Approaches for improvement in digestive survival of probiotics, a comparative study

Agnieszka Dudkiewicz[a]*, Laura Masmejean[ab], Clement Arnaud[ab], Bukola A. Onarinde[a], Ramana Sundara[c], Amir H. P. Anvarian[a] and Nick Tucker[d]

a- University of Lincoln, National Centre for Food Manufacturing, Park Road, PE12 7 PT, Holbeach, UK
b- Polytech Montpellier, University of Montpellier, Place Eugène Bataillon, CC419, 34095 Montpellier, FR
c- University of Lincoln, Lincoln Institute for Agri-food Technology, Brayford Pool, LN6 7TS, Lincoln, UK
d- University of Lincoln, School of Engineering, Brayford Pool, LN6 7TS, Lincoln, UK

*- corresponding author

Abstract

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, *L. casei* W56 and *B. lactis* W52, in the presence or absence of prebiotics, maize starch, fermented milk and upon encapsulation in calcium alginate-chitosan were evaluated. While *B. lactis* W52 was resistant to stomach juice, but sensitive to duodenal juice, *L. casei* W56 showed an exactly opposite behaviour. Overall the digestive survivability of probiotics was not improved by prebiotics, maize starch or encapsulation. A significant improvement of the overall survivability of *B. lactis* W52 (but not *L. casei* W56) during *in vitro* digestion was noted in milk and fermented milk, possibly due to reduction of the activity of bile against this probiotic. Overall no one method could be recommended universally for the improvement of probiotic survivability. Nevertheless, this research indicated that certain probiotic characteristics, such as susceptibility to bile or acid or ability to use matrix components as an energy source could perhaps be used in further research to select the most effective approaches to deliver viable cells into lower parts of the digestive tract.

Highlights

- *L. casei* W56 was sensitive to stomach and *B. lactis* W52 to duodenum juice
- *L. casei* W56 during *in vitro* digestion survived worse than *B. lactis* W52
- Fermented milk and milk facilitated survival of *B. lactis* W52 but not *L. casei* W56
- Prebiotics, starch and encapsulation did not improve survival of probiotics
- Susceptibility of probiotics to bile or acid may be indicative of the effective approach

Keywords

Probiotics, Prebiotics, Food matrices, *Bifidobacterium*, *Lactobacillus*, *in vitro* digestion

1. Introduction

Consumers are increasingly interested in the influence of diet on health (Dianawati et al., 2016). One class of “healthy ingredients” increasingly added to foods are probiotics, the global consumption of probiotics is increasing and probiotic foods are thought to comprise up to 70% of total functional foods market (Tripathi and Giri, 2014).

Probiotics are defined as a “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014; Joint FAO/WHO Working Group, 2002). Up to date research indicates that probiotics may improve wellbeing in multiple ways e.g.:

- inhibition of the growth of pathogenic bacteria via lowering intestinal pH or by competition (Oelschlaeger, 2010; Timmerman et al., 2004),
- reduction of body mass index and waist circumference (Sun and Buys, 2015)
- improvement of glucose and lipid homeostasis (Kasińska and Drzewoski, 2015; Sun and Buys, 2015),
- prevention and treatment of antibiotic-associated and infectious diarrhoea (Allen et al., 2010; Hempel et al., 2012)
- improvement of overall symptom response in irritable bowel syndrome therapy (Zhang et al., 2016)
- prevention of necrotising enterocolitis in pre-term infants (Alfaleh et al., 2011)
- prevention of infantile eczema (Zuccotti et al., 2015)
- decrease of incidence of respiratory tract infections in children (Wang et al., 2016)

To confer these health benefits, it is often assumed that probiotics need to be viable. So, if administrated orally, probiotics need to pass through a sequence of human digestive system compartments that intrinsically pose a challenge to their survival (Babiloni et al., 2004; Uriot et al., 2016). The two main obstacles hampering survival are the low pH of stomach juice and the action of bile salts (Bezkorovainy, 2001).

Published data indicate that survivability of the probiotics in the human digestive tract varies between species and even between strains of species. Published papers suggest up to 10 log reductions for probiotic populations upon exposure to digestive juices (Kingwatee et al., 2014).

To date a number of studies have investigated how the survivability of probiotics in passing through the digestive tract be improved. The results of these studies indicate that this could be achieved by application of a food matrix or addition of prebiotic to either supplement or encapsulate the formulations.

