cells need more time to reach the maximum acidification rate. A highly significant ($p < 0.01$) inverse correlation existed between acidification rate (V_{max}) and final pH, where a higher acidification rate resulted in lower final pH values. For particle size, there was a significant ($p < 0.05$) correlation between $D_{[3,2]}$ and $d_{0.1}$, related to higher particle size.

Textural parameters and viability resulted in highly significant ($p < 0.01$) correlation between resilience with viable counts and maximum acidification rate. This means that more resilient beads enhanced encapsulated lactic acid bacteria viability (as in the alginate + gellan gum microcapsules). In the same way, resilience presented a significant ($p < 0.05$) inverse correlation with maximum acidification rate and final pH. Resilience of microcapsules seems the most important factor in cells viability. Alginate + gellan gum samples were more resilient than the rest of the treatments, resulting in higher cell viability, higher milk

acidification rate with a concomitantly lower final pH. For the textural parameters and the particle size, there was a significant ($p < 0.05$) inverse correlation between hardness with $D_{[3,2]}$. Viscosity index had also a significant ($p < 0.05$) effect on surface area mean diameter. In the same manner, resilience significantly ($p < 0.05$) correlated with $d_{0.9}$ values. This means that most of the microcapsules ($d_{0.9}$ value), with lower surface area diameter, were harder, more resilient and less viscous. In the relationship between particle size and viability, $D_{[3,2]}$ presented a highly significant ($p < 0.01$) inverse correlation with cell viability, and a highly significant ($p < 0.01$) correlation with time to reach V_{max}. There was also a significant ($p < 0.05$) correlation between this parameter and the pH at the maximum acidification rate. Higher surface area affected the growth and fermentative capability (lower viable count values, longer times to reach maximum acidification rate, with higher pH at this point) of encapsulated lactic acid bacteria. The incorporation of gellan or κ-carrageenan/locust bean gum into alginate beads resulted in a lower surface area that, on one hand, enhanced viable counts and, on the other hand, slowed down milk acidification during fermentation tests. Volume median diameter $d_{0.1}$ presented an inverse highly significant ($p < 0.01$) correlation with maximum acidification rate, probably because microcapsules with higher volume decreased the acidification rate. Volume median diameter $d_{0.9}$ presented a highly significant ($p < 0.01$) inverse correlation with acidification rate. There was also a highly significant ($p < 0.01$) correlation of $d_{0.9}$ with time to reach maximum acidification rate. Since $d_{0.9}$ (smaller particle volume) represented the bulk of the microcapsules, the smaller size was related to higher viability rate displayed as faster fermentation and lower times to reach the maximum acidification.

Due to the significant correlations found between most of the analysed variables, the principal components analysis was performed with all of them. In the initial factor method of analysis of principal components, the eigenvalues showed that two components provided a good summary of the analysed data, accounting for 79.4% of the total variance (44.6% and 34.8% for factor 1 and factor 2, respectively). PCA was performed to determine the relationship among the parameters related to gel texture, cell viability and particle size, and how they were distributed in the two-dimension space. Factor 1 positively correlated with gels resilience, pH at V_{max}, final pH and volume median diameter $d_{0.9}$. This factor inversely correlated with viscosity index and final pH. Factor 2 correlated with viable counts and V_{max}, with an inverse correlation with surface mean area $D_{[3,2]}$ and volume median diameter $d_{0.1}$. In the bi-plot graph, the two components were related to elastic behaviour of gelled hydrocolloids mixtures (resilience and viscosity index) and a small particle size (inversely correlated to $D_{[3,2]}$ and $d_{0.1}$). Lower particle size ($d_{0.9}$) allowed greater cells viability

Tab. 5. Rotated factor pattern and final communality estimates.

