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Co-encapsulation of probiotic bacteria *L. rhamnosus* GG and β-carotene by a novel biphasic encapsulation technique: Stability and *in vivo* anti-inflammatory properties

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ABSTRACT

In this study, β-carotene (βC) was encapsulated in liposomes and spray-dried with the probiotic bacteria *Lactobacillus rhamnosus* GG (LGG), to obtain a biphasic structure with two functional components. Initially, the liposomes loaded with βC resulted in large multivesicular vesicles (LMVV) with spherical morphology, mean size of 1191 nm and entrapment efficiency of 81.33%. Then, the spray-drying of the mixture βC-LMVV with the LGG resulted in biphasic dried microparticles (BDM) with a spherical shape, retention of 64,48% of βC, and LGG survivor above 90%. The BDM showed high storage stability for 90 days at room temperature and, at the dose of 2000 mg/kg of BDM did not cause any acute toxicity in *Wistar* rats. In addition, at the same dose, presented significant anti-inflammatory activity in carrageenan-induced paw edema and pleurisy. Thus, the produced BDM could be an innovative ingredient with functional properties and also an efficient encapsulation strategy for βC.

1. Introduction

The acknowledgment of foods containing probiotics as functional foods that benefit the host's health beyond basic nutrition, and the emerging evidence of their potential in disease prevention have encouraged the dissemination and consumption of these products (Damián et al., 2022). However, producing foods containing specific probiotic strains in the required concentrations remains challenging due to heat, pH changes, and oxygen, which greatly reduces their viability during processing, storage, distribution, and gastrointestinal transit (Sun et al., 2023). Therefore, effective encapsulation systems can increase the tolerance of probiotic bacteria to environmental stresses. The main approach used for this purpose is polymer encapsulation, which can provide stability to probiotics, extend their survival, and protect them from exogenous factors such as high pressure, temperature changes, and oxidative environment (Manojlović et al., 2010).

Recent works have shown that co-encapsulation of bioactive compounds and probiotics in a single structure can enhance the individual ingredient's bioactivity, providing synergistic health benefits, lower cost, and improved long-term storage (Misra et al., 2021). In this regard, the co-encapsulation of probiotic bacteria with nutraceuticals that can promote human health has been reported, including omega-3 unsaturated fatty acids, polyphenols, anthocyanins, and resveratrol (Enache et al., 2020; Eratte et al., 2017; Gaudreau et al., 2016; Vazquez-Maldonado et al., 2020). Among the existing methods, microencapsulation of bioactive compounds and probiotics by the spray-drying technique is a viable alternative that provides physical protection to the probiotics against gastrointestinal (GI) conditions and during long-time storage (Colín-Cruz et al., 2019; Vazquez-Maldonado et al., 2020).

Beta-carotene (βC) is one of the most relevant provitamin A sources. It presents an antioxidant capacity that can reduce free radicals, which have the potential to damage bodily tissues accelerating the aging process and increasing the risks of several diseases (Kasperczyk et al., 2014). However, due to its hydrophobicity, βC presents low bioavailability after ingestion (Han et al., 2019). As an alternative, to improve the solubility and bioavailability efficiency of βC, micro and

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nanoencapsulation techniques including aerogels (Zhang et al., 2023), amylose microparticles (Letona et al., 2017) nanofibers (Yildiz et al., 2023), have been used as viable options for overcoming these drawbacks and others, related to adding carotenoids to foods. Among the different options of encapsulation, liposome encapsulation is an interesting approach to improve βC bioavailability, due to βC has a hydrophobic character and a partition coefficient of 17.62 log P (Octanol/H_2O), showing high compatibility with lipid biomembranes (Tan et al., 2014). Nano-colloidal delivery systems such as liposomes are lipophilic and stable structures that prevent the isomerization of β-carotene, produced during a gastric environment (Rovoli et al., 2018). The βC encapsulation into liposomes using different techniques has been reported by several researchers (Ji et al., 2023; Liu et al., 2020; Wang et al., 2023). In addition, it has been reported that spray-dried liposomes loaded with βC had good stability during storage, and the powder presented rapid dispersion after rehydration (Moraes et al., 2013). Liu et al. (2020), reported that the liposome co-encapsulation of βC and vitamin C increased the storage stability of βC and provide protection of the bioactive compounds during simulated stomach conditions, which could result in their effective release in the small intestine.

Conventional liposomes undergo physicochemical changes during processing and GI digestion through their interaction with food components and other exogenous factors which limit their industrial application, however, delaying this interaction, which can be achieved by surface modification or additional polymeric coating, is of great importance for functional liposomes development (Liu et al., 2019). Therefore, the use of polymeric microencapsulation as additional protection for the liposomes would contribute positively to stabilizing the liposome cargo, and at the same time stabilize the polymeric structure of the microparticle, which can result in better retention of other functional components such as probiotic bacteria or bioactive compounds present in the formulation, allowing the production of "multi-active" microparticles, and expanding the applications of this system in functional and nutraceutical therapy (Chai et al., 2018).

In this paper, βC and the probiotic bacteria *Lacticaseibacillus rhamnosus* GG, which demonstrated anti-inflammatory effects (Pagnini et al., 2018), were co-encapsulated in a dual-step method. First, βC was loaded in liposomes by freeze-thawing method, these liposomes were characterized by particle size, polydispersity index (PDI), zeta-potential (ZP), and Cryo-transmission electron microscope (Cryo-TEM). Second, a mixture of βC loaded liposomes and viable probiotics cells was spray-dried using whey as wall material. Then, the βC stability and bacterial viability in the microparticles were studied for 240 days. Finally, the toxicity and anti-inflammatory effects of the biphasic microparticles were evaluated *in vivo*.