Foods can protect probiotics during digestion in different ways and this is an area of ongoing research. Of importance is the buffering capacity of foods, which could ensure a safe passage of probiotics through stomach (Lo Curto et al., 2011). Further, particular food ingredients, may aid survival by various mechanisms. For example, metabolizable sugars have been shown to improve the tolerance of *L. rhamnosus* GG to acid by provision of an energy source for the exclusion of protons from cells (Corcoran et al., 2005). Soy protein added to fermented soy milk results in the aggregation of bile salts, limiting the action of bile against probiotics (Ranadheera et al., 2010). Furthermore, in several studies, the protective effect of foods containing fat has been demonstrated (Govender et al., 2013; Tompkins et al., 2011). Another factor that has been implied in the improvement of probiotic survival may be growth within the food matrix. Lo Curto *et al.* (2011) reported that probiotics challenged with digestive juices had up to 6 log cfu higher log reduction in the growth phase compared to the stationary phase. Food fermented with probiotics e.g. milk, will contain cells in the stationary phase, whilst if the probiotic powder is freshly introduced to the food, cells may only start to multiply prior to digestion. Hence, a difference would be expected between survival of probiotics administered into digestive tract in fresh and fermented milk.

Another group of compounds that has been shown to effectively improve the survivability of probiotics are prebiotics (Kingwatee et al., 2014; Sanchez et al., 2014). Prebiotics are defined as a “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al., 2017). To

date only a few compounds are considered prebiotics. Substances such as inulin and products of its degradation such as fructans (oligofructose), lactulose and trans/galacto/fructo-oligosaccharides (TOS, GOS and FOS), match these criteria (Roberfroid, 2008; Roberfroid et al., 2010).

Besides the addition of various compounds to the probiotic loaded ingestable matrix, published research also examined the limitation of exposure of probiotics to digestive juices by encapsulation. Such capsules need to be made of a food-grade material that will enable release of the probiotics in the intestines (Picot and Lacroix, 2004). There are a number of substances that may be used for the probiotic encapsulation (see review by Burgain *et al.* (2011)). Among them, a popular choice is calcium alginate. The material is available as a food grade, and it has been demonstrated to dissolve in intestinal conditions (Segale et al., 2016). However, calcium alginate 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). It has been shown that coating calcium alginate capsules with chitosan enables enhancement of the probiotic survival in digestive juices (Shori, 2017).

Besides a selection of suitable encapsulation material, an important factor is the size of produced capsules. The evidence in published literature suggests that if the capsules are too small (<100 μm) viability during digestion may not improved (Khosravi Zanjani et al., 2014).

A number of methods of encapsulation may be used (Burgain et al., 2011). One novel method being increasingly applied in research studies is electrospraying, where an electrostatic field is used to draw polymer solution through a spinneret resulting in the formation of a spray discharge of small droplets (Coghetto et al., 2016). This method is more suitable for use with probiotics than some other methods, such as spray-drying, as it does not use high temperatures.

Despite the extensive research on the improvement of probiotic survival during the passage through gastro-intestinal tract it is not clear which solutions could be practically implemented. For example, it is not known if the beneficial effects described to date would be observed in other probiotic strains. An additional complication is a lack of the uniformity in digestive models used in the various studies - dynamic and static systems with varying pH, digestion time, chemicals and their concentrations have been examined, which makes comparison of data difficult. Furthermore, studies on comparison of different approaches for the improvement of the digestive survivability of probiotics are scarce. Studies that do exist are typically focused on the investigation of combined effects of tested approaches e.g. prebiotics or other food substance combined with encapsulation or comparison of survivability of different probiotic strains in commercial formulations featuring varying matrices (Fredua-Agyeman and Gaisford, 2015; Shori, 2017). Such studies, although provide very valuable information, do not allow a conclusion as to whether there is a method of choice that could be applied to improve the digestive survivability of probiotics in general.

This research aims to compare three methods that were shown to improve probiotic survival in other studies:

1. Inclusion of the food matrix (fresh and fermented milk, maize starch)
2. Application of the probiotic at different concentration levels (inulin and FOS)
3. Encapsulation in calcium alginate coated with chitosan by means of electrospraying

For the comparison of both method efficacy, and transferability to different probiotic strains we have chosen two probiotics, *L. casei* W56 and *B. lactis* W52, which according to the manufacturer's data exhibit different responses to gastro intestinal conditions. We confirmed that these two probiotics show very different behaviour when exposed digestive liquids. While *L. casei* W56 was sensitive to stomach juice, *B. lactis* W52 was reasonably resistant to stomach juice, but unstable in duodenum juice. Thanks to these differences it was possible to test the protective effect of studied here approaches to probiotics in both stomach and duodenal conditions. These two compartments of the human digestive tract pose a great challenge to the effective survival of probiotics. Hence, the approach which would be generally

recommended as improving probiotic survival should display a good protective effect in both stomach and duodenal conditions. Testing whether such approach was available among those recommended in the literature was a subject of this study.

