

Approaches for Improvement in Digestive Survival of Probiotics, a Comparative Study

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The aim of this study was to compare approaches commonly recommended in the literature for the improvement of the survival of probiotics in the human digestive tract. The survival of two probiotics, *Lactobacillus casei* and *Bifidobacterium lactis*, in the presence or absence of prebiotics, maize starch, fermented milk and upon encapsulation in calcium alginate-chitosan was evaluated. While *B. lactis* was resistant to stomach juice, but sensitive to duodenal juice, *L. casei* showed an exactly opposite behaviour. In contrast to other published studies, here the overall digestive survivability of probiotics was not improved significantly by prebiotics, maize starch or encapsulation. However, a significant improvement of the overall survivability of *B. lactis* (but not *L. casei*) during *in vitro* digestion was noted in milk and fermented milk, possibly due to reduction of the activity of bile against this probiotic. Summarising, no one method could be universally recommended for the improvement of probiotic survivability. Nevertheless, this research indicated that certain probiotic characteristics, such as susceptibility to bile or acid or ability to utilise matrix components as an energy source, could be used in further research to select the most effective approaches to deliver viable cells into lower parts of the digestive tract.

INTRODUCTION

Delivering live probiotics to the lower part of the human digestive tract, when administered orally, presents a challenge. Low stomach pH and action of bile salt hamper their survival [Bezkorovainy, 2001].

According to multiple studies, the survivability of probiotics in the digestive tract can be improved by application of a food matrix or addition of a prebiotic, or encapsulation [Burgain *et al.*, 2011; Govender *et al.*, 2014; Kingwatee *et al.*, 2014; Sanchez *et al.*, 2014]. As yet, these three methods were not confirmed to ensure effective survival of multiple strains of probiotics. Previous studies have examined the behaviour of only one or very few probiotics without consideration for differences in their survival characteristics [e.g. Burgain *et al.*, 2011; Haghshenas *et al.*, 2015; Kingwatee *et al.*, 2014; Lo Curto *et al.*, 2011; Sanchez *et al.*, 2014; Sumeri *et al.*, 2010]. Additionally, these studies varied in the application of *in vitro* digestion models which could be dynamic or static and applied different pH, digestion time, and chemical concentrations at each stage of digestion, making comparison difficult. Therefore, based on such data it is not possible to distinguish

the method for oral route delivery of probiotics, with the best protective effect. This is important since many of the commercial probiotic formulations combine multiple strains.

The few existing studies comparing different approaches for the improvement of probiotic survivability focus on combined effects, e.g., encapsulation with addition of elements of food matrix or prebiotic [Fredua-Agyeman & Gaisford, 2015; Shori, 2017]. Such studies also do not allow a conclusion as to whether there is a general method to improve the digestive survivability of probiotics.

Therefore, in this study we compared three methods previously shown to improve probiotic survival and two probiotic strains featuring different sensitivity to digestive juices.

The three compared methods were:

- Food matrix inclusion (fresh and fermented milk, maize starch),
- Different prebiotic concentrations (inulin and FOS),
- Electrospray encapsulation in calcium alginate and chitosan.

The chosen probiotic strains were *Lactobacillus casei* W56 and *Bifidobacterium lactis* W52, which according to the manufacturer should feature different sensitivities to gastrointestinal conditions. *L. casei* W56 was sensitive to stomach juice but unstable in duodenum juice (unlike *B. lactis* W52).

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Thus, it was possible to test the protective effect of the applied methods to probiotics in stomach and duodenal conditions. Ideally, the method which would be generally recommended for the improvement of the probiotic survival should be effective in both types of digestive juices. Testing whether such an approach was available from those recommended by the literature was the subject of this study.

MATERIALS AND METHODS

Bacterial strains, materials, and reagents

Two probiotic strains used in the study, *B. lactis* W52 and *L. casei* W56, in a powdered form, were obtained from Winclove Probiotics (Amsterdam, Netherlands). Based on in-house experiments conducted by Winclove Probiotics, it was expected that *B. lactis* W52 would survive the *in vitro* digestion better compared to *L. casei* W56. Freeze-dried organisms were kept at 4°C prior to use. Winclove Probiotics supplied commercial prebiotics – Fructo-oligosaccharide (FOS P1) (hereafter FOS), and inulin containing a small quantity of FOS (Inulin and FOS P7) (hereafter inulin), and a probiotic supplement carrier material (Maize Starch) that were used as received. Full fat milk (composition per 100 mL: fat 3.6 g, sugar 4.7 g, and protein 3.4 g; pH 6.8) was purchased from a local supermarket. Sodium chloride, sodium bicarbonate, potassium chloride, calcium chloride, porcine pepsin, porcine pancreatin, and porcine bile used for the digestive assay were obtained from Fisher (Loughborough, UK). Sodium alginate, chitosan, and calcium carbonate used in probiotic encapsulation were purchased from Avonchem limited (Macclesfield Cheshire, UK), Acros Organics (Morris Plains, USA) and Fisher (Loughborough, UK), respectively.