2. Materials and methods

2.1. Preparation of beta-carotene liposomes

The βC loaded liposomes were prepared by using the freeze-thawing method. Briefly, the lipids Soy phosphatidylcholine (Avanti Polar Lipids, Alabaster, USA) 19 mM, cholesterol 3 mM, and beta-carotene (Sigma Aldrich, St. Louis, USA) 5 μM, were dispersed in chloroform. After the lipids and βC solubilization, the chloroform was evaporated under a nitrogen atmosphere to form a lipid film. Then, the flasks were subjected to vacuum drying for 24 h in a desiccator to remove the organic solvent completely. After removing the solvent, the lipid film was resuspended in Hepes buffer (60 mM) with a pH of 7.2, under constant agitation to obtain multilamellar vesicles (MLVs). Once formed, the MLVs were subjected to 4 cycles of freezing and thawing in liquid nitrogen (−196 °C) and bath at 30 °C for the formation of large multivesicular liposomes (LMVL).

2.2. Characterization of liposomes

The LMVL with β-carotene ($LMVL_B$) were characterized by their mean diameter, polydispersion index (PDI), zeta potential (ζ), and particle concentration [particle/mL] right after preparation. A formulation of LMVL without beta-carotene was also produced as a control. Measurements of mean diameter, PDI and zeta potential were taken in triplicate at 25 °C, by dynamic light scattering, in a ZetaSizer equipment (Zeta ZS90, Malvern). Particle concentration was measured in triplicate at 25 °C and determined using particle tracking analysis, NTA, (NanoSight NS300, Malvern) (Freitas de Lima et al., 2021).

2.2.1. Encapsulation efficiency of liposomes

The encapsulation efficiency (EE) of βC in the $LMVL_B$ was determined by high-performance liquid chromatography (HPLC) as previously described by Freitas de Lima et al., (2018). Before quantification by HPLC, 1 mL of liposomes were centrifuged at 4 °C for 30 min at 14,000 *g*, the supernatant was discarded, replaced by 500 μl of hexane, and agitated until the complete release of βC from the interior of the liposomes, finally, the same volume of 60 mM Hepes buffer (pH 7.4) was added. The solution was centrifuged under the same conditions mentioned above, and the supernatant containing the βC released from the liposomes was collected for its determination by HPLC.

βC concentrations were analyzed on an HPLC (Waters 2707) using a C18-reverse-phase column (250 × 4.6 mm), detection at 470 nm, with 20 μL injection, 0.70 mL min^{-1} of isocratic flow, and 25 °C. The mobile phase was a mixture of acetonitrile and ethyl acetate. The encapsulation efficiency (EE) of beta-carotene was determined according to equation (1):

$$\%EE = \frac{E}{U}\, x\, 100 \qquad \text{Eq(1)}$$

Where E is the concentration of encapsulated beta-carotene and U is the total concentration of beta-carotene introduced into the system.

2.2.2. Electronic microscopy

The morphological characteristics of the liposomes were observed by cryogenic electron microscopy (Cryo-TEM) as previously reported by Freitas de Lima et al., (2021), using a Krios G3i Cryo-TEM system (Thermo Scientific). For Cryo-TEM, the grids (mesh 300, carbon) were observed in a light discharge procedure (15 mA for 10 s). Then, the grids were inserted into a Vitrobot® and 3 μL of the sample was added, leading 20 s for sample fixation. After drying the sample excess with a negative transfer force (transfer force = −3), the samples were rapidly dipped into liquid ethane surrounded by a liquid nitrogen environment. The photomicrographs were measured at 200 kV with the TEM imaging system.

2.3. Probiotic strain and culture conditions

For the cultivation of, *Lacticaseibacillus rhamnosus* GG ATCC 53103 (LGG®, from Chr. Hansen) was used the MRS broth (De Man Rogosa Sharp). The lyophilized commercial strain was activated (1%) in MRS broth at 37 °C/16 h. After growth, was centrifuged at 4677 *g* at 4 °C for 12 min, the supernatant was discarded, and the cell pellet was resuspended and washed twice with saline (0.85%). This cell concentrate containing approximately 10 Log CFU/mL was dispersed in the encapsulating solutions.

2.4. Preparation and characterization of bifunctional microparticles by spray-drying

$LMVL_B$ freshly prepared and probiotic bacteria LGG were microencapsulated using cheese whey powder (13.5% w/w of protein, 0.5% fat, and 83% of carbohydrates) (Alibra, Campinas, Brazil) as wall

material. The formulation contained 768 mL of LMVLB, 58 g of whey protein concentrate, 20 mL of cell suspension of LGG with around 10 log CFU/mL, and 155 mL of distilled water. This mixture was homogenized in a magnetic stirrer and divided into two parts, to evaluate two different drying temperatures. The drying process was tested for the inlet temperatures of 130 °C and 170 °C in a spray dryer (B290, Büchi, Switzerland). The flow rate was adjusted to 75 °C via the aspirator and pump parameters (Alves Gragnani Vido et al., 2023). The diameter of the atomization nozzle was 0.7 mm, and the compressed air flow was 600 L/h. In this way, biphasic dried microparticles (BDM) containing βC and the LGG were produced.

