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Viability of microencapsulated *Lactobacillus acidophilus* by complex coacervation associated with enzymatic crosslinking under application in different fruit juices

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ABSTRACT

The objective of this study was to produce microcapsules containing *Lactobacillus acidophilus* LA-02 by complex coacervation followed by crosslinking with transglutaminase and to evaluate the effect of their addition on different fruit juices, as well as the probiotic viability of *L. acidophilus* and its effect on fruit juices during storage. To this end, *L. acidophilus* was microencapsulated by complex coacervation, followed by crosslinking with transglutaminase at different concentrations. Probiotics, in their free and microencapsulated forms, were added to orange juice and apple juice at concentrations of 10% and 30%. The obtained microcapsules were characterized in terms of morphology. The viability of probiotics and the effects of their addition on fruit juices were assessed and the juices characterized (with respect to pH and total soluble solids) during 63 days of storage at 4 °C. Orange juice proved to be more suitable for the addition of probiotics, and the survival of probiotics was directly related to pH. The microcapsules had a protective effect on *L. acidophilus*, prolonging their survival, and the crosslinking process proved to be adequate and promising, ensuring probiotic viability. Thus, the complex coacervation process associated with induced enzymatic crosslinking provided protection for *L. acidophilus* in different fruit juices, showing an adequate methodology for adding probiotics to this adverse food matrix, guaranteeing the survival of *L. acidophilus* for up to 63 days, and generating products with innovative and promising probiotic appeal.

1. Introduction

Functional foods have attracted increasing interest from consumers in search of healthier diets. It is proven by scientific evidence that probiotics provide innumerable health benefits to the host, being able to protect them against a wide range of diseases, from infections to psychological and even degenerative diseases, justifying the growing interest in these foods, especially in the last decades (Ester et al., 2019; Horáčková, Rokytová, Bialasová, Klojdová, & Sluková, 2018).

Probiotics are generally available in dairy products, as these foods provide excellent conditions for maintaining their viability, mainly because of their high amounts of proteins and considerable amount of lipids. However, this can be considered a limiting factor for the consumption of probiotics if we consider lactose intolerance, allergies to milk protein, the prevalence of high cholesterol, and vegetarianism (Lebaka, Wee, Narala, & Joshi, 2018). In this sense, greater attention is given to fruit juices due to their high content of bioactive compounds, for which they are considered healthy and consumed by all age groups (Horáčková et al., 2018). Thus, the development of a probiotic juice would be highly beneficial.

However, the addition of probiotics to fruit juices is difficult due to their low pH and insufficient amounts of some peptides and free amino acids needed by probiotics; therefore, fruit juices are not always suitable for the application of probiotics (Antunes et al., 2013; Nualkaekul, Cook, Khutoryanskiy, & Charalampopoulos, 2013). Thus, microencapsulation of probiotics can be a promising strategy for protecting them from the adverse conditions found in food matrices, such as fruit juices.

For probiotic microencapsulation, complex coacervation has proven

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to be an extremely relevant technique as it demonstrates high encapsulation efficiency and protection (Marques Da Silva et al., 2018). This technique consists of ionic interactions between two or more polymers of opposite charge, usually proteins and polysaccharides, which leads to the formation of coacervates and phase separation (Timilsena, Taiwo, Khalid, Adhikari, & Barrow, 2019). In addition, according to Oliveira et al. (2020), to date, few studies report the application of microcapsules by complex coacervation in tropical juices.

However, some parameters can affect the structure of the microcapsules formed by complex coacervation, and they can show fragility under certain conditions (Comunian et al., 2016; Da Silva et al., 2019). For this reason, crosslinking associated with coacervation has been applied. Among the crosslinking processes, enzymatic crosslinking has been used, and transglutaminase is among the widely used enzymatic crosslinkers. This enzyme acts by forming intra- and intermolecular crosslinks between two residual amino acids present in the protein structure, providing improved rheological and physical properties, bringing significant changes to the protein molecule, without negatively affecting the sensory and nutritional qualities of the product (Da Silva et al., 2019).

There has been recent research involving probiotics and transglutaminase. Mituniewicz-Małek, Ziarno, and Dmytrów (2014) studied the effect of transglutaminase on the viability of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. *lactis* Bb-12 in goat's milk fermented under storage at 5 °C. There was no positive influence of transglutaminase on the viability of probiotics in fermented goat's milk samples. However, probiotics remained above 6 log UFC g^{-1} after 8 weeks of storage at 5 °C. Currently, there are also investigations involving complex coacervation, in this sense. Da Silva et al. (2019) studied the effect of crosslinking with transglutaminase on the resistance of microcapsules containing *Lactobacillus acidophilus* produced by complex coacervation and on probiotic viability and evaluated these microcapsules against thermal resistance, simulated gastrointestinal conditions, and storage at various temperatures. Crosslinking with transglutaminase proved to be an effective means of increasing probiotic viability by improving the resistance of microcapsules, demonstrating its efficiency in front of the adverse conditions evaluated.

However, there are few studies on the application of encapsulated compounds in food and the release of coacervates in the food matrix. Therefore, more research is needed on the process and application of complex coacervation in the food industry (Eghbal & Choudhary, 2018). In addition, no investigations were found involving the study of the crosslinking of probiotic microcapsules produced by complex coacervation and its application in fruit juices, highlighting the relevance of our study.

Therefore, the objective of the present work was to produce microcapsules containing *Lactobacillus acidophilus* LA-02 by complex coacervation followed by crosslinking with transglutaminase and to evaluate the effect of addition to different fruit juices, as well as to evaluate probiotic and fruit juice viability during refrigerated storage.

2. Material and methods

2.1. Probiotic culture and inoculum preparation

The lyophilized probiotic commercial culture *L. acidophilus* LA-02 (Probiotical, Italy) was kindly donated by Coana Importação e Exportação Ltda. *L. acidophilus* LA-02 (1 g) was activated by incubation in MRS broth (Merck, Germany) for 17 h at 37 °C. The resulting culture was centrifuged at 2470 g for 15 min, at 4 °C. The supernatant was then discarded, and the probiotic culture was washed twice with 0.85% saline. After washing, the probiotic culture was suspended in this solution to obtain approximately 10 log CFU mL^{-1}.