Preparation of samples challenged to *in vitro* digestion

Portions of approximately 0.1 g of probiotic powders were combined with prebiotics (at four levels of concentration: 0.1, 0.5, 1, and 5%, w/w) or maize starch (5%, w/w) by mixing in sterile tap water. Probiotics were added aseptically. The count of cells in the initial sample was above 8 log cfu/mL (count of viable cells in each sample is presented in the Results and Discussion section).

To allow resuscitation of probiotics, samples were kept for 15 min at room temperature prior to analysis and commencement of the simulation of digestion. The highest concentration of prebiotic was chosen to reflect the dose that was previously shown to have a beneficial effect to the human health [Kellow *et al.*, 2014]. Fermented milk was prepared by the incubation of sterile milk (150 mL) with the addition of ~0.1 g of probiotic powder with either *L. casei* W56 or *B. lactis* W52 for 18 h at 40°C. The pH of milk following fermentation was 4.3 for *L. casei* W56 and 4.7 for *B. lactis* W52. Control samples contained only sterile tap water and probiotics.

For probiotic encapsulation, we have chosen calcium alginate additionally coated with chitosan. Calcium alginate is a frequently used encapsulation material, since it is food-grade and enables release of the probiotics in intestines [Segale *et al.*, 2016]. However, it tends to create porous capsules, which may mean that the probiotics would not be protected from the contact with digestive juices [Burgain *et al.*,

2011]. To minimise this effect and enhance the survival, calcium alginate capsules were coated with chitosan following the example of Shori [2017].

Consideration was also given to the method of capsule preparation. One of the novel methods being increasingly applied in research studies is electrospraying, where voltage is used to extrude a polymer solution through a capillary, resulting in a formation of small droplets [Coghetto *et al.*, 2016]. This method is suitable for use with probiotics as compared to some other methods, such as *e.g.* spray-drying, it does not require high temperatures.

For encapsulation, probiotics were mixed into the 22.5 g/kg sodium alginate solution. They were either added to the sodium alginate as a supplied freeze-dried powder (~0.1 g of powder to 5 mL of alginate; powder) or upon previous resuscitation (broth). The resuscitation was carried out by subsequently:

1. Inoculating 150 mL de Man Rogosa Sharpe (MRS) broth with ~0.1 g of probiotic powder and incubating for 24 h at 37°C (*B. lactis* was grown in MRS broth supplemented with 0.5 g/L L-cysteine),
2. Harvesting cells from 40 mL of the broth by centrifugation at 1500×g for 15 min at 25°C,
3. Washing and centrifuging the pellet twice with the saline solution using same settings as in 2., and
4. Re-suspending the pellet in 3 mL of the saline solution and adding to 20 mL of the alginate solution.

Suspensions of probiotics in sodium alginate were then electrosprayed. The electrospraying process has been explained in the previous publication [Zaeim *et al.*, 2017]. The equipment used for electrospraying was provided by Electrospin Ltd (Blenheim, New Zealand). The set up was composed of a polymer header tank, a hollow needle with a 0.06 mm internal diameter, and a dish collector grounded through a crocodile clip. The solution was electrosprayed at 8 kV and the distance between the needle and the collector was 8 cm. The polymer/ probiotic solution was placed in the header tank and flowed under gravity to the needle. Droplets were electrostatically attracted into a dish collector which contained 500 mM calcium carbonate solution at pH 5.2. About 0.5 g of calcium alginate droplets encapsulating probiotics were obtained during a single 2 h run. Capsules were then filtered through a filter paper (Whatman no 4, Fisher, Loughborough, UK), rinsed with sterile water, and further coated with chitosan.

For coating, 20 mg of chitosan was dissolved in 2 mL of 100 mL/L glacial acetic acid and the pH was raised to ~6.0 by adding 0.5 M NaOH. Alginate micro beads were immersed in the chitosan solution and stirred at 60 rpm for 40 min using an orbital shaker (LSE, Corning, New York, US). This procedure was adapted from a method by Sohail *et al.* [2011].

The coated capsules were then filtered, washed twice with sterile water, and placed in a fresh portion of sterile water. The capsule suspension was stored for up to 2 days at 4°C prior to digestion assay.

Capsules were prepared in triplicate and characterised by means of the optical microscope (MOTIC B1 Advanced Series with MotiC Images Plus version 3 software for im-

age analysis). An example image of these capsules is shown in Figure 1. The prepared capsules contained probiotics at a level of $>6 \log \text{ cfu/g}$.

