TABLETS OF SOY PROTEIN-ALGINATE MICROPARTICLES WITH *Lactobacillus casei* 01: PHYSICOCHEMICAL AND BIOPHARMACEUTICAL PROPERTIES

### Article Highlights
- Tablets based on probiotic microparticles were developed
- The tablets possess desirable physicochemical properties
- The tablets were able to protect the entrapped probiotic from the gastric acidity
- The tablets were able to retard probiotic release in the intestinal stage
- The storage stability of the tablets was acceptable at 25 °C/60% relative humidity for 6 weeks

### Abstract
The aim of the study was to develop direct-compress-tablets of microencapsulated probiotic *Lactobacillus casei* 01 in soy protein-alginate microparticles and excipients able to provide probiotic delivery near the colon. Considering their physicochemical properties, all series of tablets prepared met the requirements of the Ph Eur 9.0. The compaction of the probiotic loaded microparticles caused viability decrease up to 1.5 log cycles. The tablets containing Methocel K100M showed higher potential for preserving probiotic viability in simulated gastrointestinal fluids within 4 h and retarding its release in the intestinal stage, maintaining the required minimum of viable probiotic cells above $10^7$ CFU per gram of tablet. In addition, acceptable storage stability (viability of probiotic above $10^6$ CFU/g) at 25 °C/60% relative humidity for 42 days was observed. In conclusion, novel tablet dosage forms of microencapsulated *L. casei* 01 were prepared with high potential for preserving probiotic viability in simulated gastrointestinal fluids and retarding its release in the lower intestine. Further research is needed to optimize the formulation and process parameters in order to obtain tablets with probiotic viability over long storage periods.

**Keywords:** *Lactobacillus casei* 01, soy protein-alginate microparticles, tablets, physicochemical properties, biopharmaceutical properties.
However, the probiotic delivery by these dosage forms shows limited stability of a large number of probiotic cells since they often do not survive in harsh gastrointestinal conditions. From a technological point of view, it is of paramount importance to produce live microbiologically stable biotherapeutics with a sufficient number of probiotic cells per gram of product. Therefore, optimal conditions for their preparation and storage should be provided. It can be achieved by adding cryoprotectants or by microencapsulation. Microencapsulation is performed by means of electrostatic, extrusion/coacervation or emulsification (freeze-, fluidized-, or spray-drying) treatments. In this case, the bacterial survival rate depends not only on the microencapsulation method used but also on the bacterial strain involved and the chemical nature of the artificial or natural matrix in which it will be contained [7-10].

Spray drying technology offers several advantages over other microencapsulation methods, being cheap, friendly and efficacious for preparing probiotic microparticles (MPs) with preserved viability and with acceptable stability in terms of time. However, during preparation there is a possibility for some of the probiotic cells to be exposed at the surface of the MPs and for that reason, and because of the pressure and high temperature used during spray-drying, cell wall/membrane, ribosomes and DNA can be injured [11]. To overcome this disadvantage and improve storage stability, tablets as "live biotherapeutics" could be utilized. Tablets offer several advantages over other dosage forms, being easy for preparation and offering precise dosage and compliance to administration. With respect to probiotics, their viability could be preserved due to the low water-activity. Further, by a proper selection of excipients for tablet forming matrix, the entrapped bacteria could be protected against the harsh effects of the gastric environment and intestinal bile, and delivered to the colon in sufficient numbers (> 10^6 colony forming units per gram of product, CFU/g) [12,13].

In previous studies, probiotic tablets [13-15], effervescent tablets [16], probiotics-loaded pellets in a tablet form [17] and coated tablets [18] were prepared and characterized. Generally, the effects on bacterial survival in the tablets were investigated concerning compression force and matrix forming excipients, with the results showing that both the formulation and process parameters affect the properties of probiotic tablets in terms of physicochemical characteristics as well as the survival of the bacteria. The coating solution or heat during drying when coated tablets are prepared present additional stress for the probiotic [19], while direct compression of the freeze-dried probiotic may result in disintegration of the tablet in the stomach (and probiotic exposure) before reaching its site of action in the lower intestine [18]. From this point of view, direct compression of the microencapsulated probiotic might be a solution. The research data in this regard are very scarce and, to the best of our knowledge, only one study is available [12], in which *L. paracasei* L26 loaded whey protein MPs were tableted by direct compression. Approved excipients for probiotic tablet formulation were used and the effect of compaction force on viability of the probiotic strain was evaluated, showing negative impact on the probiotic viability with its increase.

