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Evaluation of Viability of *Lactobacillus fermentum* CECT 5716 in Gelatin and Gastroresistant Capsules

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Abstract: Different studies have attributed health benefits to *Lactobacillus fermentum* CECT 5716. However, the main problem associated with probiotics is their low resistance to environmental and technological factors. In this sense, capsules can provide a shell protection and a dosage form that is easy to swallow. For these reasons, the aim of this study was to evaluate the survival of *Lactobacillus fermentum* CECT 5716 in gelatin and gastro-resistant capsules during a period of 12 months at RT (room temperature) and 4 °C. The number of encapsulated cells remained relatively constant after six months of storage, since there were no statistically significant differences compared to the initial time ($p > 0.05$). Moreover, capsules are able to maintain a therapeutic level of bacteria (10^9 CFU/capsule) during the total period of storage. Gelatin capsules seem to protect worse probiotic than gastro-resistant (HPMC (hydroxy propyl methyl cellulose)) capsules. Furthermore, capsules stored at 4 °C show a low level of viability. These results suggest that HPMC capsules are an optimal dosage form for *L. fermentum* CECT 5716 and that the recommended condition of storage is room temperature rather than 4 °C.

Key words: Probiotics, gastro-resistant, gelatin, capsules, *Lactobacillus fermentum*.

1. Introduction

The intestinal microbiota plays an important role in human health as it contributes to inhibiting pathogen colonization, boosting the immune system, and metabolizing nutrients [1]. However, different factors like diet, antibiotics, and stress are reported to negatively influence bacterial population in human gastrointestinal tract and cause dysbiosis that could lead to different diseases. In this respect, the administration of probiotic could reestablish the balance in the microbiota. Probiotics are live microorganisms, which, when administered in appropriate quantities, offer health benefits to their host [2]. Several studies have attributed health benefits to probiotics [3]. Specifically, *Lactobacillus fermentum* CECT 5716 is a well-known probiotic of human origin. Its usefulness has been demonstrated in the treatment and prevention of mastitis [4], inflammatory bowel diseases, and gastrointestinal and respiratory infections [5, 6]. The problem is that, several conditions influence the bacterial survival during technological processes and the gastrointestinal transit [7]. The currently accepted definition of probiotics requires that the bacteria maintain its viability from production to consumption. Consequently, the industry demands technologies ensuring probiotic stability for both economical and functional reasons. Therapeutic live bacteria represent a major delivery challenge for pharmaceutical scientists.

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Although many formulation have been developed for oral delivery of probiotic ranging from exotic functional food to traditional dairy products (yoghurts and fermented milks). However, solid dosage forms such as capsules or tablets containing dried probiotic bacteria, offer the most control of both dose and site of delivery and also increase shelf life and stability [8]. Tablets are usually produced by pressing a powdery material in a mold. Sensitive biological materials, such as probiotics, are very sensitive to compression forces and heat that develop when tablets are formed, causing a severe reduction in viability [9]. In this sense, capsules offer many advantages over other dosage forms, like compressed solid tablets or bulk dry powder preparations. Capsules provide a shell protection and they are a dosage form is easy to swallow, without the need of being flavored. Capsules also provide a good oxygen barrier due to low oxygen permeability through its shell. Moreover, capsules can be formulated to protect the bacteria during passage through the stomach thus allowing their release in the intestine. Some of these capsules are elaborated with HPMC (hydroxy propyl methyl cellulose). HPMC capsules may offer attractive alternative to gelatin capsules because of its vegetable source. The crosslinking of gelatin and drug incompatibilities and the strict regulations regarding the use of animal derived gelatin- requiring the absence of BSE (bovine spongiform encephalopathy)/TSE (transmissible spongiform encephalopathy)-have encouraged the search for gelatin replacement. Religious, cultural and personal issues may affect patients' preference towards the medications presented in capsule dosage forms [10]. Other advantages are the reduced static, low microbial growth potential as well as no cross-linking possibility. In addition, their low moisture content makes them appropriate for administration of sensitive products, such as probiotics.