In vitro digestion model

To investigate the survivability of probiotics in the human digestive tract, a model designed based on several studies was applied. The composition and proportions of the digestive juices were adapted from studies of Marteau *et al.* [1997] and Timmerman *et al.* [2007] and were given in Table 1. The chosen transit times were 30 min in the stomach, 1 h in the duodenum and 2 h in the ileum. The pH during digestion was 2 for stomach and 6.5 for duodenum stage. Ileal juice was simulated by the addition of 11.5 mM of CaCl_2 to duodenum juice containing the sample in order to deactivate bile salts. Anaerobiosis during the digestion was created by overlying digestive liquids with 5 mL of mineral oil. Anaerobiosis seemed to be applied only in recent artificial digestion studies on the survival of probiotics, nevertheless it is a realistic condition present in the digestive tract. In our preliminary assessment, we saw a better survival of the microorganisms, especially *L. casei* W56 in the stomach juice, when anaerobiosis was applied. Stomach and duodenal juice were prepared and warmed up to 37°C prior to experimentation. Artificial digestion was carried out at 37°C with a constant agitation on an orbital shaker (LSE, Corning, New York, US) at 60 RPM.

Liquid samples were added in volumes of 5 mL, whilst capsules in quantities of $\sim 0.5 \text{ g}$ suspended in 5 mL of sterile deionised water to digestive juices. The pH of digestive juices was measured and re-adjusted upon the addition of the samples.

In vitro digestion of each sample was carried out in triplicate.

Enumeration of probiotics

Enumeration of lactic acid bacteria in the samples was carried out using the ISO 15214:1998 method. Briefly, samples were serially diluted in buffered peptone water (BPW, Oxoid, Basingstoke, England, CM0509). The diluted samples were then transferred in volume of 1 mL to empty Petri dishes and mixed with $\sim 15 \text{ mL}$ of de Man, Rogosa, Sharpe agar (MRS, Oxoid, Basingstoke, England, CM0361). Growth of *B. lactis* W52 was encouraged by the addition of 0.5 g/L of L-cysteine into MRS agar. Plates were incubated at 37°C for 72 h in 150 mL/L CO_2 , $<10 \text{ mL/L O}_2$ and N_2 atmosphere created with the MULTIVAC T200 tray sealer (Multivac, Wolfertschwenden, Germany). The limit of detection for the method was 1 cfu/mL of digestive juice. Results below the limit of detection were included in statistical analysis as 0.5 cfu/mL.

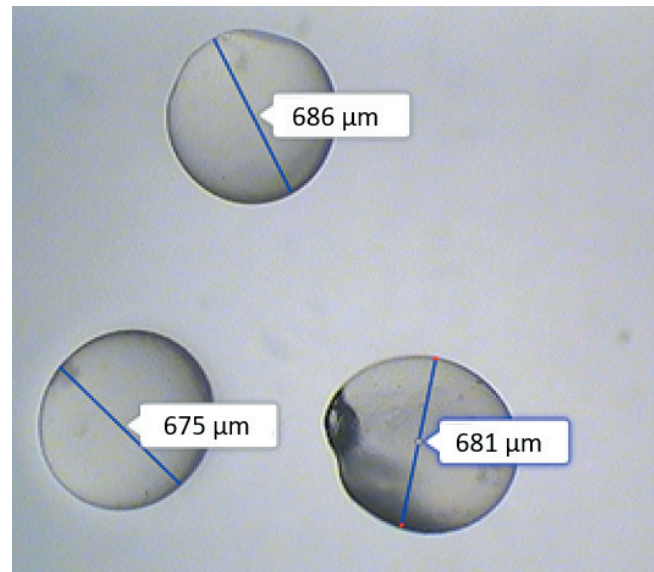


FIGURE 1. Light microscopy image of capsules obtained in the process of electro spraying.

Calcium alginate-chitosan capsules were visually intact through the entire digestion process. Hence, prior to enumeration, cells were released from the capsules. This was carried out by stirring encapsulated probiotics in 10 mL of 0.1 M phosphate buffer at pH 7 at room temperature for 30 min on a magnetic stirrer. The release of the probiotics from capsules has been confirmed by preliminary trials, where the count of viable cells was determined at different time points during mixing.

Statistical analysis

Statistical tests were performed using IBM SPSS Statistics 22. All assumed a significance level of 0.05. Particular tests are mentioned in the Results and Discussion section next to relevant data.

All log reduction values quoted in the text and shown in the figures were corrected for the dilution factors caused by the addition of stomach and duodenum juice to the samples within the digestive assay.