Samples were collected to determine the viability of LGG cells in the feed suspension before and after spray drying. The dried powders obtained were subjected to analyses of moisture content, water activity (Aw), particle size distribution, content of βC, and morphological analysis. The rest of the powder was stored in fully sealed plastic vials, in the dark at 25 °C, for the stability test.

2.4.1. Size, polydispersity (PDI), moisture and water activity (Aw)

The size distribution of the dried BDM was evaluated by laser diffraction using LA960 equipment (Horiba, Japan). For this, an aliquot of each sample was previously dispersed in absolute ethanol and added to the equipment reading chamber containing the same dispersing medium until adequate transmittance levels were reached for the measurements (Alves Gragnani Vido et al., 2023). The A_w was determined using a digital water activity meter (AquaLab®), and the moisture was determined by direct drying in a laboratory oven at 105 °C for 24 h.

2.4.2. βC encapsulation efficiency in dried microparticles

The EE of βC in the BDM was carried out following the protocol described above for the $LMVL_B$. In this case, 1 g of powder was solubilized in hexane to completely remove the encapsulated beta-carotene, centrifuged (11,000 *g*, 20 min and 4 °C) and the supernatant was collected for HPLC analysis. The values of βC obtained in the liquid formulation (wall materials, LMVLB and LGG) before the spray-drying process were analyzed using equation (1) with the values of βC in the powder after drying. Values were expressed as EE% of βC in the BDM.

2.4.3. L. rhamnosus GG cell viability

The viability of LGG after microencapsulation by drying and during the store time was determined via the agar plate culturing technique. For this, 1 g of BDM powder was serially diluted in a peptone solution (0.1%) and cultured on MRS agar plates. Then, the agar plates were incubated at 37 °C for 48h in anaerobic conditions. Bacterial counts were expressed in Log of CFU/g. The survival rate after spray drying was calculated by the number of viable cells according to follow equation:

$$SR\% = \left(\frac{N}{N_0}\right).100 \qquad \text{Eq (2)}$$

SR = survival rate %; N_0 = number of viable LGG cells (Log CFU/mL) before the drying; N = number of viable LGG cells (Log CFU/mL) in the powder.

The BDM powders dried at 130 and 170 °C were placed in hermetically sealed plastic tubes, a part was stored at 4 °C for further characterization and *in vivo* tests, and the other part was stored at room temperature (25 °C) in the dark for the stability analysis were the SR% of LGG and βC EE was monitored for 240 days.

2.5. Toxicological evaluation and in vivo bioactive effect of microparticles

2.5.1. Animals

Female Wistar rats (*Rattus norvegicus*), 6–7 weeks old, with weights around 200 and 250 g supplied from the vivarium of the Federal University of Grande Dourados, were used for the acute toxicology experiment. Male Wistar rats *(Rattus norvegicus*), 8–9 weeks old, weighing between 300 and 350 g from the vivarium of the State University of Campinas were used for the paw edema and pleurisy experiments. The experiments were carried out following the Ethical Principles in Animal Research and approved by the Animal Experimentation Ethics Committee of the Federal Universities of Grande Dourados and State University of Campinas (protocols: September 2020 and 5637–1/2020, respectively).

2.5.2. In vivo acute toxicology

BDM were administered at a concentration of 2000 mg of microparticles by Kg animal (w/w), by gavage after fasting for 8 h (Awounfack et al., 2016). Sequentially, at intervals of 48 h, the same dose was administered to four adult rats, totaling five animals treated per group (BDM 2000 mg/kg group). A control group was treated with a saline solution and established as the comparative negative control group. Ten female animals were used for the experiment (n = 5 per group).

The animals were observed periodically in the first 24 h after BDM administration, and then, daily for 14 days. During the experiment the five parameters of Hippocratic screening were evaluated: state of consciousness; activity and coordination of the motor system; reflexes; activities in the central nervous system and activities in the autonomic nervous system. Body weight, water and food intake were also verified daily. At the end of the evaluation period (15 days), all animals were euthanized with anesthetic (isoflurane, inhalation) followed by exsanguination. The organs (heart, lung, spleen, liver, kidney, uterus, and right ovary) were removed, weighed, and examined macroscopically (Freitas de Lima et al., 2018).

2.5.3. Anti-inflammatory effect, paw edema and pleurisy

In the *in vivo* evaluation of carrageenan-induced paw edema, male *Wistar* rats were randomly distributed into five groups, 7 animals/group. Group 1 (carrageenan) was treated orally with vehicle (0.9% saline solution); Group 2 (indomethacin control) was treated orally with indomethacin (5 mg/kg); Group 3 (naive) was treated orally with vehicle (0.9% saline solution); Groups 4 and 5 were treated with 1000 and 2000 mg/kg BDM, respectively. The doses used in the study were stipulated in pre-tests and established within the non-toxic limits of daily doses. After 1 h, the animals received 100 μL of 0.9% saline solution containing 1% carrageenan in the right hind paw, except for the naive group that received saline solution. The same volume of saline was administered to the left hind paw. Edema was assessed in both paws at times of 0.5; 1; 2; 4 and 6 h after carrageenan injection, with the aid of a digital plethysmometer. The anti-inflammatory effect was evaluated and expressed through the volume of edema (mL) and inhibition (%) of edema induced by carrageenan (Freitas de Lima et al., 2018).