2. Materials

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 survived the *in vitro* digestion better compared to *L. casei* W56. Freeze-dried organisms were kept at 4°C prior to use. Prebiotics FOS (FOS P1, Winclove Probiotics, Amsterdam, Netherlands) and inulin (inulin & FOS P7, Winclove Probiotics Amsterdam, Netherlands) as well as a maize starch carrier material used in commercial probiotic supplements were also supplied by Winclove Probiotics. 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 digestive assaying were obtained from Fisher (Loughborough, UK). Sodium alginate, chitosan and calcium carbonate used in probiotic encapsulation were obtained from Avonchem limited (Macclesfield Cheshire, UK), Acros Organics (Morris Plains, USA) and Fisher (Loughborough, UK) respectively.

3. Methods

3.1 Preparation of samples challenged by *in vitro* digestion

Probiotic powders were combined with prebiotic or maize starch by mixing in sterile tap water. Probiotics were added aseptically in a quantity allowing to obtain concentration of cells in the sample prior to digestion above 8 log cfu/ ml (count of viable cells in each sample was presented in the result section). This is the level recommended as a probiotic daily therapeutic dose (Kailasapathy and Chin, 2000).

To allow resuscitation of probiotics, samples were kept for 15 min at room temperature prior to analysis and the start of the digestion simulation. The concentrations of prebiotics in water suspensions were 0.1, 0.5, 1 and 5% and maize starch 5%. 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 encapsulation, probiotics were mixed into a 2.25% sodium alginate solution. Probiotics 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 hours at 37°C (B. lactis was grown in MRS broth supplemented with 0.05% 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 saline solution using same settings as in step 2 and

4. Re-suspending the pellet in 3 ml of saline solution and adding to 20 ml of alginate solution.

Suspensions of probiotics in sodium alginate were then electrosprayed. The electrospraying process has been explained in the previous publications (Zaeim *et al.*, 2017). The equipment used for electrospraying was shown in Figure 1a and was provided by Electospinz Ltd (Blenheim, New Zealand). The set up was composed of a polymer dispenser, a needle with a 0.06 mm 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 reservoir 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 10% 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.*, (2010) (Sohail et al., 2011).

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 image analysis). An example image of these capsules is shown in Figure 1b. Capsules measured between ~500 to 800 µm and on average 660 µm. This was much greater than 100µm, the size below which survival could not be facilitated (Khosravi Zanjani et al., 2014). Prepared capsules contained probiotics at a level of > 6 log cfu/g.