In this study, direct-compress-tablets of previously optimized *Lactobacillus casei* 01 loaded soy protein-alginate MPs (SPI-ALG MPs) [20] were prepared. In preparation of probiotic loaded MPs, ALG has been used as a protective polymeric carrier due to its confirmed favorable properties, including biocompatibility, biodegradability, safety, and ease of gelation in the presence of divalent cations. SPI served as a cell adhesion ligand to increase the cell loading, enhance mechanical properties of the MPs and thus, to preserve viability of the probiotic at low pH. As added values, biodegradability, low immunogenicity, and similarity with the components of the tissue extracellular matrix of SPI were considered [20]. For tablet preparation, different matrix forming excipients were applied and their effects on resistance of probiotic to simulated gastrointestinal conditions and storage stability were evaluated. Based on a simple and friendly scale-up method, a new favorable dosage form for delivery of the probiotics in the human lower intestine was developed.

**EXPERIMENTAL**

**Materials**

For preparation of the probiotic loaded MPs, as a bacterial strain FD-DVS nu-trish® *L. casei* 01, freeze-dried probiotic culture from Chr. Hansen, Copenhagen, Denmark, was used, while the polymer carriers SPI and ALG (Protanal® LF 10/60 LS sodium alginate, guluronic acid content 35-45%) were purchased from Sojaprotein a.d. Becej (Becej, Serbia) and FMC Biopolymer UK Ltd. (Girvan, UK), respectively. MRS (de Man, Rogosa, Sharpe) agar and broth, as well as peptone water, used for enumeration of *L. casei* 01 were purchased from Merck KGaA (Darmstadt, Germany), while for the resistance test, bile salts (ox gall) were supplied from Sigma-Aldrich (Poole, UK), and α-amylase, pepsin from porcine gas-
tric mucosa (800-2500 U/mg) and pancreatin (>100 U/mg) from porcine pancreas from Sigma-Aldrich (St. Louis, MO, USA). For tablets preparation, the following excipients were used: hydroxypropyl methylcellulose (HPMC) (23% methoxy substitution and 6.5% hydroxypropyl substitution; Methocel™ Series K100M, Colorcon Ltd., Dartford Kent, UK), cellulose acetate phthalate (CAP, G.M. Chemie Pvt. Ltd., Navi Mumbai, India), lactose (Meggle, Wasserburg am Inn, Germany), Aerosil 200 (Evonik Industries AG, Darmstadt, Germany), magnesium stearate (Faci S. p. A., Carasco GE, Italy), and talc (Alkaloid AD Skopje, Skopje, Macedonia). All other reagents were of analytical grade. Solution reagents and the laboratory equipment were autoclaved before usage.

Preparation and characterization of soy protein-alginate microparticles with L. casei 01

For microencapsulation, the probiotic cells were activated in 5 mL MRS broth at 37 °C, during 24 h, under aerobic conditions, as previously described [20-22]. Afterwards, the cells were harvested by centrifugation at 1500 g for 10 min and washed twice with sterile 0.1% peptone water and subjected to microencapsulation. With that aim, 100 mL of aqueous mixture of ALG (4%) and SPI (1%, pH 7.0) was inoculated with 1% bacterial suspension (cell load approx. 12 log CFU/g) and homogenized by magnetic stirrer (600 rpm, model 4803-02, Cole-Palmer, Vernon Hills, USA) at room temperature for 30 min. The resultant mixture kept under low speed agitation on a magnetic stirrer (200 rpm) was subsequently infused into a spray-dryer nozzle unit of mini spray dryer B-290 (Büchi Labortechnik AG, Flawil, Switzerland) and continuously sprayed using an automatic infusion/withdrawal pump (model Sonceboz 6530R096 3.1 A/ph 9407, Sonceboz SA, Switzerland). The conditions of the spray drying process were: nozzle diameter 0.7 mm, aspirator pressure 90%, atomizer pressure 600 nL/h and flow rate 5 mL/min. The critical material attributes, process parameters and responses of interest were previously identified. The optimal formulation and conditions for the preparation of probiotic ALG-SPI MPs were determined using surface methodology-central composite face-centered design of experiments (Design-Expert™ software v.8 trial, Stat-Ease, Inc., Minneapolis, MN, USA).