In this sense, the encapsulation of probiotics in these different types of capsules may provide an approach for protecting probiotics.

Based on these considerations, the goal of the present study was to evaluate the survival of *Lactobacillus fermentum* CECT 5716 loaded in gelatin and gastro-resistant capsules during a period of 12 months at RT (room temperature) and 4 °C.

2. Materials and Methods

2.1 Materials

Freeze-dried *Lactobacillus fermentum* CECT 5716 and maltodextrine were kindly provided by Bioserch Life (Granada, Spain). Gelatin capsules were obtained from Guinama (Valencia, Spain). Gastro-resistant capsules were purchased from Fagron Iberica S.A. (Barcelona, Spain).

2.2 Preformulation Assay: Determination of Flow Properties

The flow properties of freeze-dried probiotic and a combination of maltodextrine and freeze-dried probiotic were analyzed in accordance with the USP method. A 250 mL graduated cylinder was submitted to 10, 500 and 1,250 taps on the same powder sample and read the corresponding volumes. The Hausner and Carr ratio were calculated as follow:

$$\text{Tapped density} = \text{mass} / \text{tapped volume}$$

$$\text{Pour (or bulk) density} = \text{mass} / \text{untapped volume}$$

$$\text{Hausner ratio} = \text{tapped density} / \text{pour density}$$

$$\text{Carr ratio} = (\text{tapped density} - \text{bulk density} / \text{tapped density}) \times 100$$

2.3 Capsule Preparation

Capsules with a constant weight of 300 mg (dose = 10) of a homogeneous mixture of maltodextrine and freeze-dried *Lactobacillus fermentum* CECT 5716 (LAB) using a manual filling machine (Capsunorm®, Burgos, Spain). All the pieces were disinfected with 70% ethanol before each capsule were produced.

Capsules were dosed aseptically by means of Capsunorm®, with a cell density of 10^9 CFU (colony forming units) per capsule. After that some of them

were placed in flasks containing silica-gel desiccants and stored at RT under darkness and at 4 °C. The rest were submitted to the technological assays.

2.4 Capsules Evaluation

The probiotic capsules obtained were evaluated for their mass uniformity, content uniformity and disintegration, according to USP. Disintegration of caspules was examined by means of a disintegration device (Erweka, Heusenstamm, Germany).

Hard gelatin capsules were placed separately in the test chamber, and then immersed in water maintained at 37 °C. Meanwhile, gastro-resistant were immersed in PBS pH 1 and subsequently in pH 6.8 as the disintegration medium at 37 °C.

2.5 Stability of the Probiotic Capsules

For stability testing, capsules were stored in light-resistant containers at 4 ºC in a refrigerator and at room temperature for a period of twelve months. The stability of the bacterial cells in terms of cells viability in the capsules across the storage period was examined. The plating procedure method used to determine cell viability was described in Section 2.4.1. The percentage of survival after time was calculated as follows (Eq. (1)):

$$\text{Survival} = (\text{number of cells after time} / \text{number of cells at time}) \times 100 \quad (1)$$

2.6 Data Analysis

All analyses were performed using SPSS Version 19.0 for windows (SPSS, Chicago, Ilinois, USA). Values are expressed as mean ± standard deviation. Data were compared using the Student *t*-test. Statistical significance was set at $p < 0.05$.

3. Results and Discussion

3.1 Preformulation Assay: Flow Properties

For a successful capsule filling operation and the production of capsules with uniform fill weight, it is essential that the powder have optimal flow and packing properties [11]. The Hausner and Carr ratio are measures of the flow properties of powders. They are an indirect measurement of bulk density, shape and size, surface area, moisture content and cohesiveness of the materials, since all these parameters can influence these ratios. Moreover, these flow parameters may also be used to predict capsule fill weight uniformity; better flow ability tends to yield more uniform fill weights.