In the *in vivo* evaluation of carrageenan-induced pleurisy, male Wistar rats were randomly distributed into four groups, 7 animals/group. Group 1 (carrageenan) was treated orally with vehicle (0.9% saline solution); Groups 2 (indomethacin control) were treated orally with indomethacin (5 mg/kg); Group 3 (naive) was treated orally with vehicle (0.9% saline solution); Group 4 treated with 2000 mg/kg BDM. After 1 h, the animals received 100 μL of 0.9% saline solution containing 1% carrageenan in the pleural cavity, except for the naive group that received saline solution. After 4h, the animals were euthanized, and the leukocyte migration and total protein volume were measured; leukocyte counting was performed in an automatic cell counter and protein dosage was performed using the Bradford method. The anti-inflammatory effect was evaluated through the inhibition of leukocyte migration (neutrophils, basophils, lymphocytes and macrophages) and a decrease in protein volume, after pleurisy induction with carrageenan (Freitas de Lima et al., 2018).

2.6. Statistical analysis

The results are shown as the mean values and standard deviations. The reference and treatment groups were analyzed according to their

variance (ANOVA). Bonferroni and Tukey's tests were also applied considering a 95% confidence level ($p < 0.05$), using the software Statistica V.12.

3. Results and discussion

3.1. Production and characterization of liposomes produced by freeze-thawing

The particle size is a key property of any delivery system, due to this property is related to the surface-to-volume ratio and may influence the release kinetics of the active compound. (Brandelli et al., 2023). The values of mean diameter (MD), polydispersity (PDI) and zeta potential of $LMVL_C$ (control without βC) and $LMVL_B$ (with βC) are shown in Table 1. In $LMVL_C$ the MD values were 913.10 ± 63.66 nm, while for $LMVL_B$ the DM values were 1191.00 ± 50.66 nm. As expected, the LMVL presented high MD values, this result is due to the freeze-thawing method, which does not use high-energy homogenization systems or extrusion with membranes. Similar values of average size were reported by the liposome encapsulation of isoniazid in liposomes by the freeze-thawing method (Nkanga et al., 2017). The increase in particle size after a bioactive insertion is quite common for encapsulating systems, especially when we have a large portion of the active ingredient in the lipid bilayer (Couto et al., 2018). The polydispersity index (PDI) was 0.515 ± 0.036 for $LMVL_C$ and 0.458 ± 0.032 for $LMVL_B$, indicating a wide size particle distribution. Besides, PDI values between 0.2 and 0.3 are considered as optimal in the production of liposomes with narrow size distribution (Danaei et al., 2018), these values could vary according to the liposome production method. However, no aggregation was observed in the liposome suspension, indicating good colloidal stability, probably favored by the electrostatic repulsion between the LMVL.

The surface charge values were −24.20 ± 1.08 mV for $LMVL_C$ and −24.60 ± 0.27 mV for $LMVL_B$. Zeta potential (ζ) values remained negative even after βC encapsulation. Values of ζ far from zero are important for the physical stability of the formulations, as the surface charges of the liposomes repel each other, preventing the aggregation process (Lowry et al., 2016). These results indicate that loading the βC did not affect the surface charge of liposomes.

To complement the data from DLS and estimate particle concentration, particle tracking analysis (NTA) was used. As result, was obtained particle concentration (particles/mL) values of $0.70 \times 10^{13} \pm 0.01$ for $LMVL_C$ and $0.97 \times 10^{13} \pm 0.03$ for $LMVL_B$ (Table 1). The particle concentration is an important parameter in the biological evaluation of micro and nanostructures for food and pharmacy applications. Also, it helps to detect changes in the physical instability of the system (Ribeiro et al., 2018). Therefore, it will be an important parameter to consider in the *in vivo* experiments.

The $LMVL_B$ showed an encapsulation efficiency value of 81.33% ± 1.33% (Table 1). In contrast, other works have reported the liposome encapsulation of βC with EE around 86% and 97% using the ethanol injection method (Bai et al., 2019; Liu et al., 2020), however, those formulations included Coix seed oil and tween 80 as additional stabilizing ingredients, respectively. Besides the relatively low EE, we consider as a successful encapsulation and results indicate that most of the βC was located deeply within the hydrophobic cavity of the liposome.

Cryo-Transmission electron microscopy (Cryo-TEM) is one of the suitable techniques as it preserves the hydrated structure of the MVLs and provides an accurate high-resolution representation of the LMVL structure. Direct cryo-TEM observations in the nanometric scale of $LMVL_C$ and $LMVL_B$ are shown in Fig. 1. In LMVL Cryo-TEM image analysis, large liposomes with multivesicular content, circular projection, and a considerably polydisperse system were observed. The electron microscopy technique used in the work showed that the system still presented adequate morphology and no changes in the lipid bilayer even after the insertion of beta-carotene.

Table 1
Values of mean diameter (MD), polydispersity (PDI), zeta potential (ζ) and particle concentration values [particles/mL] of control liposomes and βC loaded liposomes produced by freeze-thawing.

Liposomes	MD (nm)	PDI	ζ (mV)	EE (%)	[particles/mL x10^{13}]
$LMVL_C$	913.10 ± 63.66	0.515 ± 0.036	−24.20 ± 1.08		0.70 ± 0.01
$LMVL_B$	1191.00 ± 50.66	0.447 ± 0.012	−24.51 ± 0.25	81.33 ± 1.33	0.97 ± 0.03

Measurements in triplicates ± standard deviation.