RESULTS AND DISCUSSION

The survival of probiotics during *in vitro* digestion

Survival curves of *L. casei* W56 and *B. lactis* W52 during passage through digestive liquids in control sample, as well as in the presence of prebiotics, food matrices, and in encapsulates was presented in Figure 2. Statistical tests (repeated measures

TABLE 1. Composition of simulated stomach and duodenal juice.

	NaCl	NaHCO_3	KCl	CaCl_2	Porcine pepsin	Porcine pancreatin	Porcine bile
	(g/L)						
Stomach	6.2	1.2	2.2	0.22	3.2	–	–
Duodenum	5	–	0.6	0.25	–	9	14

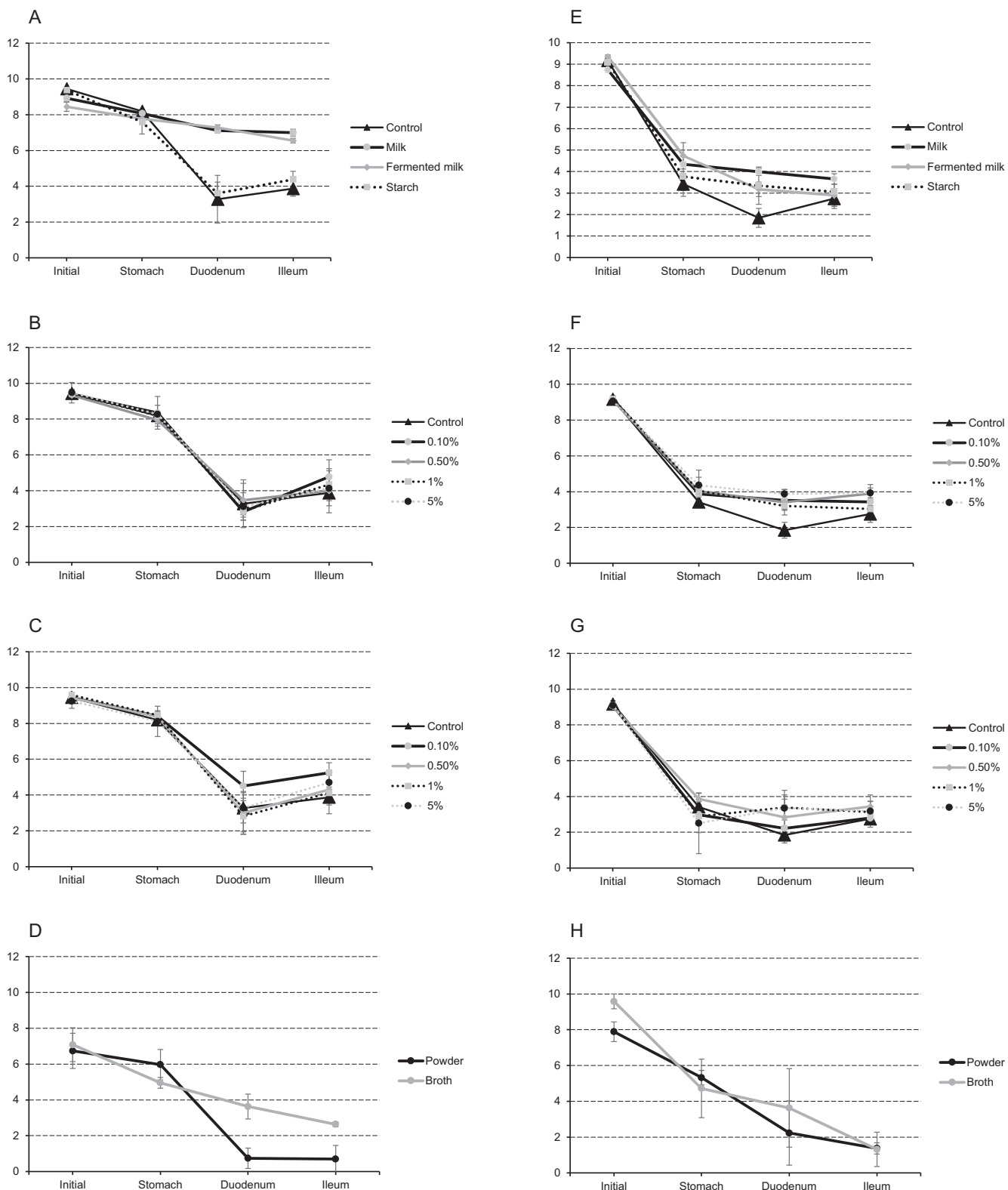


FIGURE 2. Survival during *in vitro* digestion of *B. lactis* W52 (graphs A, B, C and D) and *L. casei* W56 (graphs E, F, G and H) in presence of (A and E) food matrices, (B and F) inulin, (C and G) FOS, and (D and H) in capsules. Errors bars correspond to standard deviation. Counts are expressed per mL of initial solution. Dilutions factors from the addition of juices during the experiment were not compensated for.