Full physicochemical and biological characterization of the prepared probiotic loaded MPs was conducted and the data are presented in Hadzieva et al. [20]. Before their incorporation into tablet dosage form, MPs were characterized in term of their flowability and compressibility. Bulk and tapped volumes were determined using the methods outlined in the Ph. Eur. 9.0. (2.9.36. method) [23]. A sample (30 g) of MPs was transferred into a pre-weighed 50 mL graduated cylinder with 1 mL markings. The bulk volume (V₀) was measured after manually tapping the cylinder two times on a flat table top surface without compacting the material. The tapped volume (Vₜ) was measured with Tapped Volumeter SVM 102 (Erweka GmbH, Germany) after tapping in increments of 1250 taps. The bulk and tapped volumes were used to calculate the Carr compressibility index (as a measure of powder bridge strength and stability) and the Hausner ratio (a measure of the interparticle friction) as follows:

\[
\text{Compresibility index} = 100 \frac{V₀ - Vₜ}{V₀}
\]

\[
\text{Hausner ratio} = \frac{V₀}{Vₜ}
\]

Preparation of tablets

In order to prepare suitable tablet dosage form for effective colon delivery of viable probiotic cells, two series of direct compression tablets were prepared (series I and II), with the composition presented in Table 1. Briefly, a homogeneous blend of a required amount of SPI-ALG MPs, Aerosil 200, magnesium stearate, and talc (mass ratio = 1:1:2.7) was

Table 1. Formulation of different series of tablets prepared with L. casei 01 loaded soy protein-alginate microparticles with different matrix excipients

<table>
<thead>
<tr>
<th>Constituents, mg</th>
<th>Series</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Probiotic loaded SPI-ALG MPs(^a)</td>
<td>500</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>CAP(^b)</td>
<td>-</td>
</tr>
<tr>
<td>HPMC(^c)</td>
<td>-</td>
</tr>
<tr>
<td>Aerosil 200(^d)</td>
<td>3</td>
</tr>
<tr>
<td>Magnesium stearate</td>
<td>3</td>
</tr>
<tr>
<td>Talc</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^a\) Soy protein-alginate microparticles; \(^b\) cellulose acetate phthalate; \(^c\) hydroxypropyl methylcellulose (‘K’ grade); \(^d\) hydrophilic fumed silica with a specific surface area of 200 m\(^2\)/g
prepared. Its flow and compressibility characteristics were determined according to the Ph. Eur. 9.0. (2.9.36.) method described above [23]. To this mixture, CAP (series I) or HPMC (series II), respectively, were added as additional excipients. The tableting blends were compressed into round 12 mm tablets using a hydraulic single punch tablet press (Veb Maschinen-V. Mühlenbau, Lutherstadt Wittenberg, Germany), with a compaction force of 11 kN. Tablets containing equivalent amount of SPI-ALG MPs, and mixture of Aerosil 200, magnesium stearate and talc in the same mass ratio, with lactose (series III) and without lactose (series IV) were also prepared for comparison (Table 1).

Viability of *L. casei* 01 loaded in soy protein-alginate microparticles and probiotic tablets

The viability of *L. casei* 01 encapsulated in the SPI-ALG MPs was assessed after dissolution of 1 g of probiotic cells and 1 g of MPs, respectively, in 9 g of phosphate buffer saline (PBS 1 mol/L, pH 7.0), using the plate count method as previously described [20-22]. The cell counts were determined by sequential dilutions using 0.1% peptone water to achieve countable cell numbers. Quantitative determination of viable cells was performed in triplicate on selective MRS agar after incubation for 72 h at 37 °C in aerobic conditions, counting the plates with 30-250 colonies. The average results were expressed in CFU per g of sample, converted to log CFU/g.

For enumeration of viable cells of *L. casei* 01 into tablets, the method described by e Silva et al. was used [12]. Shortly, the tablets were grounded in a mortar and suspended in PBS (pH 6.8) in a 1:9 (g/ml) ratio. The resulting suspension was subjected to agitation at room temperature until complete disintegration. Afterwards, the viability of *L. casei* 01 was determined by the enumeration method described above.

Physical properties of tablets

Uniformity of mass

For this procedure, 20 tablets were individually and randomly weighed on an analytical balance (Ainsworth DE-100, Germany) to determine their average mass according to the Ph. Eur. 9.0. (2.9.5.) method [23].

Tablet hardness

The hardness of tablets/resistance to crushing, measured by the force needed to disrupt the tablets, was determined with ten analyzed tablets, using a hardness tester (erwekatablet hardness tester type TBH 425 TD (Erweka GmbH, Germany) according to the Ph. Eur. 9.0. (2.9.8.) method [23].

Tensile strength

The tensile strength (TS) was calculated according to the equation:

$$TS = 2F \pi dt$$

where *F* is hardness of the tablet (N), *d* is the tablet diameter (mm) and *t* is the tablet thickness (mm) [24].

Disintegration

The disintegration time of a sample of six tablets was determined using a disintegration apparatus (Erweka DZT, ERWEKA GmbH, Germany), at a temperature of 37.0±2.0 °C. As a disintegration media for the first 2 h, 0.1 M HCl (pH 1.5) was used, subsequently substituted with PBS (pH 6.8) under the same conditions for the remaining time.