The Hausner ratio (1.35 ± 0.05) and the Carr ratio (26 ± 0.3) for freeze-dried powder are related with poor flow properties (Table 1). Meanwhile, the mixture of maltodextine and freeze-dried probiotic showed a Hausner ratio of 1.29 ± 0.001 and a Carr ratio of 22.4 ± 0.2, so the addition of maltodextrine leads to results that are considered as good flow properties. As Tan and Newton [11] demonstrated these flow parameters derived from packing characteristics of powders during tap consolidation also indicate coarse powders to have good flow ability whilst fine powders exhibit very poor flow. Apart from particle size, there are others factors that can affect to powders flow ability, such as moisture content, humidity, temperature, pressure, fat and flow agents [12].

3.2 Capsules Evaluation

Technological properties (mass uniformity, content uniformity and disintegration) were determined at time cero (Table 2). The results showed that gelatin capsules and low moisture capsules fulfill all the requirements. The deviation in the mass uniformity test is below 7.5%, as it indicated for capsules of ≥ 300 mg in all of the cases. Moreover, content uniformity was between 85% and 115% for all the capsules [13]. However gastro-resistant capsules released their content before 1 h at gastric pH. The experiment was repeated several times and in each case the capsule is opened before 1 h.

HPMC have a lower moisture permeability compared to gelatin, since the polymer's hydratation

Table 1 Flow properties of the powders.

Items	Hausner ratio	Carr ratio	Flow properties
Freeze-dried probiotic	1.35 ± 0.05	26 ± 0.3	Poor 1.35~1.45 26~31
Maltodextrine Freeze-dried probiotic	1.29 ± 0.001	22.4 ± 0.2	Adequate 1.26~1.34 21~25

Table 2 Technological properties of capsules.

Capsules	Mass uniformity	Content uniformity	Desintegration
Gelatin	Desviation < 7.5%	85~115%	5 min
Gastro-resistant	Desviation < 7.5%	85~115%	30 min

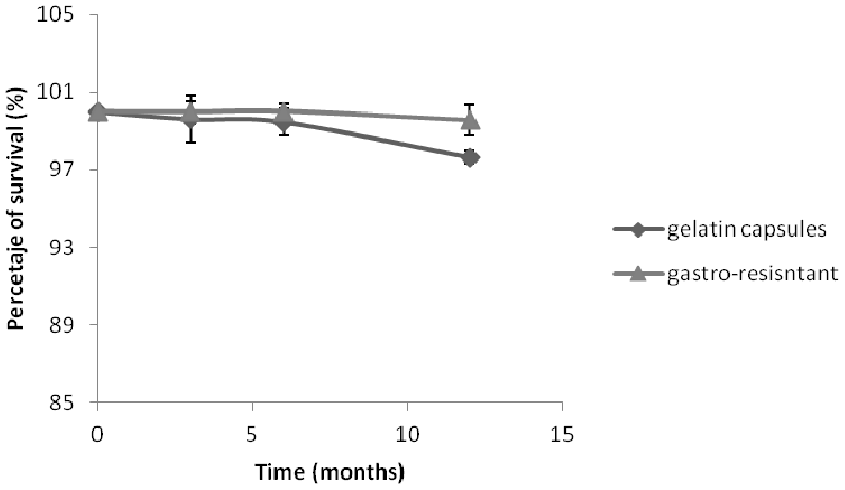

Fig. 1 Percentage of survival of Lactobacillus fermentum encapsulated in capsuules stored at RT.