ANOVA with Bonferroni post-hoc, results were not presented), indicated that stage in the digestion process had a significant effect on the log reduction of probiotic population. The digestion stage where the largest log reduction occurred was stomach for *L. casei* W56 (mean log reduction \pm standard deviation, 4.4 ± 1.1 log cfu) and duodenum for *B. lactis* W52 (3.9 ± 1.9 log cfu). At the same time, *L. casei* W56 seemed relatively resistant to duodenum juice (0.6 ± 1.4 log cfu) and *B. lactis* W52 to stomach juice (0.5 ± 0.5 log cfu). The ileum juice offered the gentlest conditions for both probiotics (0.2 ± 1.1 and -0.7 ± 1.2 log cfu for *L. casei* W56 and *B. lactis* W52 respectively, negative log reduction indicated growth).

For *L. casei* W56, the survival curves representing control, food matrices, and all levels of prebiotics followed a similar pattern. Encapsulated, freeze-dried *L. casei* W56 seemed to reduce at similar rates through all digestive stages, indicating that the approach was able to minimise the effect of stomach juice on the probiotic. However, this trend was not observed in broth-grown, encapsulated *L. casei* W56, where a greater decline of probiotic population in the stomach was observed. The result indicates that the form in which *L. casei* W56 was encapsulated, rather than the encapsulation, had an influence on its survivability in the stomach. In turn, *B. lactis* W52 behaved similarly to the control when probiotic was challenged to digestive assay in the presence of maize starch, prebiotics at all concentration levels as well as within capsules containing probiotic powder. The survival curves of *B. lactis* W52 in the presence of milk and fermented milk as well as upon encapsulation of the broth-grown probiotics, resembled a straight line, meaning that the decline of the probiotics was similar through all the digestion stages. Out of these three treatments, encapsulation of the broth-grown probiotics seemed to feature a steeper decline for the population of *B. lactis* W52 compared to when the probiotic was challenged in milk or fermented milk.

Presented survival curves are real log cfu/mL counts disregarding the dilution of the probiotics by the addition of stomach and duodenum juices. To compare the effectiveness of different approaches, total log reductions were calculated based on the concentration of probiotics in the first (initial) and after passage through the last (ileum) stage correcting for dilution factors resulting from the addition of digestive juices (see Figure 3).

Total log reductions for *L. casei* W56 ranged from 4.2 to 7.4 log cfu (milk and capsules containing broth-grown probiotics, respectively) and for *B. lactis* W52 from 1.0 to 5.1 log cfu (fermented milk and capsules containing probiotic powder, respectively). For *L. casei* W56, there was no treatment which decreased the total log reduction significantly compared to the control. However, encapsulation of broth-grown probiotics caused a significant ($p < 0.05$) increase of the total log reduction compared to the control and all other treatments. On the other hand, a significantly ($p < 0.05$) greater survival of *B. lactis* W52 was obtained in the presence of milk and fermented milk compared to the control and all the other treatments.

Overall, mean total log reductions were significantly higher for *L. casei* W56 compared to *B. lactis* W52 (paired for treatments t-test, $p < 0.05$), indicating that *B. lactis* W52 was more resistant to conditions of the digestive tract than *L. casei* W56.

This was in line with the suggestion given by the probiotic provider, as specified in the method section.

The effect of probiotic type on the survival during *in vitro* digestion

This study evaluated survivability of two different probiotic bacteria, *L. casei* W56 and *B. lactis* W52, in human digestive juices. Other works suggest that these two probiotic species might exhibit contrasting survival in human digestive tract, although it should be noted that each of these works used a different *in vitro* digestion design [Fávaro-Trindade & Grosso, 2002; Kingwatee et al., 2014; Lo Curto et al., 2011].

Here, by application of a single *in vitro* digestion design for these two probiotic species, we had an opportunity to verify the difference in their survival. We have found that overall *B. lactis* W52 survived better compared to *L. casei* W56. Nevertheless, the magnitude of the differences in survival was affected by the type of the matrix surrounding probiotics.