Storage stability

For storage stability testing, tablets were kept in light-resistant high-density polyethylene bottles and stored in a stability test chamber (Köttermann 2391, Köttermann, Germany) under ambient conditions 25±2.0 °C and relative humidity (RH) 60±5% for 60 days [17]. The viable cells of *L. casei* 01 in the tablets over storage period were enumerated as described previously, while stability was expressed as:

$$\text{Viability} (%) = \frac{100 \times (\text{CFU at } n \text{ weeks of storage})}{\text{initial CFU}}$$

Viability of probiotic in simulated gastrointestinal conditions

The method originally developed by Madureira et al. [25] and modified by e Silva et al. [12] was used to assess the viability of *L. casei* 01 cells in the tablets exposed to simulated gastrointestinal conditions: mouth, esophagus-stomach, duodenum and ileum. The mouth conditions were simulated by a synthetic saliva solution prepared of 100 IU/ml of α-amylase in 1 mM CaCl₂. pH 6 was adjusted with 1 M NaHCO₃. Salt solution was added to the tablets at a rate of 0.6 ml/min during 2 min. The esophagus-stomach conditions were simulated by adding equal sized aliquots of 25 mg/ml pepsin in 0.1 M HCl, 0.05 mL per g of sample coming from the “mouth” phase and gradually decreasing the pH to 2 using 1 M HCl. Duodenum conditions were simulated with a solution of 2 g/L pancreatin and 12 g/L bile salts, dissolved in 0.1 M of NaHCO₃ added at a rate of 0.25 mL per g of sample coming from the “stomach” phase. The increase of pH to reach that corresponding to the ileum was simulated by adding 0.1 M of NaHCO₃. All enzyme solutions were freshly prepared for each experiment and filter-sterilized through 0.22 µm membrane filter (Milli-
pore, USA). Thermostatic water bath at 37 °C, with a horizontal shaker was used to simulate the temperature and peristaltic movements in the gastrointestinal tract (horizontal shaker, 75 rpm; SWB 20 HAAKE, Waltham, MA, USA). Eight flasks for each of the series containing 25 mL MRS broth and two tablets were subject to the viability test procedure. In two flasks, the viable cells of L. casei 01 in the tablets were enumerated. Two flasks were withdrawn after being exposed to simulated mouth and esophagus-stomach conditions; two flasks were withdrawn after being exposed to duodenum conditions, and finally, two flasks were withdrawn after being exposed to ileum conditions. In each sampled flask, the viable cells of L. casei 01 in the tablets as well as in the MRS solution were assessed.

Statistical analysis

The results obtained for the physical properties of probiotic tablets, viability test in simulated gastrointestinal conditions, and storage stability were analyzed using one-way ANOVA and unpaired t-test (IBM SPSS Statistics for Windows, version 19.0). Differences were considered statistically significant when \( p < 0.05 \).

RESULTS AND DISCUSSION

Properties of probiotic microparticles

The systems developed for the delivery of probiotics to the lower GIT include both conventional pharmaceutical and non-conventional commercial products. The conventional pharmaceutical products have lower potential for variations and are much more characterized compared to the commercial food-based carrier systems, tending to be more efficient for specific therapeutic applications [26]. The usual pharmaceutical products vary in their effectiveness to deliver a sufficient number of functional bacteria cells which will exert the health benefits to the body. These discrepancies are due to different formulations and processes applied for their preparation. With these issues, various pharmaceutical changes during formulation processes could be incorporated into formulations to improve the survival rate of probiotic bacteria. Among them is microencapsulation of probiotics.

Microencapsulation tends to stabilize cells, potentially enhancing their viability and stability during production, storage, and handling, with the potential benefit of maintaining higher cell viability in the harsh environment of the GIT [27]. This was confirmed in our previous study, in which L. casei 01 was micro-encapsulated in a mixture of ALG and SPI, constituents generally recognized as safe, using spray-drying. Stable MPs with high percent yield (approx. 64.4±5.4%) and probiotic loading (approx. 11 log CFU/g) were prepared, with narrow size distribution (\( d_0 = 9.7 \mu \text{m}, \text{span factor} = 0.47 \)). The probiotic loaded SPI-ALG MPs obtained from the optimal formulation had a spherical shape but a disk shape as well, showing low level of humidity (0.8±0.1%), solubility of approx. 95.0±0.2%, wettability of 100% in 5 min, as well as swelling and mucoadhesion properties, and in vitro release profile suitable for colon delivery [20].

Tablets formulation and evaluation: probiotic viability after tableting and physicochemical properties

The next step was to design tablet formulations able to protect entrapped probiotic bacteria from the gastric acidity and provide release near or in the colon. For that reason, MPs were characterized for their flow and packing properties. The results from the flowability characterization of the MPs indicated a fair flow character, with a compressibility index and Hausner ratio being 19% and 1.23, respectively. Therefore, excipients such as Aerosil, magnesium stearate and talc were added to the MPs, having in regard their function as gliding/anti-caking agents and lubricants [28]. In this manner, good flowability and compressibility properties of the blends were achieved, with the compressibility index and Hausner ratio being 14 and 1.16%, respectively.