2.2. Encapsulation and crosslinking process

Microencapsulation and crosslinking were carried out according to the methodology of Da Silva et al. (2019). The probiotic suspension was added to a 2.5% gelatin solution (Gelita, Germany), under stirring and heating (48 ± 2 °C). Then, the 2.5% gum Arabic solution (CNI, Brazil) was added together with the distilled water, maintaining agitation and heating, and the pH was adjusted to 4.0. After pH adjustment, stirring was continued, and natural cooling was carried out to 30 °C, after which an ice bath was added to rapidly lower the temperature to 10 °C. To carry out the crosslinking process, the enzyme transglutaminase (100 U/g of activity, Ajinomoto, Brazil) was added to the microcapsules produced in concentrations of 2.5 and 5.0 U/g of protein, separately, corresponding to Treatments 1 and 2, respectively. The reactions were carried out at 25 °C for 15 h, under constant agitation.

2.3. Incorporation of probiotics in fruit juices

Commercially available orange and apple fruit juices (Natural One, Brazil) were obtained from the local market in Santa Maria, RS, Brazil and were evaluated as a vehicle for supplying probiotics. The incorporation of probiotic in fruit juices was carried out in accordance with Rodrigues et al. (2012), with modifications. For each juice (orange and apple), 10 mL were transferred to sterile capped tubes, along with the addition of probiotics: 1 g in 10 mL of fruit juice, which corresponds to a 10% concentration of probiotics and, 3 g in 10 mL of fruit juice, which corresponds to a concentration of 30% probiotics, followed by storage at 4 °C. Thus, for each concentration studied there were five treatments: Control (Control samples were composed of only 10 mL of juice), Free cell (free cells correspond to non-microencapsulated probiotics), Microcapsules without crosslinking (microcapsules without crosslinking correspond to microcapsules obtained by coacervation complex without association with crosslinking), Treatment 1 (treatment 1 corresponds to the addition of 2.5 U/g of transglutaminase to the microcapsules) and Treatment 2 (treatment 2 corresponds to the addition of 5.0 U/g of transglutaminase to the microcapsules). Table 1 shows all the studied combinations.

2.4. Probiotic viability during storage for 63 days at 4 °C

Probiotics, both in free form and in different microcapsule treatments, were stored in fruit juices at 4 °C for 63 days. Probiotic viability analysis was performed at 0, 35, and 63 days of storage.

To determine the viability of free cells in fruit juices, the fruit juices were slightly agitated, 1 mL was removed, and dilutions were performed. To determine viability from the microcapsules, the fruit juices were first centrifuged in a refrigerated centrifuge (Hitachi, Japan) at 1,088 g and 4 °C for 7 min, to separate the microcapsules from the juices; after centrifugation, 1 g of microcapsules was weighed, and 9 mL of sodium phosphate buffer solution (pH 7.5) was added, followed by disruption on a shaker with heating at 37 °C for 10 min. After this process, 1 mL was removed, and viability determination analysis was

Table 1
Adding probiotics to fruit juices (orange and apple).

Treatment	Juice with 10% probiotic		Juice with 30% probiotic	
	Probiotic (g)	Juice (mL)	Probiotic (g)	Juice (mL)
Control	0	10	0	10
Free cell	1	10	3	10
Microcapsules without crosslinking*	1	10	3	10
Treatment 1(2,5 U/g)*	1	10	3	10
Treatment 2 (5,0 U/g)*	1	10	3	10

* For all formulations, microcapsules were used in the wet form.

performed. The determination of probiotic viability was carried out according to the methodology described by Sheu, Marshall, and Heymann (1993). From the initial dilutions, serial dilutions were performed, and the appropriate dilutions were transferred in triplicate to sterile Petri dishes, followed by the addition of MRS agar (Kasvi, Brazil). The plates were incubated at 37 °C for 72 h in anaerobic jars containing anaerobic generators (Anaerobac, Probac, Brazil). The results were expressed in log CFU mL^{-1}.

2.5. Morphological analysis of microcapsules

To monitor the morphological changes in microcapsules during storage for 63 days at 4 °C in fruit juices, together with probiotic viability analyses, optical microscopy was performed using an optical microscope (Scope A.1, Zeiss, Germany) coupled with a digital camera AxioCam MRc according to Rodrigues et al. (2012).

2.6. Monitoring of fruit juice pH and total soluble solids

To assess whether fruit juices underwent any changes during storage with the addition of probiotics, the pH was measured with a pH meter (Digimed, Brazil) and total soluble solids (TSS) with a bench refractometer (Nova, Brazil), at 0, 35, and 63 days of storage according to Antunes et al. (2013), with modifications.

2.7. Statistical analysis

The results were subjected to analysis of variance (ANOVA), and the differences between the means of the triplicates were verified by the Tukey test ($p < 0.05$) using Statistica software 7.0® (Tulsa, OK, EUA). Multivariate cluster analysis was used to determine the similarity between fruit juices. The analysis used the single bond as an amalgam rule and Euclidean distances as a measure of similarity.

3. Results and discussion

3.1. Viability of probiotics added to fruit juices during storage at 4 °C for 63 days

Table 2 shows the results obtained for the analysis of the viability of *L. acidophilus* during storage for 63 days at 4 °C.

It was observed from the results shown in Table 2, the higher viability of *L. acidophilus* during the storage period present in orange juices stands out (8.12 log CFU mL^{-1}). According to Champagne and Gardner (2008), it is believed that the survival of probiotic strains in fruit juice is related to pH, with values close to 4.0 being more appropriate. Therefore, orange juice (pH 3.99) proved to be more suitable for probiotics than apple juice (pH 3.65). Furthermore Horáčková et al. (2018) claim that the selection of the type of juice is a determining factor in the stability of the added probiotics. In their study, they observed that pineapple juice (pH 3.8) proved to be more suitable than strawberry juice (pH 3.2) for the survival of *Bifidobacterium lactis* over 28 days of cold storage.