The effect of the presence of food matrices on the survival of probiotics during *in vitro* digestion

The presence of a food matrix, such as milk, may substantially improve the survival of probiotics. Several authors noted that fat could potentially enhance probiotic survival. For example, Tompkins et al. [2011] showed that probiotics (ProtecFlor®, commercial supplement containing 4 probiotic strains) survived better in 1% fat milk and oats compared to fruit juice and spring water. Furthermore, Lo Curto et al. [2011] showed that the digestive survivability of two different probiotics: *L. casei immunitas* and *L. acidophilus johnsonii*, was improved in the presence of the whole milk matrix compared to water by 6.5 and 1 log cfu, respectively. This finding indicated that different probiotics may not be equally protected by fat. In the present study, food matrices containing fat, milk, and fermented milk, improved significantly the survival of *B. lactis* W52 (on average by 3.6 and 3.7 log cfu, respectively), but not that of *L. casei* W56 (on average by 1.3 and 0.0 log cfu, respectively). Since *B. lactis* W52 was sensitive to duodenum juice, while *L. casei* W56 was comparatively resistant to it, results might point at the role of fat in the protection of probiotics from bile. Given that the task of bile is to emulsify the fat to aid its digestion, inclusion of fat into probiotic matrix could mean that the bile salts would not be free to interact with probiotic cells [Begley et al., 2005].

Based on the published literature, it was expected that fermentation of milk might add to the protective effect of the food matrix through:

1. Possible acid adaptation, especially of stomach juice sensitive *L. casei* W56, and
2. Propagation of the probiotic population from logarithmic growth phase into stationary phase.

Improvement of acid resistance might be expected, since the pH of fermented milk was lower than that of fresh milk (4.3 and 6.8, respectively). Nevertheless, in our study, the digestive survival of *L. casei* W56 was on average worse (although not significantly) in fermented milk compared to milk (total log reduction 5.5 and 4.2 log cfu, respectively). In contrast, the development of acid adaptation was demonstrated for *L. acidophilus* LA-5 and *L. rhamnosus* GG which after ex-

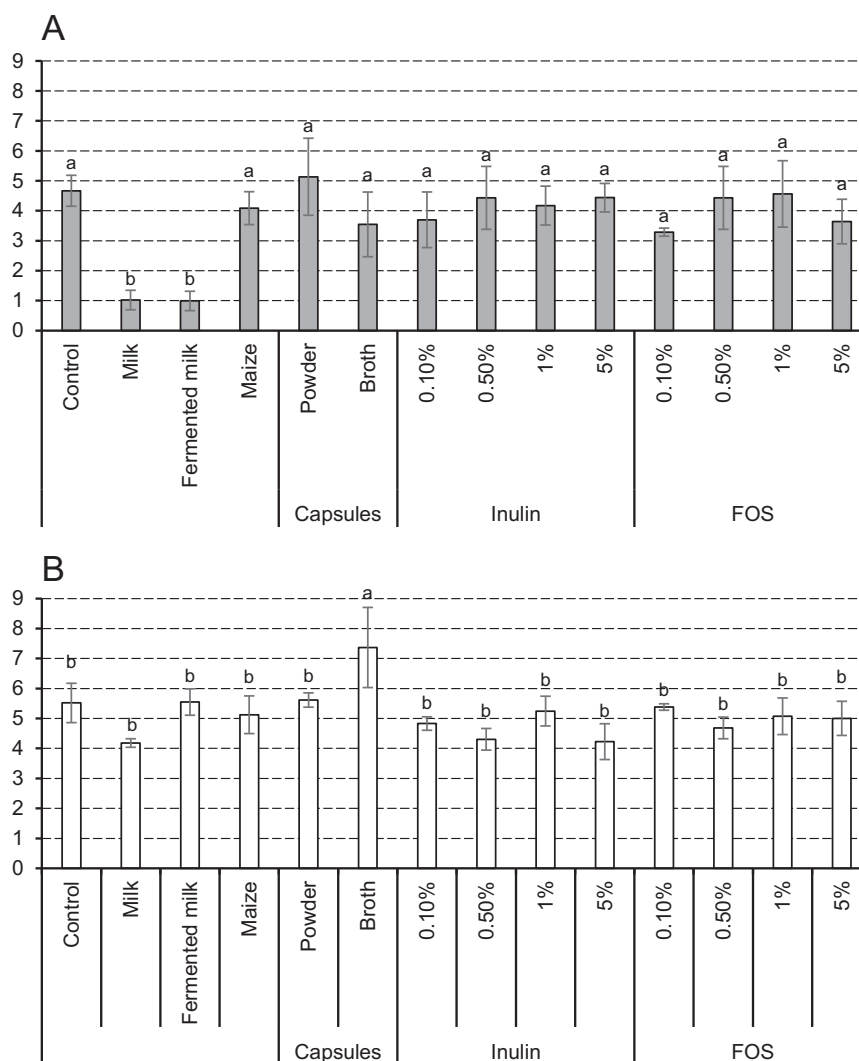


FIGURE 3. Total log reduction for (A) *B. lactis* W52 and (B) *L. casei* W56 in the *in vitro* assay. Columns note mean values and error bars standard deviations. Different letters above different columns in the same graph mean significant differences between different matrices and/or control (ANOVA and Tukey post-hoc, significance level of 0.05).

posure to low pH (3.5) prior to digestion, showed a slightly better survival in the stomach acid compared to the control (by 0.31 and 0.30 log cfu, respectively [Sumeri *et al.*, 2010]). The same study did not report acid adaptation in these strains, when probiotics were adapted at pH 4.5, which was closer to pH of fermented milk here, and hence supports our finding [Sumeri *et al.*, 2010].