Two series of tablets were prepared containing as additional excipients cellulose ester, CAP (series I), and cellulose ether, HPMC (series II), respectively. For comparison, a series of tablets with and without lactose as additional excipient were also prepared (series III and IV, respectively). Lactose preserves high probiotic viability during storage and administration based on the evidence that most of the probiotics utilize lactose as an energy source for their growth [29].

The probiotic viability in the different series of tablets and their physicochemical properties are presented in Table 2. In the series of tablets designed to retard the probiotic release in the intestinal stage (I and II), the compaction of the probiotic loaded SPI-ALG MPs caused viability decrease of 1.5±0.08 log cycles, from 10.63±0.28 log CFU/g (average value of viability in the powders for tableting) to 9.01±0.32 and 9.17±0.28 log CFU/g viability determined for series I and II, respectively (Table 2). A lower viability decrease was observed in the series of tablets containing or not lactose as excipient (series III and IV, respectively; average value of 0.92±0.13 log cycle).
Generally, monolithic matrix type tablets were prepared, with the probiotic MPs evenly distributed through them. The decrease of the void spaces and fragmentation of some MPs, which probably occurred during the compaction, may explain the decrease of up to 1.5 log cycle in the number of viable cells of \(L.\) casei 01 in the tablets. The compaction had a slightly more negative impact on the probiotic viability in comparison with the previously reported results for tab-leted whey protein MPs with Lactobacillus strain [12], where lower decrease in the viability of the probiotic was observed (up to 1 log unit), with no increase in the damages on the bacterial cells with increasing compaction force from 10 to 39 kN. Moreover, when bacteria-loaded pellets, primarily prepared with hydroxypropyl methylcellulose acetate succinate, were compressed into multi-unit tablets, no significant difference in the probiotic viability was observed before and after compression [17]. Similar results to this study were obtained by Nagashima et al. [16], where the viability of \(L.\) acidophilus in the probiotic effervescent tablets decreased for 2 log units when the compres-sion force reached over 20 kN. Brachkova et al. [30] reported similar decrease in the viability (< 2 log units) of Lactobacillus spp. loaded in mini-tablets, pre-prepared with or without microcrystalline cellulose and inulin by applying compaction force from 1 to 5 kN.

Considering the physical properties, all series of tablets met the requirements of Ph. Eur. 9.0 [23]. The values for the mean mass of series of tablets I-III (prepared of equal amounts of excipients) were between 599 and 617 mg, lower mean mass was understandable measured for series IV (488 mg). In all series, none of the individual tablet masses deviated from the average mass by more than 5%.

The mean hardness values exceeded 30 N as a minimum recommended hardness, being between 69 N (series III) and 96 N (series II) and again, much lower for series IV (31 N). These results kept correl-ation with the lowest porosity and highest resistance, which was confirmed by the disintegration time for series II and III as well. The tablets from series II required longer time to disintegrate into the PBS (pH 6.8), 2.75 h vs. 0.75 h for the tablets in series IV.

### Viability/resistance of probiotic

As expected, the disintegration profiles correlated with the probiotic release/resistance to simulated gastrointestinal conditions in the different series of tablets. In Table 3, the number of viable cells of \(L.\) casei 01 in the tablets when exposed to simulated gastrointestinal conditions is presented. The number of viable cells of \(L.\) casei 01 in the tablets labeled as series II did not change significantly upon exposure to simulated gastrointestinal conditions throughout 2 h (between 0 and 120 min). Similar data after 4 h exposure were obtained, with non-significant change of the probiotic viability. The number of viable cells decreased for approx. 0.5 log cycle, however, the tablets maintained the required minimum of viable probiotic cells for exerting the health effects \((10^7\ \text{CFU/g at a point of delivery})\). The same viability was preserved with the series of tablets prepared of CAP as excipi-ent (series I), although significant decrease in viability upon exposure to simulated gastrointestinal conditions was observed throughout 4 h \((p < 0.05)\) unlike this, the probiotic viability in the tablets loaded with SPI-ALG MPs alone (series IV) and those with SPI-ALG MPs and lactose (series III), significantly decreased upon exposure to simulated gastrointestinal fluids throughout 2 h \((p < 0.05)\), being near or below \(10^7\ \text{CFU/g}\).