It can also be considered that in addition to the probiotic viability being directly related to the characteristics of the food matrix, it is also related to the probiotic strain. Ding & Shah (2008) analyzed the viability of *L. acidophilus* in orange juice at 4 °C, observing the survival of probiotics for approximately 14 days; Nualkaekul, Lenton, Cook, Khutoryanskiy, and Charalampopoulos (2012) analyzed the viability of *L. plantarum* in pomegranate juice at 4 °C, obtaining 21 days of survival; and Sohail, Turner, Prabawati, Coombes, and Bhandari (2012) analyzed the viability of *L. acidophilus* in orange juice at 4 °C, also observing the survival of probiotics for 21 days.

Regarding the free and microencapsulated forms, it was observed that the majority of *L. acidophilus* showed greater viability when added to fruit juices in the microencapsulated form, with significant differences ($p < 0.05$), demonstrating that the packaging of *L acidophilus* in a microcapsule formed by complex coacervation with gelatin–gum Arabic as coatings, is protective enough to prolong its survival. Through the complexation between gelatin and gum Arabic by electrostatic interactions, a protective barrier is formed against *L. acidophilus*, reducing their interaction with the external environment and, consequently, favoring their survival. In addition, in the case of encapsulated cells, the pH does not appear to significantly affect bacterial cells. In low pH media, amino groups on the side chain of proteins are protonated and the percentage of carboxylic groups dissociated from polysaccharides is decreased. Therefore, electrostatic interactions between these biopolymers are strengthened, so that cells are less susceptible to being severely affected by ambient pH (De Almeida et al., 2019). This result was expected, since microcapsules are used to improve the survival of probiotics in food products (Horáčková et al., 2018; Nualkaekul et al., 2012), acting as a protective barrier against the adverse conditions present fruit juices, such as acidity and oxygen. Similarly, Calabuig-Jiménez et al. (2019) observed that microcapsules formed with alginate were able to protect *L. salivarius*, increasing its survival in tangerine juice. Rodrigues et al. (2012) encapsulated *L. paracasei* in alginate using the extrusion technique and added microcapsules to orange and peach fruit juices, analyzing the protection provided by the microcapsules and the viability of the probiotics for 50 days. It was observed that the microcapsules protected the probiotics during the 50 days, providing high

Table 2
Probiotic viability (log CFU mL^{-1}) during 63 days of storage at 4 °C in different fruit juices.

Orange juice								
Time (d)	CL(L10)	C(L10)	T1(L10)	T2(L10)	CL(L30)	C(L30)	T1(L30)	T2(L30)
0	$9,60 \pm 0,01^{aB}$	$8,91 \pm 0,11^{Ac}$	$8,70 \pm 0,23^{aCD}$	$8,00 \pm 0,09^{aE}$	$9,88 \pm 0,05^{aA}$	$8,49 \pm 0,05^{aD}$	$7,92 \pm 0,02^{aE}$	$8,64 \pm 0,01^{aCD}$
35	$7,95 \pm 0,44^{bA}$	$8,35 \pm 0,04^{Ba}$	$8,31 \pm 0,02^{aA}$	$6,56 \pm 0,01^{bBC}$	$6,95 \pm 0,11^{bB}$	$7,96 \pm 0,02^{bA}$	$6,36 \pm 0,04^{bC}$	$8,05 \pm 0,05^{bA}$
63	$3,94 \pm 0,14^{cE}$	$5,93 \pm 0,23^{Cc}$	$7,20 \pm 0,17^{bB}$	$6,23 \pm 0,05^{cC}$	$5,41 \pm 0,04^{cD}$	$7,41 \pm 0,06^{cB}$	$2,66 \pm 0,10^{cF}$	$8,12 \pm 0,02^{bA}$
APPLE JUICE								
Time (d)	CL(M10)	C(M10)	T1(M10)	T2(M10)	CL(M30)	C(M30)	T1(M30)	T2(M30)
0	$7,01 \pm 0,09^{aE}$	$9,05 \pm 0,08^{Aa}$	$7,11 \pm 0,25^{aE}$	$8,44 \pm 0,02^{aB}$	$8,34 \pm 0,03^{aBC}$	$8,10 \pm 0,09^{aC}$	$7,69 \pm 0,04^{aD}$	$7,50 \pm 0,02^{aD}$
35	$5,95 \pm 0,02^{bD}$	$8,27 \pm 0,34^{bA}$	$5,27 \pm 0,08^{bE}$	$5,31 \pm 0,27^{bE}$	$6,57 \pm 0,11^{bC}$	$5,00 \pm 0,15^{bF}$	$7,68 \pm 0,10^{aB}$	$6,63 \pm 0,02^{bC}$
63	$3,92 \pm 0,03^{cC}$	$6,10 \pm 0,13^{Ca}$	$2,40 \pm 0,17^{cE}$	$3,32 \pm 0,11^{cD}$	$4,01 \pm 0,19^{cC}$	$5,00 \pm 0,22^{bB}$	$5,09 \pm 0,19^{bB}$	$3,36 \pm 0,04^{cD}$

Each value is the mean ± SD of experiments performed in triplicate.
Means followed by the same letter, uppercase on the line and lowercase on the column, do not differ statistically from each other by the Tukey test at 5% significance. CL(L10) – Free cell at 10% concentration, CL(L30) – Free cell at 30% concentration, C(L10) - Microcapsules without crosslinking at 10% concentration, C(L30) – Microcapsules without crosslinking at 30% concentration, T1(L10) – Treatment 1 at 10% concentration, T1(L30) – Treatment 1 at 30% concentration, T2(L10) – Treatment 2 at 10% concentration, T2(L30) – Treatment 2 at 30% concentration, CL(M10) – Free cell at 10% concentration, CL(M30) – Free cell at 30% concentration, C(M10) - Microcapsules without crosslinking at 10% concentration, C(M30) – Microcapsules without crosslinking at 30% concentration, T1(M10) – Treatment 1 at 10% concentration, T1(M30) – Treatment 1 at 30% concentration, T2(M10) – Treatment 2 at 10% concentration, T2(M30) – Treatment 2 at 30% concentration.

viability in both fruit juices (9–10.5 log CFU mL^{-1}).