In fermented milk, cells should have reached the stationary growth phase. According to Lo Curto *et al.* [2011], probiotics challenged with digestive juices survive better when in the stationary phase compared to the logarithmic growth phase. Here, this effect was not observed for either of the two tested probiotics. Some possible reasons for different results between current and the Lo Curto *et al.* [2011] study are differences in used probiotic strains, digestive assay design as well as the way in which cells have been grown into the stationary phase. In the cited study, after the addition of probiotics to milk or water, samples were maintained at 4–6°C for 6 days, while here, the milk was fermented for 18h at 40°C and samples were subjected to the digestive as-

say, without chilling. It is not clear whether cold storage could improve the resistance of probiotics to digestive juices, nevertheless some information in support of this hypothesis can be found in the literature. It is known that the temperature of cell growth will affect the expression of genes and the physiological characteristics of microorganisms [Spano & Massa, 2006]. Additionally, cases of the resistance to multiple stressors upon adaptation to a single stressor have been documented in the literature. For example, acid adaptation of *Bifidobacterium breve* resulted in a better survival during cold storage of probiotic as well as during exposure to bile and hydrogen peroxide [Park *et al.*, 1995]. Overall, these data suggested that adaptation to cold stress could be beneficial in aiding probiotic survival during passage through the upper digestive tract.

Maize starch is used by probiotic manufacturers as a carrier material at a concentration of approx. 90% in the powdered product formulation (Winclove probiotics, private communication). In this study, digestive survival of *L. casei* W56 and *B. lactis* W52 in the presence of maize starch was not improved compared to the control. However, high amylose

maize starch has been shown to enhance the survival of *Bifidobacterium* Lafti™ 8B and 13B during exposure to *in vitro* as well as *in vivo* (mice) digestion [Wang *et al.*, 1999]. Significantly, both of these probiotics had an ability to utilise amylose from maize starch. The mechanism of the improvement of the digestive survival of probiotics in the presence of food source was well explained by Corcoran *et al.* [2005]. These authors found that metabolizable sugars have been shown to improve the tolerance of *L. rhamnosus* GG to acid by provision of the energy for the exclusion of protons from cells. Summarising, studies suggest that the ability of probiotics to use a present matrix as an energy source might be a factor allowing to improve their digestive survival. This was partly supported by findings in our study. The total log reduction of stomach juice-sensitive *L. casei* W56 was on average lowest (although not significantly) in the presence of milk compared to all other treatments (including fermented milk where the lactose concentration would be lower), suggesting that the lactose present in milk could have aided survival of this probiotic in the stomach juice.

The effect of the encapsulation on the survival of probiotics during *in vitro* digestion

In the present study, we have compared two encapsulation approaches – powder and broth. Most of the reviewed studies have used freshly grown and harvested cells for encapsulation (broth method). Encapsulation of the powder might however make a commercial sense, since the population of encapsulated probiotics declines during storage [Yeung *et al.*, 2016].

Capsules obtained in this study measured between ~500 to 800 μm and on average 660 μm . This was much greater than 100 μm , the limiting size below which survival could not be facilitated [Khosravi Zanjani *et al.*, 2014]. Nevertheless, we found that the applied encapsulation did not improve the overall survival of the studied probiotics using neither of the probiotic strains nor the encapsulation approaches. These data do not generally agree well with the literature reviewed by Shori [2017]. Also, in recently published research papers [Yeung *et al.*, 2016; Zaeim *et al.*, 2017], alginate-chitosan encapsulation is claimed an effective means of protecting probiotics. Nevertheless, overall results show that this protection is limited. For example, Yeung *et al.* [2016] encapsulated *B. infantis* UMA299 into alginate-chitosan using injection-gelation method and observed that the encapsulation provided improved protection to stomach juice (by 1.3 log cfu compared to control), but not duodenum juice. In the present study, we also noted a significant improvement of protection of *L. casei* W56 in stomach juice compared to control when probiotic powder was encapsulated (by 3.2 log cfu; calculated based on data from Figure 2 when corrected for the dilution factor resulting from the addition of the digestive juice to the sample). Nevertheless overall survival of *L. casei* W56 did not improve due to increased sensitivity of the encapsulated probiotic to duodenal and ileal juice (increase of log reduction compared to control by 1.5 and 3.2 log cfu, respectively; calculated based on data from Figure 2 when corrected for the dilution factor resulting from the addition of the digestive juice to the sample). Furthermore, for encapsulates containing broth-grown

L. casei W56, survival in the stomach or any other digestive stage was not improved, and total log reduction was significantly greater compared to the control (Figure 3).