At the end of the 4th hour, a complete disinteg-ra- tion of the tablets was observed. However, viable cells of \(L.\) casei 01 in the tablets after exposure to simulated ileum conditions (at the end of the 4th hour) were detected only in series I and II, confirming that the designed tablets with CAP or HPMC are suitable to protect the probiotic from gastric pH and harsh con-ditions in duodenum and ileum. Low diffusion of the

<table>
<thead>
<tr>
<th>Physical properties and probiotic viability</th>
<th>Series</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average tablet mass, mg, mean ± SD</td>
<td></td>
<td>599±3.67</td>
<td>617±3.24</td>
<td>603±4.31</td>
<td>488±3.07</td>
</tr>
<tr>
<td>Diameter, cm, mean ± SD</td>
<td></td>
<td>1.22±0.02</td>
<td>1.23±0.01</td>
<td>1.22±0.01</td>
<td>1.22±0.01</td>
</tr>
<tr>
<td>Height, cm, mean ± SD</td>
<td></td>
<td>0.54±0.01</td>
<td>0.54±0.02</td>
<td>0.52±0.01</td>
<td>0.51±0.01</td>
</tr>
<tr>
<td>Hardness, N, mean ± SD</td>
<td></td>
<td>90.2±0.5</td>
<td>95.6±0.6</td>
<td>68.9±0.6</td>
<td>31.2±0.6</td>
</tr>
<tr>
<td>Tensile strength, mean ± SD</td>
<td></td>
<td>0.96±0.01</td>
<td>1.01±0.01</td>
<td>0.73±0.01</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td>Disintegration in 0.1 M HCl, pH 1.5, h</td>
<td></td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Disintegration in PBS, pH 6.8, h</td>
<td></td>
<td>1.75</td>
<td>2.75</td>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>Viability of (L.) casei 01, log CFU/g</td>
<td></td>
<td>9.01±0.32</td>
<td>9.17±0.28</td>
<td>9.58±0.13</td>
<td>9.84±0.25</td>
</tr>
</tbody>
</table>
gastric medium into the tablets due to CAP (series I) or HPMC (series II), the lower specific area of the tablets in comparison with the probiotic MPs, and subsequently the reduced contact between the probiotic cells and the medium probably contributed to preserve the probiotic viability within the required minimum of viable probiotic cells (10^6 CFU/g) at a point of delivery. Slightly longer disintegration time and higher viability of L. casei 01 throughout 4 h in series of tablets II was observed, supporting the findings of Klayraung et al. [13], where disintegration time of approx. 5 h (2 h in acidic medium and 3 h in PBS pH 6.8) was most suitable for probiotic delivery.

The differences in disintegration time/probiotic resistance can be explained by the differences in the disintegration mechanisms and/or excipients added to control the probiotic delivery. Cellulose esters, such as CAP, play a vital role in the development of modern drug delivery technology, addressing critical patient needs. Properties of CAP, such as very low toxicity, endogenous and/or dietary decomposition products, stability, high water permeability, high T_g, film strength, compatibility with a wide range of actives, and the ability to form micro- and nanoparticles have enabled creation of a wide range of drug delivery systems employing it as a key ingredient. It was included in the composition of the tablets due to its resistance to acidic gastric fluid and solubility in mildly alkaline medium of the intestine (pH > 6), i.e., to prevent probiotic release in the gastric medium and provide delivery near the colon. As an excipient that does not swell (no swelling of the tablet matrix was observed), it imparts its disintegrating/release action through porosity and capillary action. Therefore, when placing the probiotic tablet into the gastric medium (pH 1.5), the medium penetrated insignificantly into the tablet due to CAP. However, when the tablet was placed into the PBS pH 6.8, the intermolecular bonds of CAP started to weaken (erosion of matrix without swelling was visible) and the tablet broke into fine particles, thus releasing the probiotic/probiotic MPs.

On the other hand, HPMC is a nonionic carbohydrate polymer which dissolves in water by swelling and subsequent hydration, with no sharp solubility limit. The presence of HPMC throughout the tablet caused hydration on the outer tablet surface and forming of a hydrogel layer. Upon complete hydration, chain disentanglement occurred, i.e., erosion of the matrix, facilitating the probiotic/probiotic MPs release. In addition, enzyme resistance and stability of HPMC over a pH range of 2.0 to 13.0 contributed to prolonged tablet disintegration and retardation of probiotic release in comparison with the tablets containing CAP and also in comparison with the series of tablets III and IV, in which no gastro-resistant excipients were present.