In addition, the results found for the crosslinked microcapsules were initially similar to those found for the microcapsules without crosslinking, with no significant differences ($p > 0.05$). However, over the storage period, crosslinking proved to be essential to prolong probiotic viability, compared with other treatments. This is a key point, because until now, the protection of probiotic bacteria in microcapsules of gelatin and gum Arabic formed by the complex coacervation technique associated with crosslinking, and the subsequent application in fruit juices is an innovative alternative. Thus, the crosslinking process was proven adequate in association with complex coacervation, ensuring probiotic viability. This result was corroborated by Da Silva et al. (2019), who used crosslinking with transglutaminase to improve the resistance of microcapsules containing *L. acidophilus* produced by complex coacervation, as well as protection against probiotics. The authors observed that *L. acidophilus* remained viable for 60 days in cold storage.

This protection, initially similar to microencapsulation without crosslinking ($p > 0.05$), may have occurred due to some limiting factors in relation to transglutaminase, such as the amount of enzyme used, the complexation of gum Arabic and gelatin, the gelation state of gelatin, and consequently, its low mobility in the temperature of the crosslinking reaction process. The formation of a discontinuous protein network after enzymatic crosslinking, characteristic of films composed of gelatin and treated with transglutaminase, can also be taken into account to justify this behavior (Prata, Zanin, Ré, & Grosso, 2008). Furthermore, according to Prata et al. (2008) and Lv, Yang, Li, Zhang, and Abbas (2014), crosslinking by transglutaminase is considered weak and is not as effective in controlling the release rate. However, it can be observed that crosslinking with transglutaminase was efficient in our work, with emphasis on Treatment 2 at a concentration of 30% in orange juice, as it protected the probiotics until the end of the storage period (63 days), with very high viability (8.12 log CFU mL^{-1}). This result is in accordance with the multivariate cluster analysis that defined this treatment as the most suitable for probiotic viability (Fig. 1).

It is still possible to point out that, in orange juices, with the exception of Treatment 1 at a concentration of 30%, microcapsules were efficient in protecting *L. acidophilus*, ensuring their survival for 63 days, with significant differences ($p < 0.05$) and that although Treatment 1 did not maintain probiotic viability for 63 days, it was able to protect probiotics for up to 35 days, with 6.36 log CFU mL^{-1}. In contrast, in apple juices, only the treatment without crosslinking at a concentration of 10% was able to maintain probiotic viability until the end of storage (63 days), with a viability of 6.10 log CFU mL^{-1}, showing a significant difference ($p < 0.05$) (Table 2).

In addition, in relation to the concentrations of *L. acidophilus* added to fruit juices, we can emphasize that the increase in concentration is not linked to the increase in probiotic viability, with no significant differences ($p > 0.05$) and that a concentration of 10% would already be sufficient for the supply of probiotics in the amount stipulated by the legislation (>6 log CFU mL^{-1}) so that the consumer obtains the benefits derived from the probiotics (Brasil, 2008). However, in apple juice, the higher concentration of probiotics associated with crosslinking (Treatment 1) favored the increase in probiotic viability, with a significant difference ($p < 0.05$), resulting in 7.68 log CFU mL^{-1} in 35 days of storage (Table 2).

3.2. Comparisons: Multivariate analysis

Multivariate statistical cluster analysis has been widely used to develop taxonomies, in order to organize the data observed in significant structures (Francisco, Neves, Jacob-Lopes, & Franco, 2010).

Fig. 1 shows the probiotic viability dendogram for the different treatments in fruit juices. The dendogram shows two sets of clusters that are visibly apparent: cluster 1 (CL(L10), CL(M10), CL(M30), CL(L10), T2 (M10), T2(M30), T1(L30), T1(M10)) and cluster 2 (C(L10), T2(L10), C (M10), CL(L30), C(M30), T1(M30), (T1(L10), C(L30), (T2(L30). However, in cluster 1 it is possible to observe the formation of three subclusters: subcluster 1 (CL(L10), CL(M10), CL(M30)), subcluster 2 (T2 (M10), T2(M30)), and subcluster 3 (T1(L30), T1(M10)), and in cluster 2, two subclusters are formed: subcluster 4 (C(L10), T2(L10), C(M10), (CL (L30), C(M30), T1(M30)) and subcluster 5 (T1(L10), C(L30), (T2(L30)).

Thus, it was observed with multivariate cluster analysis that treatment 2 at a concentration of 30% in orange juice (T2(L30)) has some characteristic that clearly differentiates it from the others in relation to viability. Thus, this characteristic is related to the greater viability of probiotics found for this treatment (T2(L30)) until the end of the storage