3.2. Production and characterization of bioactive microparticles

The mean diameters of the samples, represented by the D50 values in Table 2, are close and within the range of results observed in the literature at similar drying parameters (Alves Gragnani Vido et al., 2023; Fadini et al., 2018). The SPAM values observed in the samples (0.80 and 0.79) are slightly below those observed in the literature (values between 1.0 and 2.0 are commonly observed) indicating a lower polydispersity for these samples than observed in other studies. This is important due to spray-dried powders with span values below 2.0 are considered monomodal and homogenously distributed; while span values above two indicate probable particle agglomeration (Tonon et al., 2011). Thus, the particle size distribution of both BDM dried at 130 and 170 °C present monomodal behavior and mean particle size (D50) of around 10 μm.

The microparticles obtained at 130 °C presented a higher A_w value compared to those obtained at 170 °C (Table 2), however both samples presented values below 0.2. Water activity values between 0.11 and 0.23 prevent cell death during storage, maintaining a high survival rate of the microencapsulated probiotic bacteria (Dianawati et al., 2016). In addition, the values of moisture were below 4%, indicating good stability of the dry products in both inlet temperatures. Usually, atomization conditions, inlet, and outlet temperatures of the drying air can affect the final moisture content of spray-dried powders (Pinto et al., 2014). In our study, besides the different inlet dry temperatures, the moisture and A_w were kept very close for both BDMs.

The morphology of the main components used to prepare the BDM was obtained by using optical microscopy (Fig. 2), including the liposomes containing βC (LMVL); the probiotic LGG; the mixture of active ingredients in the liquid formulation before drying; the spray dried microparticles at 130 °C (BDM) and an image of the same sample generated after dissolving the dried microparticles in water. As observed, the produced BDM has a spherical shape and the typical appearance of products obtained by spray drying (Alvim et al., 2016; Fadini et al., 2018). After hydration of BDM with water, it is possible to identify intact liposomes and microorganisms, indicating that the drying and microencapsulation stage of these components using a spray dryer allowed the maintenance of the liposome structures.

The microencapsulation of βC and LGG cells by spray-drying at 130 °C and 170 °C and outlet temperature of 75 °C resulted in an entrapment efficiency of βC of 56.92% and 64.48%, respectively. For the probiotic *L. rhamnosus* (LGG) the survival after the drying process was 98.50% (7.50×10^9 CFU/g) and 92.99% ($2{,}10x \times 10^9$ CFU/g), for 130 °C and 170 °C, respectively (time 0 in Fig. 3). The greater retention of βC by the higher temperature process (170 °C) is probably due to the shorter drying time of microparticles but is also highly dependent on the wall material. Corrêa-Filho et al. (2019), based on a response surface methodology (RSM), found a higher EE (16 %) of βC and a drying yield (43.9%) using 11.9% of Arabic gum as wall material and a drying temperature of 173 °C. More recently, the spray-drying microencapsulation of βC using octenyl succinic anhydride (OSA)-starch and trehalose at an inlet temperature of 165 °C, was reported; this methodology resulted in an EE of 99.06% (Zhang et al., 2021) .

Regarding the survivor of LGG after microencapsulation by spray

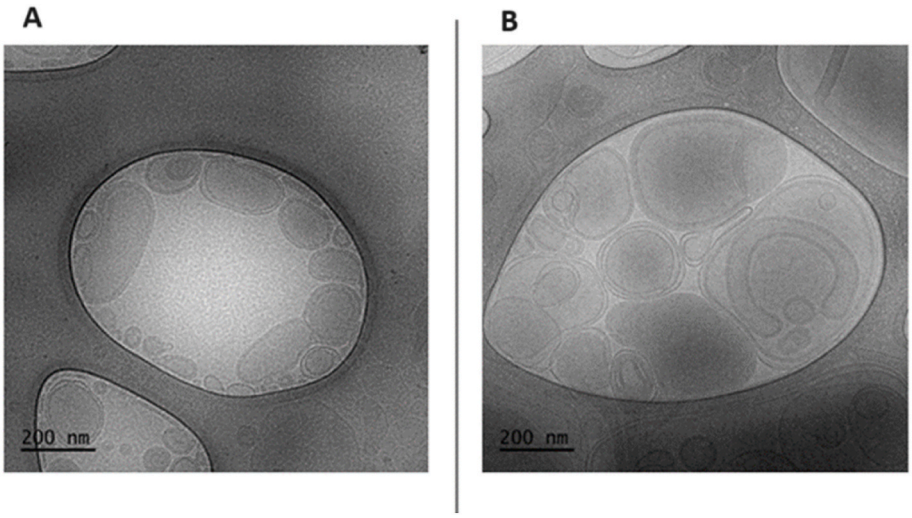

Fig. 1. Photomicrographs of $LMVL_C$ (A) and $LMVL_B$ (B) by transmission cryo-microscopy (10000x). Bar = 0.2 μm.

Table 2
Size and physical characterization of produced bioactive microparticles.