In another study, authors investigated the survival of *L. plantarum* ATCC 8014 encapsulated into alginate-chitosan using electrospraying [Zaeim *et al.*, 2017]. They have found that overall the survival of probiotic was improved compared to free cells, but only by ~0.9 log cfu. This improvement was of a similar magnitude to the one observed for broth-grown, encapsulated *B. lactis* W52 (total log reduction decreased compared to control by 1.1 log cfu).

The effect of the presence and concentration of prebiotics on the survival of probiotics during *in vitro* digestion

It has been shown that probiotic survival in the digestive juices may be improved using prebiotics proportionally to the applied prebiotic concentration [Haghshenas *et al.*, 2015; Kingwatee *et al.*, 2014; Sanchez *et al.*, 2014]. In the present study, we did not observe the improvement of survival in digestive liquids with increased concentration of either inulin or FOS. Furthermore, the total log reduction seen upon application of prebiotics in our *in vitro* digestion, decreased by the maximum of 1.4 log cfu compared to the control (for *B. lactis* W52 with 0.1% FOS). Clearly, this decrease was lower compared to what could be expected based on the literature (upon application of prebiotic concentration $\geq 0.1\%$ the log reduction decreased from 2 to ~4 log cfu in studies by Haghshenas *et al.* [2015], Kingwatee *et al.* [2014], and Sanchez *et al.* [2014]).

It is not clear why in this study we have not seen substantial improvement of probiotic survival in the presence of prebiotics. One of the possible reasons could be the ability of probiotics to metabolise substances as an energy source. In this study, we did not focus on probiotic metabolism but on the evaluation of different approaches for the improvement of probiotic survival. Nevertheless, obtained results and published literature data highlight that probiotic metabolism could be one of the factors contributing to probiotic stress resistance and should be a subject of further research [Wang *et al.*, 1999; Corcoran *et al.*, 2005].

CONCLUSIONS

In the present study we have reported survivability of *L. casei* W56 and *B. lactis* W52 in the presence and absence of food matrices, prebiotics and upon encapsulation during simulated passage through selected parts of the human digestive tract. Although improvements in the digestive survival of *B. lactis* W52 were achieved by application of milk and fermented milk, no solution seemed to improve viability of *L. casei* W56. Hence, neither of the examined methods could be recommended as a universal solution for the improvement of probiotic survival during passage through upper parts of the digestive tract.

Findings presented in this work suggested that in a choice of suitable method for the digestive survival improvement, probiotic characteristics play an important role. In the course of this study we have found that the studied probiotics featured a different survival behaviour. While *L. casei* W56 was

sensitive to stomach juice, *B. lactis* W52 was relatively resistant to stomach juice but declined in the presence of duodenum juice. Interestingly, *B. lactis* W52 survived the digestion well in the presence of matrices that contained fat (milk and fermented milk, 3.6%). This finding indicated that the interaction of bile with fat might have minimised the losses of *B. lactis* W52 in the duodenum juice.

Another important characteristic of probiotics that may improve their ability to survive through upper digestive tract is the utilisation of the matrix components as an energy source. Prebiotics, starch as well as metabolizable sugars may be used by probiotics as food and consequently provide energy for the removal of protons from cells (as shown for glucose by Corcoran *et al.* [2005]), improving resistance to gastric acid. Although the study presented here did not focus on the characterisation of probiotic metabolism, obtained results highlighted that the ability to utilise surrounding matrix as a food source might be of key interest if the improvement of the probiotic survivability in the human digestive tract is sought.

Our results suggested that probiotic manufacturers could consider focusing on the development of suspension protocols for probiotic powders. Currently, the general guidance for a suspension of probiotic powder is to mix it with water prior to ingestion. Based on the results presented here, we could recommend using whole milk instead. Further research into optimisation of such protocols looking at different, acid-sensitive probiotics may be of benefit.

This research highlighted knowledge gaps in understanding mechanisms governing probiotic survival in the upper gastrointestinal tract. Optimisation of probiotic survival in studies investigating health benefits of probiotics could address to date observed discrepancies between the reports (as noted by *e.g.* Kasińska & Drzewoski [2015]).

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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