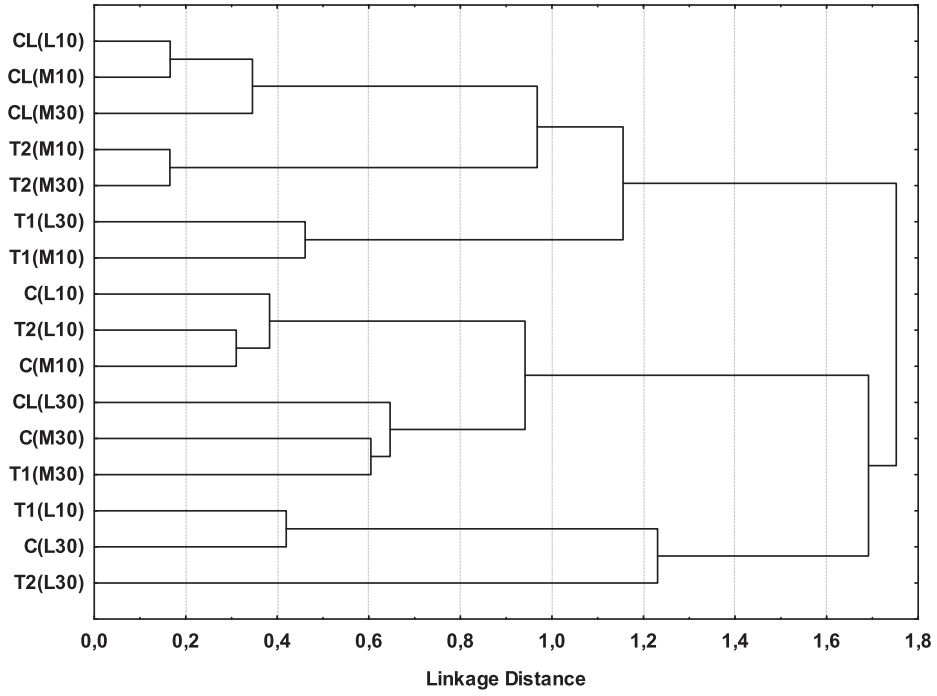

Fig. 1. Cluster dendrogram for probiotic viability on the 63rd day of storage at 4 °C in different fruit juices. CL(L10) – Free cell at 10% concentration in orange juice, CL(L30) – Free cell at 30% concentration in orange juice, C(L10) - Microcapsules without crosslinking at 10% concentration in orange juice, C(L30) – Microcapsules without crosslinking at 30% concentration in orange juice, T1(L10) – Treatment 1 at 10% concentration in orange juice, T1(L30) – Treatment 1 at 30% concentration in orange juice, T2(L10) – Treatment 2 at 10% concentration in orange juice, T2(L30) – Treatment 2 at 30% concentration in orange juice, CL(M10) – Free cell at 10% concentration in apple juice, CL(M30) – Free cell at 30% concentration in apple juice, C (M10) - Microcapsules without crosslinking at 10% concentration in apple juice, C(M30) – Microcapsules without crosslinking at 30% concentration in apple juice, T1(M10) – Treatment 1 at 10% concentration in apple juice, T1(M30) – Treatment 1 at 30% concentration in apple juice, T2(M10) – Treatment 2 at 10% concentration in apple juice, T2 (M30) – Treatment 2 at 30% concentration in apple juice.

period, as can be seen in Table 2. However, when the subclusters are compared, Treatment 1 at a concentration of 10% in orange juice (T1 (L10) and treatment with microcapsules without crosslinking at a concentration of 30% in orange juice (C(L30) can also be considered adequate for probiotic viability because they are similar to the T2(L30).

3.3. Optical microscopy of microcapsules in fruit juices during storage

Regarding the optical microscopy of the microcapsules in the different treatments (Figs. 2 and 3), we can observe that the microcapsules were multinucleated, with rounded shapes, of varying sizes, being smaller when crosslinked, in some cases, in both fruit juices. According to Rojas-Moreno, Osorio-Revilla, Gallardo-Velázquez, Cárdenas-Bailón, and Meza-Márquez (2018), the coacervate layer is formed around oil globules, leading to the formation of multinucleated capsules, and the same behavior is observed for probiotics. Also, according to these authors, some microcapsules are large, round in shape and have a smooth surface, and some particles are apparently crushed and smaller, which is also in line with our results. Furthermore, it reinforces that the behavior was similar for treatments with and without crosslinking.

However, according to Prata et al. (2008) the crosslinking process reduces the mobility of macromolecular chains, decreasing the swelling capacity of the reticulated microcapsules in relation to microcapsules without crosslinking. This justifies the smaller sizes observed in the present work for the reticulated microcapsules.

In addition, in orange juice, the microcapsules were only broken in Treatment 1 at a concentration of 30% during the 63 days of storage (Fig. 2J); this result was in agreement with the viability analysis in which the probiotics remained viable for 35 days under these conditions.

As for apple juice, it was possible to observe a similar behavior in all treatments studied: in 63 days of storage, the amount of microcapsules was reduced considerably, and it was not possible to detect broken microcapsules (Fig. 3), indicating their total dispersion in apple juice, with consequent loss of probiotic viability, as shown in the viability analysis (Table 2).

3.4. pH and TSS content of fruit juices during storage

The variations in pH and TSS in orange and apple juices containing *L. acidophilus* in the free and microencapsulated forms during the storage period of 63 days are described in Table 3.

According to Rodrigues et al. (2012), changes in pH values, with significant differences ($p < 0.05$), during the storage of fruit juices occur due to the presence of sugars, which can be fermented by *L. acidophilus* in both forms, free and microencapsulated. According to Sohail et al. (2012), probiotic bacteria ferment sugars, producing organic acids and, as a consequence of this fermentation process, the pH is reduced. In addition, according to Rodrigues et al. (2012), the variations are also related to the metabolic activity of probiotics. In this sense, we can see that in orange juices the pH reductions were more pronounced, indicating that there was no metabolic inactivation of the probiotics. This fact is also justified by the feasibility analysis, which showed high survival of probiotics during storage (Table 2).

On the other hand, in apple juices, we observed smaller variations in pH, which were related to the metabolic inactivation of most probiotic bacteria, the same result being observed in the probiotic viability analysis (Table 2).

In addition, the greatest pH variations were also observed in treatments with the addition of free cells, indicating that microcapsules were efficient in reducing the interactions of probiotics with the environment of fruit juices, highlighting the importance of microencapsulation in probiotic viability. We can also point out that the crosslinking process proved to be adequate in this sense, as it was in the treatments with the addition of reticulated microcapsules that the smallest pH reductions were observed, with some exceptions that will be discussed below.

In orange juices, we can highlight Treatment 2 at a concentration of 30%, which showed the greatest pH reductions, indicating the intense metabolic activity of probiotics, corroborating the justifications previously presented by Rodrigues et al. (2012) and Sohail et al. (2012), together with the high cell counts in the feasibility analysis (Table 2). The same is valid for Treatment 1 at a concentration of 30%, in which the pH variations did not show significant differences ($p > 0.05$), indicating the metabolic inactivation of probiotics, which can also be justified by viability analysis.

According to the literature, pH is the most relevant factor for probiotic viability, however, factors such as titratable acidity, molecular oxygen, water activity, presence of salt, sugar and chemicals, such as hydrogen peroxide, bacteriocins, flavorings and artificial colors can influence the survival of probiotics in fruit juices (Perricone, Bevilacqua, Altieri, Sinigaglia, & Corbo, 2015). Furthermore, as mentioned earlier, pH is the main factor in fruit juices related to probiotic viability. Thus, the pH of apple juice is not adequate for *L. acidophilus* and interferes with its survival until the end of the storage period.