Sample	Microparticle diameter (μm)				Aw	M (%)
	D10	D50	D90	SPAM		
BDM 130	6.48 ± 0.07[b]	9.76 ± 0.04[b]	14.32 ± 0.03[b]	0.80 ± 0.01[a]	0,146 ± 0,002[a]	1,21 ± 0.02[b]
BDM 170	6.78 ± 0.03[a]	10.18 ± 0.03[a]	14.81 ± 0.01[a]	0.79 ± 0.01[a]	0,125 ± 0,001[b]	1,88 ± 0.02[a]

Biphasic dried microparticles (BDM) dried at 130 or 170 °C. Measurements in triplicates ± standard deviation. D10, D50 and D90 correspond to the diameters of 10, 50, and 90% of the accumulated distribution. SPAN represents the polydispersity index and is calculated by (D90 - D10)/D50. A_w: Water activity; M (%): Total moisture. Different superscript letters in the same column denote significant differences ($p < 0.05$).

drying, there are several reports with high variable values according to the wall material, and drying temperatures. Close to our results, a recent work reported a survivor of 65.16% and improved integrity of the subcellular structure using an emulsion W/O/W + inulin as wall materials for spray-dried microencapsulation of LGG at 150 °C of inlet temperature (Yin et al., 2024). However, there are no previous reports of the spray drying of a mixture of liposomes and free bacterial cells; instead, some works that use liposomes as a coating for the probiotic bacteria, usually by self-assembly techniques to produce giant unilamellar vesicles (Pinilla et al., 2023) or double emulsion systems (W/O/W) containing the probiotics (Camelo-Silva et al., 2022).

Considering the good results of βC retention and survival of LGG, the use of whey as wall material could favor the structural stability of the BDM due to the interaction of liposomal phospholipids and the whey components. Recently, Wang et al., (2022), reported that hydrophobic interactions drive the mixtures of phospholipids and whey protein isolate (WPI) in an emulsion model, those interactions change the charge distribution of the particles in the mixtures improving the stability of phospholipid-WPI emulsions. In addition, lactose (a whey component) has been reported in liposome technology as a cryo/-lyoprotective agent, since this disaccharide can enhance the integrity of liposomal membranes during dehydration/rehydration processes (Guimarães et al., 2019).

3.3. Long-term stability of bioactive microparticles

As part of the physicochemical characterization, stability tests were

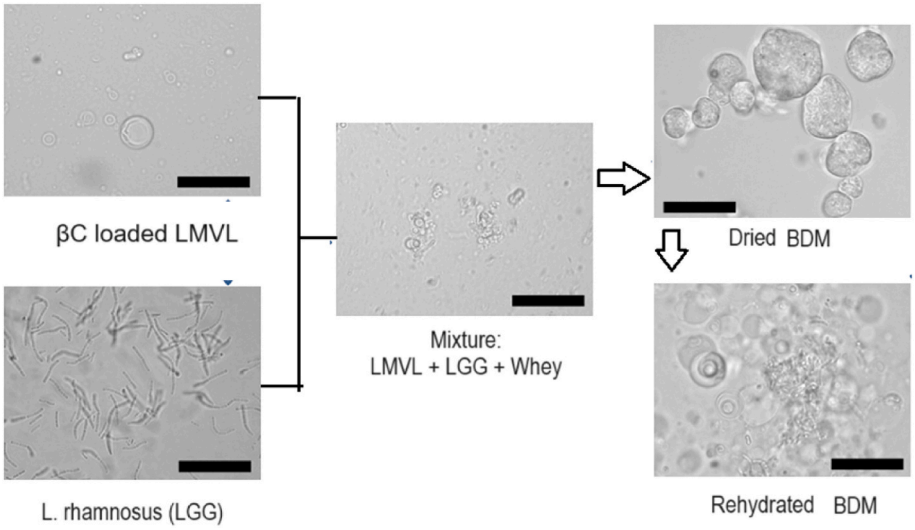

Fig. 2. Morphological aspect of the microstructures during the production of BDM. All images were captured at the same 1000× magnification. The bars represent 20 μm.

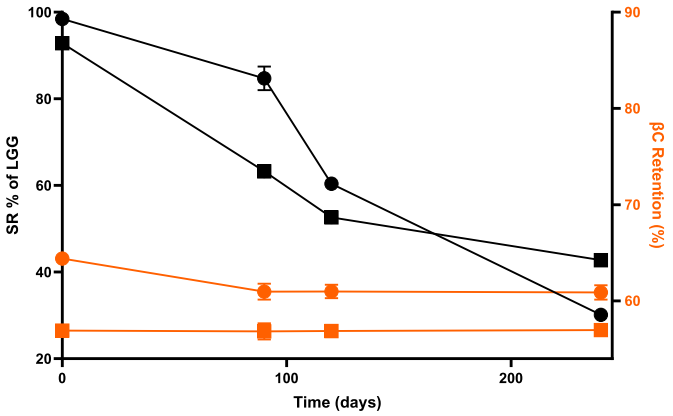

Fig. 3. Survival rate percentage (SR%) of *L. rhamnosus* LGG and retention (%) of βC in BDM produced at 130 °C (circle symbol) and 170 °C (square symbol), during 240 days of storage at 25 °C. Measurements in triplicates ± standard deviation.

performed by evaluating the cell viability of LGG and the beta-carotene retention during storage for 240 days (Fig. 3). As result, after 120 days of storage, the BDM dried at 130 °C and 170 °C showed a gradual decrease in LGG survival rate (SR%) until around 60%; at the end of the storage period, the SR% was 30.11% and 42.75% for the BDM dried at 130 °C and 170 °C, respectively. A similar decrease in probiotic SR% at room temperature of storage was previously reported in other works (Alves Gragnani Vido et al., 2023; Gul, 2017). The reduced viability of dried lactobacilli could be related to the accumulation of toxic waste from cell metabolic activity during storage and the higher reactivity and diffusivity of intracellular oxygen species (Ranadheera et al., 2015).