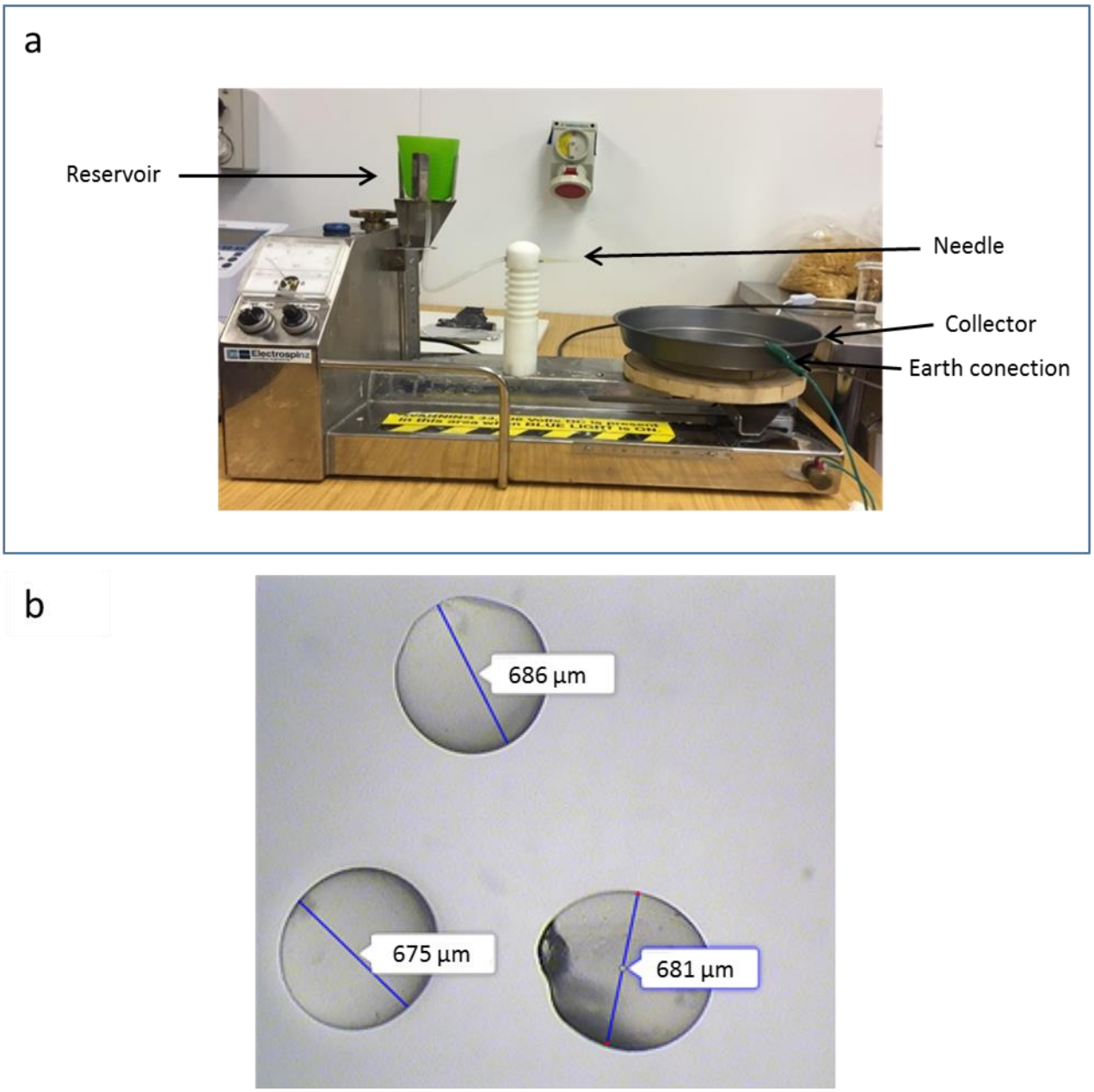

Figure 1- a. electrospinning equipment and b. light microscopy image of capsules made by electrospraying.

3.2 *In vitro* digestion model

To investigate the survivability of probiotics in the human digestive tract in the presence or absence of prebiotic maize starch and fermented milk, 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) (Marteau et al., 1997; Timmerman et al., 2007) and are given in Table 1. Stomach and duodenal juice were prepared and warmed to 37 °C prior to experimentation. Artificial digestion was carried out at 37 ˚C with a constant agitation using an orbital shaker (LSE, Corning, New York, US) at 60 RPM.

Table 1. Composition of simulated stomach and duodenal juice

Chemicals	g/l	NaCl	$NaHCO_3$	KCl	$CaCl_2$	porcine pepsin	porcine pancreatin	porcine bile

Stomach		6.2	1.2	2.2	0.22	3.2	X	X
Duodenum		5	x	0.6	0.25	x	9	14

Several preliminary trials of the digestion design were performed to ensure survivability of the probiotic suspended in water (control) on a level above the limit of detection for our enumeration method.

In the selected protocol, settings were chosen to reflect a wide range of realistic conditions in the human digestive tract – as described for example by Guerra *et al.*, (2012) (Guerra et al., 2012) as well as these used in other *in vitro* digestion studies that trialled probiotic survivability (Kingwatee et al., 2014; Timmerman et al., 2004). The model is shown in Figure 2. 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 addition of 11.5 mM of $CaCl_2$ to the duodenum juice to deactivate bile salts. Anaerobiosis during the digestion was created by overlying digestive liquids with 5 ml of mineral oil. Anaerobiosis has 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 is that we saw a better survival of the microorganisms, especially *L. casei* W56 in the stomach juice, when anaerobiosis was applied.

Samples were added in 5 ml aliquots, where ~ 0.5 g of the capsules were weighed and added to digestive juices with 5 ml of sterile deionised water. The pH of digestive juices was measured and re-adjusted upon the addition of the samples.

In vitro digestion for each sample was repeated 3 times.