With regard to TSS analysis, for free cells at concentrations of 10% and 30%, for both fruit juices, in general, there was a reduction in SST (Table 3), with significant differences ($p < 0.05$), which was expected due to the fermentation of sugars present in fruit juices by *L. acidophilus*. Hruyia, Deshpande, and Bhate (2018) also observed more marked reductions with the addition of different species of *Lactobacillus* in sweet orange juice. However, for treatments with the addition of 10% microcapsules, the TSS showed an increase during the storage period. However, for treatments with the addition of 30% microcapsules, the SST showed reductions during the storage period. These results were observed in both fruit juices, orange and apple, and are in accordance with Calabuig-Jiménez et al. (2019), who observed reductions in TSS when more microcapsules were added to tangerine juice.

4. Conclusion

This study showed that the complex coacervation technique associated with crosslinking with transglutaminase is adequate for the protection of *L. acidophilus* in fruit juices. In apple juice, probiotics were inactivated when added in free form; thus, microencapsulation associated with crosslinking provided an increase in probiotic viability. In orange juice, microencapsulation also showed satisfactory results, as only microencapsulated probiotics were able to survive for 63 days, showing high viability. In addition, orange juice provided more suitable conditions for the survival of probiotics, highlighting pH as a determining factor for probiotic viability.

In addition, even if the microcapsules do not appear to interfere with the quality attributes (color, aroma, flavor, etc.) of fruit juices, sensory studies and microbiological evaluation are considered important and will be carried out subsequently. It is also worth noting that they were not realized because they are not within the proposed objective, but their need is recognized.

Furthermore, it is worth highlighting the pioneering nature of the present study, since the addition of microencapsulated probiotics by complex coacervation associated with transglutaminase crosslinking in fruit juices has not been reported until now.

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CRediT authorship contribution statement

Thaiane Marques Silva: Conceptualization. **Vandré Sonza Pinto:** Resources. **Vítor Ramires Fonseca Soares:** Resources. **Débora Marotz:** Resources, Methodology. **Alexandre José Cichoski:** Methodology. **Leila Queiroz Zepka:** Methodology. **Eduardo Jacob Lopes:** Methodology, Formal analysis. **Cristiane Bona Silva:** Methodology, Investigation. **Cristiano Ragagnin Menezes:** Project administration.