Concerning the βC retention during the storage was observed a slight decrease at the end of the storage time, from 64.4% to 60.88% in the BDM dried at 130 °C (5.4% of βC loss), and no significative difference was found in the βC retention of the BDM dried at 170 °C. These values of βC retention could be related to reduced leakage of encapsulated βC, which is located in the bulk phase of the liposomes. Incorporation of βC in liposomes decreases the membrane fluidity, leading to a more compacted structure (Chen et al., 2017). In addition, the presence of whey protein lactose in the formulation could act as an additional layer of protection for the liposomes, reducing the βC leakage and the permeability of oxygen-reactive substances. In this regard, was reported that lactose prevented the aggregation of lecithin nanoliposomes after spray drying and also reduced the lipid oxidation, maintaining the peroxide values of dried nanoliposome formulations below 3 meq/kg, during the storage and no change in the color parameters (Chun et al., 2017). Thus, our encapsulation techniques result in a rigid and stable structure that allows extending the half-life for encapsulated βC.

In contrast with our results, Chuyen et al. (2019) reported losses of 65.3% and 41.5% of carotenoid content after 6 months at 5 °C and 20 °C, respectively, in the spray-dried powder of carotenoid-rich oil from Gac peel using the mixture of whey protein concentrate and gum Arabic as wall materials. The low stability of βC is due to rapid chemical degradation when exposed to light, high temperatures, oxygen, free radicals, and transition metals (Boon et al., 2010). In this sense, the biphasic microparticles produced in this work present great potential to increase the storage stability of βC.

3.4. Toxicological evaluation and in vivo bioactive effect of microparticles

3.4.1. Acute toxicology

Oral toxicity tests are necessary in the program for a complete panel of possible toxicological effects. The most commonly used standard toxicological tests for chemicals are those recommended by the Organization for Economic Co-operation and Development (OECD) guidelines.

In the present study, safety evaluation was performed by acute oral toxicity assay in male mice with a dose of 2000 mg/kg of BDM dried at 130 °C, this amount represents a concentration of 1.5×10^{10} CFU/kg of viable cells of LGG and 2.84 μM of βC, considering the EE % values obtained after drying. This sample was selected due to their content of βC and the high viability of LGG. As result, the BDM did not cause death or change in the evaluated parameters when compared to the control group. The exposed animals showed no statistical changes in weight gain, food and water consumption, or behavior. Macroscopic analysis and the relative weight of the organ did not show any changes that indicate signs of toxicity, based on the acute toxicity model for *Wistar* rats (data not shown). The determination of acute clinical signs is important to establish a lethal dose that can cause the death of 50% of the animals tested (LD50), as a parameter for further toxicity trials, such as subacute, subchronic and chronic toxicity. Therefore, in the present work, it is assumed that the LD50 is above 2000 mg/kg of BDM.

3.4.2. In vivo anti-inflammatory evaluation

In this test, the BDM was evaluated as a single functional product, thus, the anti-inflammatory evaluation of their separated components was not included. The use of carrageenan (Carr) is applied in several experimental models of inflammation and helps in the development of anti-inflammatory drugs. The model of paw edema induced by carrageenan is a classic model of vascular inflammatory response related to the formation of edema (Silva et al., 2010). The effects of BDM dried at 130 °C on inflammation in the paw edema induced by Carr of mice were investigated. Indomethacin 10 mg/kg, a reference drug, was used as a positive control. As shown in Fig. 4, the BDM at 1000 and 2000 mg/kg had a similar effect to the indomethacin in inhibiting edema, with no significant difference ($p < 0.05$ compared to the indomethacin group) until de first 2 h. It is important to highlight that after 6 h of applying carrageenan and administering BDM 2000 mg/kg, it was possible to observe an anti-inflammatory effect of 78% inhibition, demonstrating the high anti-inflammatory potential of the BDM.

This assay was used to observe possible activity against pro-inflammatory mediators in rats. After the carrageenan injection, the formed edema is mediated by histamine, serotonin, and increased biosynthesis of prostaglandins (Silva et al., 2010). Using this model, Oladejo et al. (2023) studied the anti-inflammatory effect of different lactobacilli isolated from traditional fermented products of Nigeria, they found a significant decrease in paw thickness and upregulation of serum

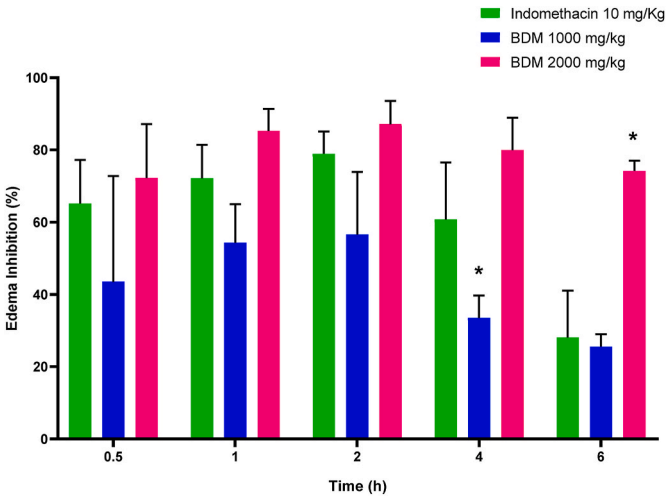

Fig. 4. Inhibition of paw edema induced by carrageenan (1%) measured at 30 min, 1 h, 2 h, 4 h and 6 h. Values expressed as mean ± standard deviation (n = 7 per group). ANOVA – Bonferroni. (*$p < 0.05$ compared to indomethacin group).

levels of IL-10 and TGF-β using bacterial concentrations of 5×10^7 CFU/ml, however, the main effects were observed after 4 h of treatment. Similar anti-inflammatory effects have been reported in animal models and clinical trials regarding the *L. rhamnosus* GG in doses of around 10^8 CFU (Amdekar & Singh, 2016; Di Caro et al., 2005; Kekkonen et al., 2008).