Parameters	Rotated factor pattern		Final communality estimates (Total = 13.6983)
	Factor 1	Factor 2	
Hardness	–0.42475	0.0815	0.9602
Cohesiveness	–0.2895	0.2192	0.9951
Resilience	–0.7245	–0.1518	0.9952
Apparent elasticity	0.4845	0.5694	0.9112
Viscosity index	0.9384	–0.0904	0.9783
Viable counts	0.1189	0.8443	0.9556
Maximum acidification rate, V_{max}	–0.8689	–0.4082	0.9877
Time to reach V_{max}	0.0784	0.9949	0.9973
pH at V_{max}	0.8543	–0.4928	0.9922
Final pH	0.9019	0.3622	0.9890
Surface area mean diameter, $D_{[3,2]}$	0.1876	–0.9754	0.9967
Volume median diameter, $d_{0.1}$	–0.3716	–0.8997	0.9875
Volume median diameter, $d_{0.5}$	0.2580	–0.0905	0.9556
Volume median diameter, $d_{0.9}$	0.7338	0.6558	0.9959

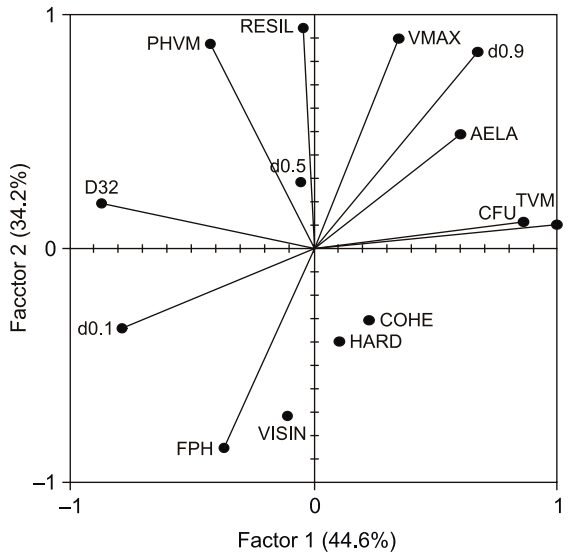

Fig. 4. Bi-plot curve of principal component 1 versus principal component 2 for the analysed variables.

HARD – hardness, COHE – cohesiveness, RESIL – resilience, AELA – apparent elasticity, VISIN – viscosity index, CFU – viable count, VMAX – maximum acidification rate V_{max}, TVM – time to reach V_{max}, PHVM – pH at V_{max}, FPH – final pH, D32 – surface area mean diameter $D_{[3,2]}$, d0.1, d0.5 and d0.9 – volume mean diameters $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$.

(viable counts, time to reach V_{max}, and pH at V_{max}) reducing the acidification rate (inverse correlation with final pH). The longer lines and closer proximity suggest a higher correlation between parameters. The final communalities showed that all the variables were well accounted for by the two factors, with final communality estimates over 0.9100 (Fig. 4 and Tab. 5).

CONCLUSIONS

The incorporation of other gelling hydrocolloids modified the texture, viability and particle size of sodium alginate gels at emulsion microencapsulation of lactic acid bacteria. As compared with sodium alginate microcapsules, it seems that the compatibility or interaction with hydrocolloids like gellan gum resulted in a harder and more resilient less viscous texture of microcapsules, with low particle size diameter, enhancing viability of the microencapsulated bacteria. Correlation analysis showed that microcapsules with lower surface area diameter were harder, more resilient and less viscous, enhancing cells viability and reducing acidifications rates. Higher surface area affected negatively the growth and fermentative capability of encapsulated lactic acid bacteria. The incorporation of gellan or κ-carrageenan/locust bean gum into alginate beads resulted in a lower surface area that, on one hand, enhanced viable counts and, on the other hand, slowed down milk acidification during fermentation tests. The use of alginate with gellan gum represents a good alternative to protect lactic acid bacteria in order to be inoculated in functional processed foods.

Acknowledgements

Ariza-Ortega thanks to National Council of Science and Technology, Mexico (CONACYT) the grant for her graduate studies at Tecnológico Estudios Superiores Ecatepec, Mexico. Authors express gratitude to Dr. José D. Sepulveda-Sánchez, at Universidad Autonoma Metropolitana-Centro Nacional Investigacion y Capacitacion Ambiental (UAM-CENICA) Electron Microscopy Laboratory, for the preparation and processing scanning electron microscopy samples. Thanks to Dr. Aurora Martínez-Trujillo for her advice in statistical analysis.

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Received 28 January 2013; revised 12 April 2013; accepted 17 April 2013.