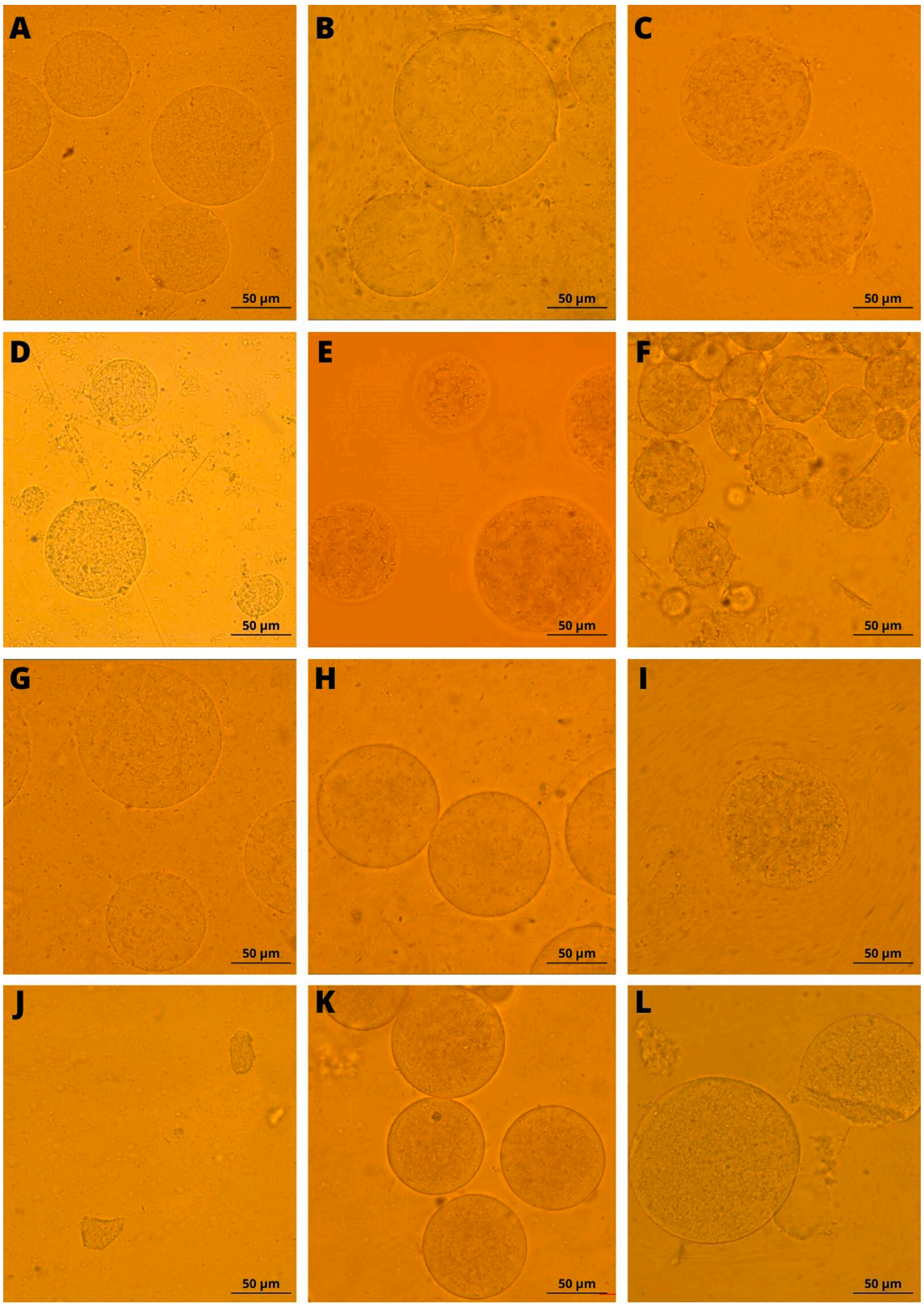

Fig. 2. Optical microscopy of microcapsules in orange juice in storage for 63 days at 4 °C. A) Microcapsules without crosslinking at 10% concentration (day 0) 20x B) Microcapsules without crosslinking at 10% concentration (day 63) 40x C) Treatment 1 at 10% concentration (day 0) 40x D) Treatment 1 at 10% concentration (day 63) 40x E) Treatment 2 at 10% concentration (day 0) 40x F) Treatment 2 at 10% concentration (day 63) 40x G) Microcapsules without crosslinking at a concentration of 30% (day 0) 40x H) Microcapsules without crosslinking at 30% concentration (day 63) 40x I) Treatment 1 at 30% concentration (day 0) 40x J) Treatment 1 at 30% concentration (day 63) 40x K) Treatment 2 at 30% concentration (day 0) 40x L) Treatment 2 at 30% concentration (day 63) 40x. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

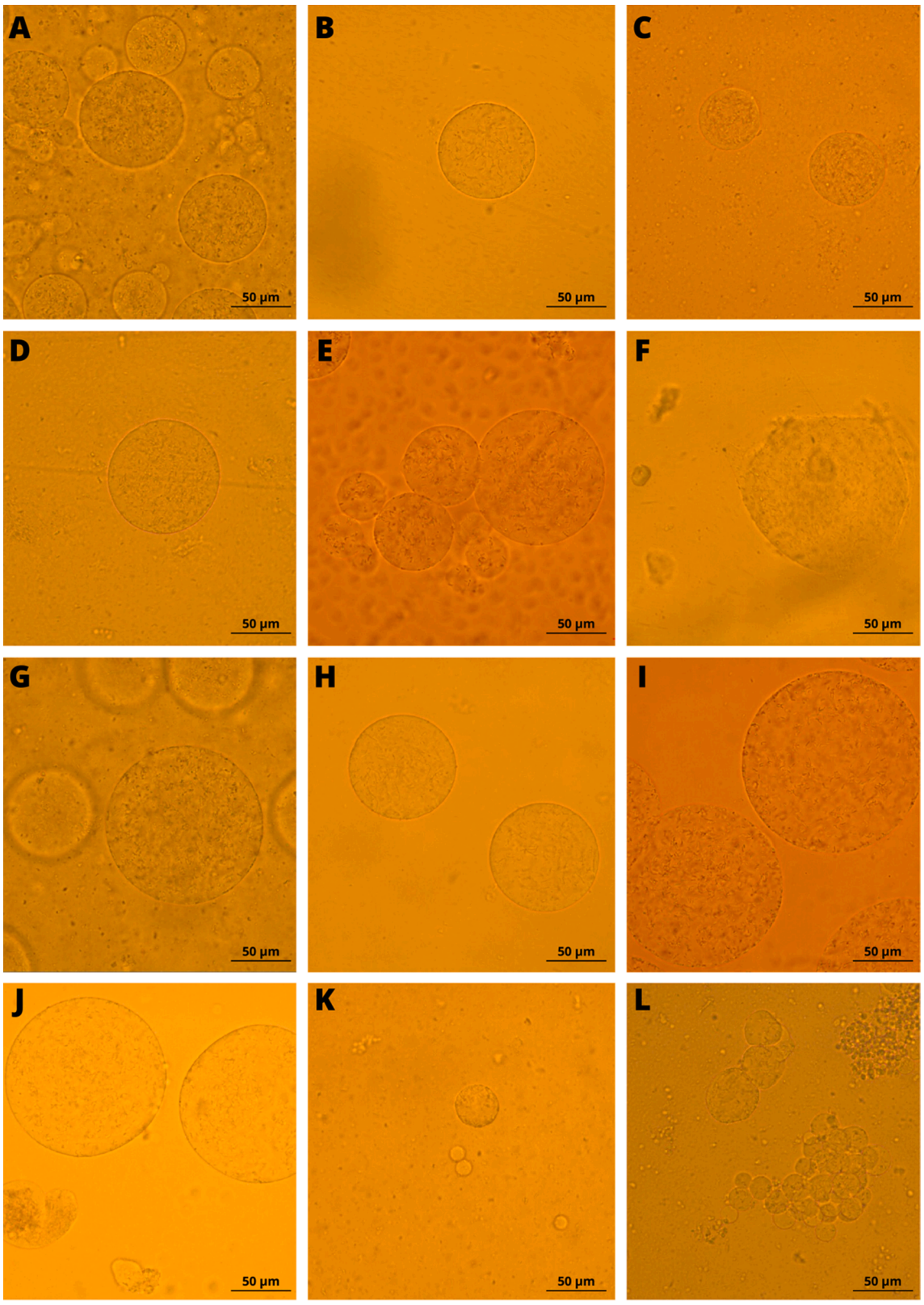

Fig. 3. Optical microscopy of microcapsules in apple juice in storage for 63 days at 4 °C. A) Microcapsules without crosslinking at 10% concentration (day 0) 40x B) Microcapsules without crosslinking at 10% concentration (day 63) 40x C) Treatment 1 at 10% concentration (day 0) 40x D) Treatment 1 at 10% concentration (day 63) 40x E) Treatment 2 at 10% concentration (day 0) 40x F) Treatment 2 at 10% concentration (day 63) 40x G) Microcapsules without crosslinking at a concentration of 30% (day 0) 40x H) Microcapsules without crosslinking at 30% concentration (day 63) 40x I) Treatment 1 at 30% concentration (day 0) 40x J) Treatment 1 at 30% concentration (day 63) 40x K) Treatment 2 at 30% concentration (day 0) 40x L) Treatment 2 at 30% concentration (day 63) 40x.

Table 3
pH and TSS results obtained from fruit juices during storage for 63 days at 4 °C.