β-carotene is a versatile molecule that interacts with multiple inflammatory molecular targets: acts in tissue metabolism, regulating gut microbiota, oxidative stress, and reducing inflammatory damage (Grar et al., 2020). Besides the anti-inflammation underlying mechanisms remain unknown, it has been reported that βC in concentrations of 50, 100, and 150 μM reduced the production of nitric oxide (NO), prostaglandin (PG)E2, tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β), and the expression of IL-1β and TNF-α (Yang et al., 2021). Thus, the results obtained from the carrageenan-induced paw edema show that the oral administration of the BDM markedly inhibited paw edema in a dose-response relationship. We hypothesize that the anti-inflammatory effect is mediated in the initial hours by the βC released from the BDM, even at low concentration (2.84 μM), and after this initial stage, the controlled release of βC plus the LGG gut adhesion, could favor a prolonged anti-inflammatory effect. However, more studies must be conducted to verify the effect of microencapsulated βC and LGG separately, to determine their individual anti-inflammatory effects, some additive or synergetic effects, and the protective effect of the encapsulation.

3.4.3. *Carrageenan-induced pleurisy*

The evaluation of leukocyte migration was performed using the carrageenan-induced pleurisy method. The results are presented in Table 3. This test evaluates the migration of leukocytes and the protein extravasation, both important parameters in the inflammatory process by inducing an acute inflammatory reaction by carrageenan injection in the pleural cavity (Lescano et al., 2014). Considering this experimental model of pleurisy, was observed that the results corroborate those obtained in the paw edema test; showing that BDM at 2000 mg/kg is capable of reducing leukocyte migration in the region where the inflammatory process is induced. Protein extravasation was reduced in the group treated with BDM with not statistically different ($p < 0.05$) from the group with indomethacin. Both groups, indomethacin and BDM, differed statistically from the group treated with carrageenan alone (1%). Similar results were obtained in the number of leukocytes per ml of pleural fluid, where the BDM and indomethacin presented significant difference ($p < 0.05$) with the positive control, and presented values close to those obtained for the naive group.

4. Conclusions

The produced βC liposomes presented adequate physicochemical characteristics, such as size, PDI, zeta potential, and encapsulation efficiency. After the microencapsulation by spray drying using whey as wall material and including viable cells of L. rhamnosus LGG, it was possible to obtain bioactive microparticles with physicochemical characteristics within the proposed standards (size, span, and morphology). Furthermore, βC retention and cell viability were high after the spray-drying process, and during long-term storage. The BDM preserved the βC of degradation, possibly for their interaction with the PC, the whey proteins, and the lactose resulting in a more rigid and low permeable structure. The toxicological studies revealed that the BDM at 2000 mg/kg does not present *in vivo* toxic effects and, at the same dose, presented anti-inflammatory effect in carrageenan-induced paw edema and pleurisy *in vivo* experiments, which could be the result of the combined anti-inflammatory effect of βC and LGG gut adhesion. Therefore, the BDM containing βC and the probiotic bacteria LGG is a promising and innovative approach to producing a dried and stable ingredient with anti-inflammatory properties and diverse applications in food and pharmacy.

Table 3
Values of total proteins and total leukocyte cells in pleural lavage after 4 h of intrapleural administration of carrageenan in male *Wistar* rats.

Groups	Proteins (g/dL^{-1})	Totals Leukocytes (cells $x10^6.mL^{-1}$)
Naive	0.31 ± 0.02^{b}	8.28 ± 0.26
Carrageen	$1.82 \pm 0.28^{a\ b}$	$21.97 \pm 8.87^{a\ b}$
Indomethacin	0.83 ± 0.15^{a}	9.16 ± 0.39^{a}
BDM (2000 mg/kg)	0.99 ± 0.11^{a}	8.60 ± 0.51

Values expressed as mean ± standard deviation (n = 7 per group). ANOVA – Bonferroni.
[a] $p < 0.05$ compared to naive group and.
[b] $p < 0.05$ compared to indomethacin group.

Industrial relevance

Co-encapsulation addresses the protection of multiple compounds, some of them with low stability, from harmful conditions. Here, for the first time was presented an innovative method for the co-encapsulation of β-carotene and probiotics. The by-phasic structure includes the stabilization of β-carotene by multilamellar liposomes followed by a spray dried, using a mixture of whey and the probiotic strain. This novel approach provides simultaneous protection and delivery of multiple functional components in a single system, which is of great significance for the development of new foods with functional features.

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Ethics approval and consent to participate

The *in vivo* tests were approved by the Animal Experimentation Ethics Committee of the Federal Universities of Grande Dourados and the State University of Campinas (protocols: September 2020 and 5637–1/2020, respectively).

CRediT authorship contribution statement

Fernando Freitas de Lima: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Talita Cesarim Mendonça:** Methodology, Investigation, Formal analysis, Data curation. **Cristian Mauricio Barreto Pinilla:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Izabela Dutra Alvim:** Methodology, Investigation, Formal analysis. **Mariana Alves Gragnani Vido:** Methodology, Formal analysis, Data curation. **Eneida de Paula:** Supervision, Methodology, Funding acquisition. **Leila Maria Spadoti:** Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Adriana Torres Silva e Alves:** Supervision, Project administration, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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