**Storage stability**

Storage stability/viability of probiotic bacteria is one of the major criteria governing the efficiency of pharmaceutical excipients and probiotic dosage forms. In all series of tablets, microencapsulated L. casei 01 manifested significant susceptibility to the storage conditions over the storage period tested. Namely, relatively low viability of L. casei 01 in the tablets after 60 days of storage was observed, not exceeding 10^6 CFU/g (data not shown!). The evolution of viable cells of L. casei 01 in the tablets throughout 42 days at 25±2 °C (RH = 60±5%) is presented in Fig. 1. Over this period of storage, the number of viable cells of L. casei 01 in the tablets significantly decreased (p < 0.05), for an average of 3

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Table 3. Viability of L. casei 01 (mean ± SD; n = 3) in tablets and in MRS broth after tablet preparation throughout the simulated gastrointestinal conditions

<table>
<thead>
<tr>
<th>Gastrointestinal compartment</th>
<th>Sampling min</th>
<th>Series I Tablets log CFU/g</th>
<th>MRS solution log CFU/mL</th>
<th>Series II Tablets log CFU/g</th>
<th>MRS solution log CFU/mL</th>
<th>Series III Tablets log CFU/g</th>
<th>MRS solution log CFU/mL</th>
<th>Series IV Tablets log CFU/g</th>
<th>MRS solution log CFU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0</td>
<td>9.01±0.32</td>
<td>ND^a</td>
<td>9.17±0.28</td>
<td>ND^a</td>
<td>9.58±0.13</td>
<td>ND^a</td>
<td>9.84±0.25</td>
<td>ND^a</td>
</tr>
<tr>
<td>Mouth (pH 6, 37 °C, 200 rpm)</td>
<td>90</td>
<td>8.39±0.24</td>
<td>ND^a</td>
<td>8.64±0.22</td>
<td>ND^a</td>
<td>7.47±0.12</td>
<td>ND^a</td>
<td>7.15±0.23</td>
<td>ND^a</td>
</tr>
<tr>
<td>Duodenum (pH 2-5, 37 °C, 45 rpm)</td>
<td>120</td>
<td>7.47±0.31</td>
<td>3.55±0.28</td>
<td>8.73±0.28</td>
<td>5.12±0.23</td>
<td>7.06±0.28</td>
<td>4.17±0.63</td>
<td>6.65±0.18</td>
<td>4.35±0.27</td>
</tr>
<tr>
<td>Ileum (pH 5-6.5, 37 °C, 45 rpm)</td>
<td>240</td>
<td>7.11±0.22</td>
<td>7.30±0.14</td>
<td>8.64±0.27</td>
<td>7.47±0.21</td>
<td>ND^a</td>
<td>6.39±0.56</td>
<td>ND^b</td>
<td>6.08±0.11</td>
</tr>
</tbody>
</table>

^aNo viable cells detected in first decimal solution; ^btablet completely dissolved/disintegrated
log cycles. Non-significantly higher viability of \textit{L. casei} 01 in tablets of series II was observed, where at the end of the noted period of storage, approx. 76% of initial viability was preserved. The values of viable cells in other series of tablets at the end of the storage period were similar, with 63-66% preserved viability from the initial cell count. High correlation (0.969 ≤ \( R \) ≤ 0.999) between the values of viable cells (log CFU/g) vs. storage time (days) was determined in all series of tablets, demonstrating that further research should be done in order to optimize \textit{L. casei} 01 viability over longer storage periods.

Many other studies have shown that the majority of probiotics lose their feasibility over time, even at room temperature, and especially in the case of bare bacteria or compressed uncoated living bacteria with other compounds. This was observed in a study by Park et al. [17], in which the survival rate of \textit{L. acidophilus} after one-month storage was only 47% and <0.1% when compressed with several pharmaceutical cushioning agents, such as microcrystalline cellulose, lactose monohydrate, corn starch and calcium silicate. However, when enteric coated pellets of \textit{L. acidophilus} embedded in tablets were exposed to 25 °C/60% RH, the survival rate was 99 and 42% after 1 and 6 months of storage, respectively.