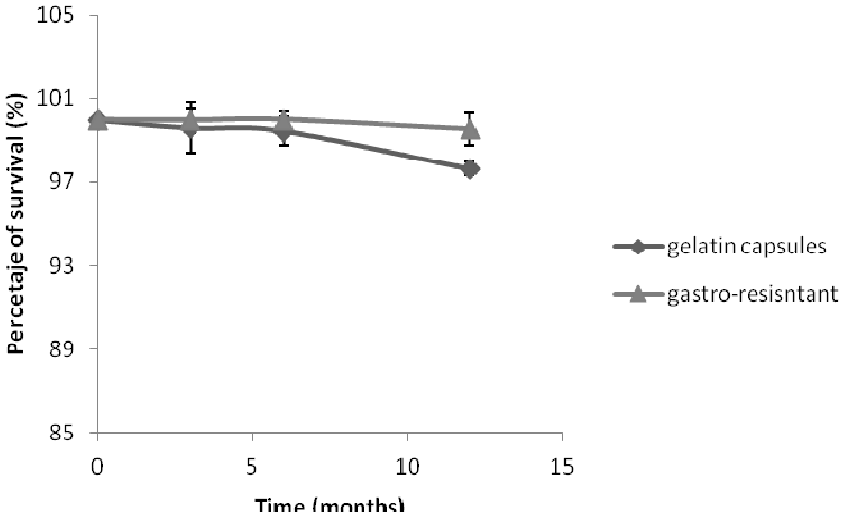

Fig. 2 Percentage of survival of Lactobacillus fermentum encapsulated in capsules stored at 4 °C.

takes longer before it starts to dissolve. Mahbubani et al. [8] showed that delivery of dried live probiotic microrganisms to the intestine may be improved by providing protection from bile by addition of bile adsorbing resins and the use of HPMC capsules. So, despite the fact that HPMC capsules were opened in simulated gastrointestinal conditions, it could be a good option try to add this bile absorbing resins like cholestyramine to the formulation in order to ensure the arrival of a higher dose of bacterias until intestine. According to Cole et al. [14], another good option could be coating HPMC capsules with enteric polymers so as to achieve intestinal targeting, thus, for HPMC capsules coated with Eudragit® FS 30 D, complete disintegration did not occur until the distal small intestine and proximal colon in an average time of 6.9 h post dose.

Jones et al. [15] studied the disintegration of gelatin and HPMC capsules in fed and fasted state in order to increase their disintegration time and both capsules showed an increase in their disintegration times in the fed state, so it could be a good solution to improve our results. Even, it was recommended that HPMC capsules should be taken with a cold drink and gelatin capsules with a hot drink.

3.3 Stability of the Probiotic in the Studied Capsules

The viability was evaluated throughout a period of twelve months. Capsules were stored at RT and 4 °C in a refrigerator. When capsules are stored at RT, the number of encapsulated cells remained relatively constant after a period of six months both in gelatin and gastro-resistant capsules. No significant differences ($p > 0.05$) in the number of viable bacteria were found between t_0 and t_6. Moreover, these capsules are able to maintain a therapeutic level of bacteria (10^9 CFU/capsule) during this period of storage. The best viability is reported by the gastro-resistant capsules (HPMC), which could be due to the composition of the capsule. Furthermore, gelatin capsules stored at 4 °C showed a lower viability. Probiotics microorganisms are very sensitive to humidity, so the less survival of probiotic at 4 °C could be related to the storage on the refrigerator where the level of humidity is higher.

Barham et al. [16] studied the sorption charasteristics of HPMC and hard gelatin capsules and their ability of protect capsule contents. Moisture uptake of gelatin humidity tested, thus suggesting that HPMC is a better choice than gelatin when making probiotic capsules. This is interesting and new because most of the capsules on the market are stored at 4 °C [17].

4. Conclusions

Maltodextrine is an adequate excipient for the preparation of capsules of *L. fermentum* CECT 5716 since it confers adequate flow properties. Moreover, both capsules meet the Spanish Pharmacopeia quality criteria referring to mass uniformity and content uniformity. Gastro-resistant capsules made of HPMC showed a better viability that gelatin capsules at both temperatures. The storage of gelatin capsules at 4 °C showed a lower level of viability than RT, so this seems to be the best way to storage them. However, both kinds of capsules were able to maintain a therapeutic level of bacteria (10^9 CFU/capsule) during the total period of storage. Further studies are actually in course in order to test if capsules provide protection against gastrointestinal tract.

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