Orange juice										
Time (days)	L		CL(L10)		C(L10)		T1(L10)		T2(L10)	
	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS
0	3,99 ± 0,01^{c}	16,50 ± 0,01^{c}	3,98 ± 0,01^{a}	16,75 ± 0,01^{c}	4,10 ± 0,01^{a}	17,75 ± 0,01^{b}	4,05 ± 0,01^{a}	16,50 ± 0,01^{c}	4,07 ± 0,01^{a}	16,75 ± 0,01^{c}
35	4,09 ± 0,01^{a}	18,55 ± 0,01^{a}	3,89 ± 0,01^{b}	17,62 ± 0,01^{a}	3,88 ± 0,03^{b}	17,75 ± 0,01^{b}	4,03 ± 0,01^{b}	17,25 ± 0,01^{b}	4,02 ± 0,01^{b}	16,80 ± 0,01^{b}
63	4,02 ± 0,01^{b}	18,00 ± 0,01^{b}	3,86 ± 0,01^{c}	17,00 ± 0,01^{b}	3,88 ± 0,01^{b}	17,80 ± 0,01^{a}	3,99 ± 0,01^{c}	17,75 ± 0,01^{a}	3,93 ± 0,01^{c}	17,75 ± 0,01^{a}
	L		CL(L30)		C(L30)		T1(L30)		T2(L30)	
	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS
0	3,99 ± 0,01^{c}	16,50 ± 0,01^{c}	3,85 ± 0,01^{b}	16,00 ± 0,01^{b}	3,98 ± 0,01^{b}	17,65 ± 0,01^{a}	4,07 ± 0,01^{a}	15,75 ± 0,01^{c}	4,05 ± 0,01^{a}	14,75 ± 0,01^{b}
35	4,09 ± 0,01^{a}	18,55 ± 0,01^{a}	3,80 ± 0,01^{c}	16,50 ± 0,01^{a}	3,95 ± 0,02^{b}	17,17 ± 0,01^{b}	4,03 ± 0,01^{b}	16,75 ± 0,01^{a}	3,75 ± 0,01^{b}	15,50 ± 0,01^{a}
63	4,02 ± 0,01^{b}	18,00 ± 0,01^{b}	3,93 ± 0,01^{a}	14,75 ± 0,01^{c}	4,01 ± 0,01^{a}	15,75 ± 0,01^{c}	4,06 ± 0,01^{a}	16,50 ± 0,01^{b}	3,67 ± 0,01^{c}	15,50 ± 0,01^{a}
Apple juice										
Time (days)	M		CL(M10)		C(M10)		T1(M10)		T2(M10)	
	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS
0	3,65 ± 0,01^{a}	16,50 ± 0,01^{c}	3,65 ± 0,01^{a}	16,50 ± 0,01^{c}	3,68 ± 0,01^{a}	16,00 ± 0,01^{c}	3,84 ± 0,01^{a}	16,00 ± 0,01^{c}	3,70 ± 0,01^{a}	16,50 ± 0,01^{c}
35	3,64 ± 0,01^{a}	18,75 ± 0,01^{b}	3,62 ± 0,02^{a}	18,75 ± 0,01^{a}	3,67 ± 0,01^{a}	16,75 ± 0,01^{b}	3,78 ± 0,01^{b}	16,40 ± 0,01^{b}	3,71 ± 0,01^{a}	17,80 ± 0,01^{a}
63	3,55 ± 0,01^{b}	18,25 ± 0,01^{a}	3,51 ± 0,01^{b}	17,50 ± 0,01^{b}	3,64 ± 0,03^{a}	18,00 ± 0,01^{a}	3,72 ± 0,01^{c}	16,50 ± 0,01^{a}	3,72 ± 0,02^{a}	17,65 ± 0,01^{b}
	M		CL(M30)		C(M30)		T1(M30)		T2(M30)	
	pH	TSS	pH	TSS	pH	TSS	pH	TSS	pH	TSS
0	3,65 ± 0,01^{a}	16,50 ± 0,01^{c}	3,63 ± 0,02^{a}	17,65 ± 0,01^{a}	3,64 ± 0,01^{b}	16,50 ± 0,01^{b}	3,65 ± 0,01^{b}	16,25 ± 0,01^{a}	3,70 ± 0,01^{b}	17,20 ± 0,01^{a}
35	3,64 ± 0,01^{a}	18,75 ± 0,01^{a}	3,51 ± 0,01^{b}	17,55 ± 0,01^{b}	3,65 ± 0,01^{b}	16,50 ± 0,01^{b}	3,77 ± 0,01^{a}	16,25 ± 0,01^{a}	3,68 ± 0,01^{b}	16,75 ± 0,01^{b}
63	3,55 ± 0,01^{b}	18,25 ± 0,01^{b}	3,63 ± 0,01^{a}	16,50 ± 0,01^{c}	3,71 ± 0,01^{a}	17,25 ± 0,01^{a}	3,78 ± 0,01^{a}	16,20 ± 0,01^{b}	3,74 ± 0,01^{a}	16,25 ± 0,01^{c}

Each value is the mean ± SD of experiments performed in triplicate.
Means followed by the same letter in the column do not differ statistically from each other by the Tukey test at the level of 5% significance.
L – Orange juice, CL(L10) – Free cell at 10% concentration, CL(L30) – Free cell at 30% concentration, C(L10) - Microcapsules without crosslinking at 10% concentration, C(L30) – Microcapsules without crosslinking at 30% concentration, T1(L10) – Treatment 1 at 10% concentration, T1(L30) – Treatment 1 at 30% concentration, T2(L10) – Treatment 2 at 10% concentration, T2(L30) – Treatment 2 at 30% concentration, M – Apple juice, CL(M10) – Free cell at 10% concentration, CL(M30) – Free cell at 30% concentration, C(M10) - Microcapsules without crosslinking at 10% concentration, C(M30) – Microcapsules without crosslinking at 30% concentration, T1 (M10) – Treatment 1 at 10% concentration, T1(M30) – Treatment 1 at 30% concentration, T2(M10) – Treatment 2 at 10% concentration, T2(M30) – Treatment 2 at 30% concentration.

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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