Lots of studies prove that the microencapsulation can extend the storage time, pointing out that lower temperatures and values of relative humidity probably extend the storage time. This was confirmed in a study by Rodrigues et al. [31], in which high survival rate within a storage period of 180 days of \textit{L. paracasei} L6 in whey-protein MPs was provided (viable cells above 10^7 CFU/g) when exposed to 25 °C/12% RH. However, when the direct compression tablets contained of these MPs, croscarmellose sodium and cellulose phthalate were exposed to 23 °C/33% RH, the number of viable cells decreased by approx. 70% (2 log cycles). The same result was observed with the actual \textit{L. casei} 01 loaded in SPI-ALG MPs. The survival rate in the MPs was maintained nearly 80% after four-month cold storage, decreasing by 2 log units [20]. However, when the tablets of the probiotic SPI-ALG MPs were stored at 25 °C/60% RH, the survival rate of \textit{L. casei} 01 after 42 days was between 63 (series I and IV) and 76% (series II), decreasing by averagely 3 log units, as pointed out. Again, the highest survival (≥ 10^6 CFU/g) during this period of storage was observed in tablets of series II, the series in which HPMC as excipient was included. Whether these results are related to the “antibacterial effect” of the polymers used remains to be explored. As literature data indicate, cellulose-based polymers substituted with carboxylic acids such as CAP manifest antimicrobial and antiviral properties [32]. CAP appeared effective on HIV-1 and several herpes viruses (HSV), and in micronized form on cytomegalovirus, \textit{Neisseria gonorrhoeae}, \textit{Trichomonas vaginalis}, \textit{Haemophilus ducreyi} and \textit{Chlamydia trachomatis}. However, it did not affect \textit{Lactobacilli} of the natural vaginal flora [33]. No antimicrobial effect was observed with HPMC when, for example, the activity of HPMC films was tested using \textit{L. monocytogenes}, \textit{E. coli} O175:H7 and \textit{S. aureus} as test microorganisms [34,35].

Specifically, high probiotic viability in the series of tablets containing lactose (series III) at the end of the 2nd week of storage was observed, approx. 87.5% from the initial cell load. This is not surprising, knowing the role of lactose in growth of \textit{Lactobacilli} species. Namely, lactose is transferred into the bacteria’s cell by membrane bounded enzyme β-galactoside permease, and there it is hydrolyzed to glucose and galactose by β-galactosidase [36]. Once lactose is hydro-
lyzed, several processes occur: glucose is glycosylated to pyruvate by lactic dehydrogenase and converted to lactic acid, while on the other hand, galactose is phosphorylated to galactose 1-phosphate by galactokinase and converted to glucose 1-phosphate and galactose 6-phosphate. For most species, lactic acid and galactose come out of the cell, but for some strains galactose is metabolized to lactic acid as well. This was confirmed in many studies in which different probiotic formulations were prepared containing lactose [19,37].

CONCLUSION

Novel direct-compress-tablets of microencapsulated probiotic bacteria and CAP or HPMC as excipients were prepared in order to increase the viability in the acidic conditions of probiotic cells during administration and providing delivery near the colon. Direct compression of probiotic MPs into tablets provided the survival rate of bacterial cells at least 10⁹ per dose unit during compaction and satisfactory functional tablet parameters. The tablets containing L. casei 01 loaded SPI-ALG MPs and HPMC showed higher potential for preserving probiotic viability in simulated gastrointestinal fluids and retarding its release in the intestinal stage as well as the most acceptable storage stability at 25°C/60% RH for 42 days. However, further research should be done to optimize the probiotic viability over long storage periods. According to the presented results, a combination of the excipients used, including lactose, and modification of the compaction force could be a suitable approach for optimizing probiotic tablet formulation.

REFERENCES

TABLETE MIKROČESTICA SOJIN PROTEIN-ALGINAT SA Lactobacillus casei 01: FIZIČKO-HEMIJSKA I BIOFARMACEUTSKA SVOJSTVA

Cilj ovog rada bio je da se razviju direktno-komprimovane tablete mikrokapsuliranog probiotika Lactobacillus casei 01 u mikročesticama sojin protein-alginat i pomoćnih supstanci koje mogu da obezbede probiotičku isporuku u blizini debelog creva. S obzirom na njihova fizičko-hemijska svojstva, sve serije pripremljenih tableta ispunjavale su zahteve Ph Eur 9.0. Kompaktnost mikročestica sa probiotikom odvela je do smanjenja viabilnosti do 1,5 log ciklusa. Tablete koje sadrže Methocel K1 00M pokazale su veći potencijal za očuvanje viabilnosti probiotika u simuliranim gastrointestinalnim tečnostima u roku od 4 h i njegovo odloženo oslobađanje u crevima održavajući potrebnim minimum živih probiotskih čelija iznad 10^7 CFU/g tablete. Pored toga, primećena je prihvatljiva stabilnost skladištenja (održivost probiotika iznad 10^6 CFU/g) pri 25°C 60% relativne vlažnosti tokom 42 dana. Kao zaključak, pripremljeni su novi tabletni oblici mikrokapsulisanog L. casei 01 sa visokim potencijalom za očuvanje viabilnosti probiotika u simuliranim gastrointestinalnim tečnostima i zaustavljanjem njegovog otpuštanja u donjem crevu. Potrebna su dalja istraživanja da bi se optimizovali formulacija i parametri procesa kako bi se dobile tablete sa viabilnošću probiotika tokom dugog perioda skladištenja.

Ključne reči: Lactobacillus casei 01, mikročestice sojin protein-alginat, tablete, fizičko-hemijska svojstva, biofarmaceutska svojstva.