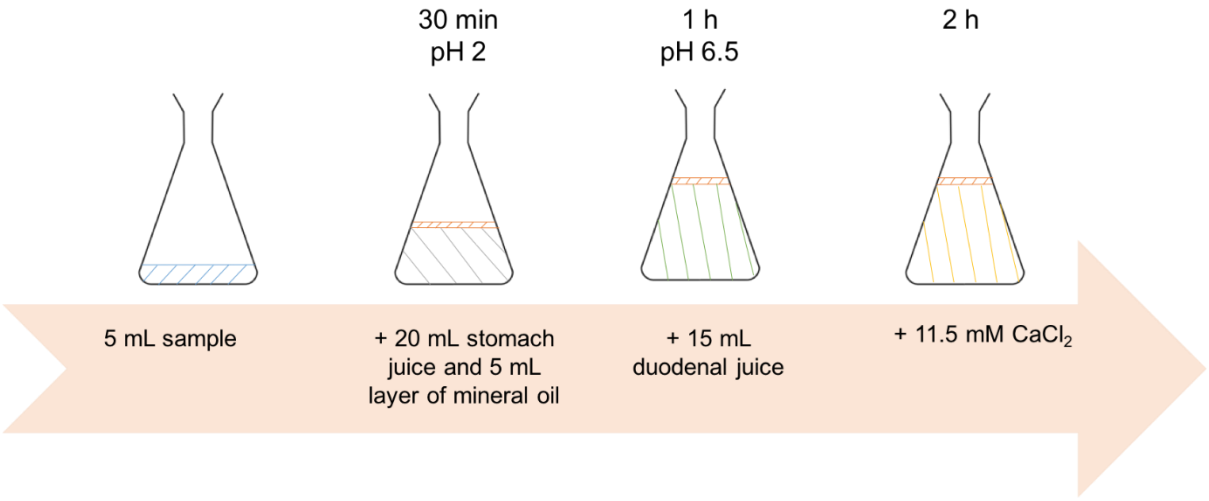

Figure 2. Schematic representation of the design of the in vitro digestion assay used in this study

3.3 Enumeration of probiotics

Samples were analysed using ISO 15214 (1998). First, samples were serially diluted in buffered peptone water (BPW, Oxoid, Basingstoke, England, CM0509) and targeted dilutions were the transferred in volume of 1 ml to empty petri dishes. Then, ~ 15 ml of de Man, Rogosa, Sharpe agar (MRS, Oxoid, Basingstoke, England, CM0361), which had been sterilised and cooled to below 50 ˚C, was poured to the petri dishes and mixed with the sample aliquots. Unsupplemented MRS agar was used for *L. casei*. For *B. lactis* MRS agar contained 0.05% of L-cysteine. Medium was allowed to solidify and plates were then incubated at 37°C for 72h in 15% CO_2, <1% 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.

The 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 minutes. The release of the probiotics from capsules has been confirmed by preliminary trials, where count of viable cells was determined at different time points during mixing.

3.4 Data analysis

Statistical tests were performed using IBM SPSS Statistics 22. All assumed a significance level of 0.05. Particular tests were mentioned in the result section with the 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.

4. Results

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 is shown in Figure 3. Based on the presented graphs for control samples it is clear that the population of *L. casei* W56 declined sharply in stomach and *B. lactis* W52 in duodenum juice.

Statistical tests (repeated measures ANOVA with Bonferroni post-hoc, for all measurements disregarding matrix, data were performed, but are not presented in the figures), indicated that stages 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 ± 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 most gentle conditions for both probiotics (0.2 ± 1.1 and -0.7 ± 1.2 log cfu for *L. casei* W56 and *B. lactis* W52 respectively, the negative log reduction indicates growth).

For *L. casei* W56 survival curves representing control, food matrices and all levels of prebiotics followed a similar pattern. Encapsulated *L. casei* W56 reduced at similar rates through all digestive stages. 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 curve of *B. lactis* W52 in the presence of milk and fermented milk as well as upon encapsulation of the broth-grown probiotics, was linear, 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.

The presented survival curves are real log cfu/ml counts disregarding the dilution of the probiotics by addition of stomach and duodenum juices. To compare the effectiveness of different approaches total log reductions (upon challenge to complete *in vitro* digestion assay), where these dilution factors were corrected for, were subjected to statistical evaluation (see Figure 4).

Total log reduction (upon challenge to complete *in vitro* assay) 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 reduced the total log reduction significantly compared to control. However, encapsulation of broth-grown probiotics caused a significant increase of the total log reduction compared to control and all other treatments. On the other hand the a significantly greater

survival of *B. lactis* W52 was obtained in the presence of milk and fermented milk compared to 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 to *L. casei* W56. This was in line with the suggestion given by the probiotic provider as specified in the method section.

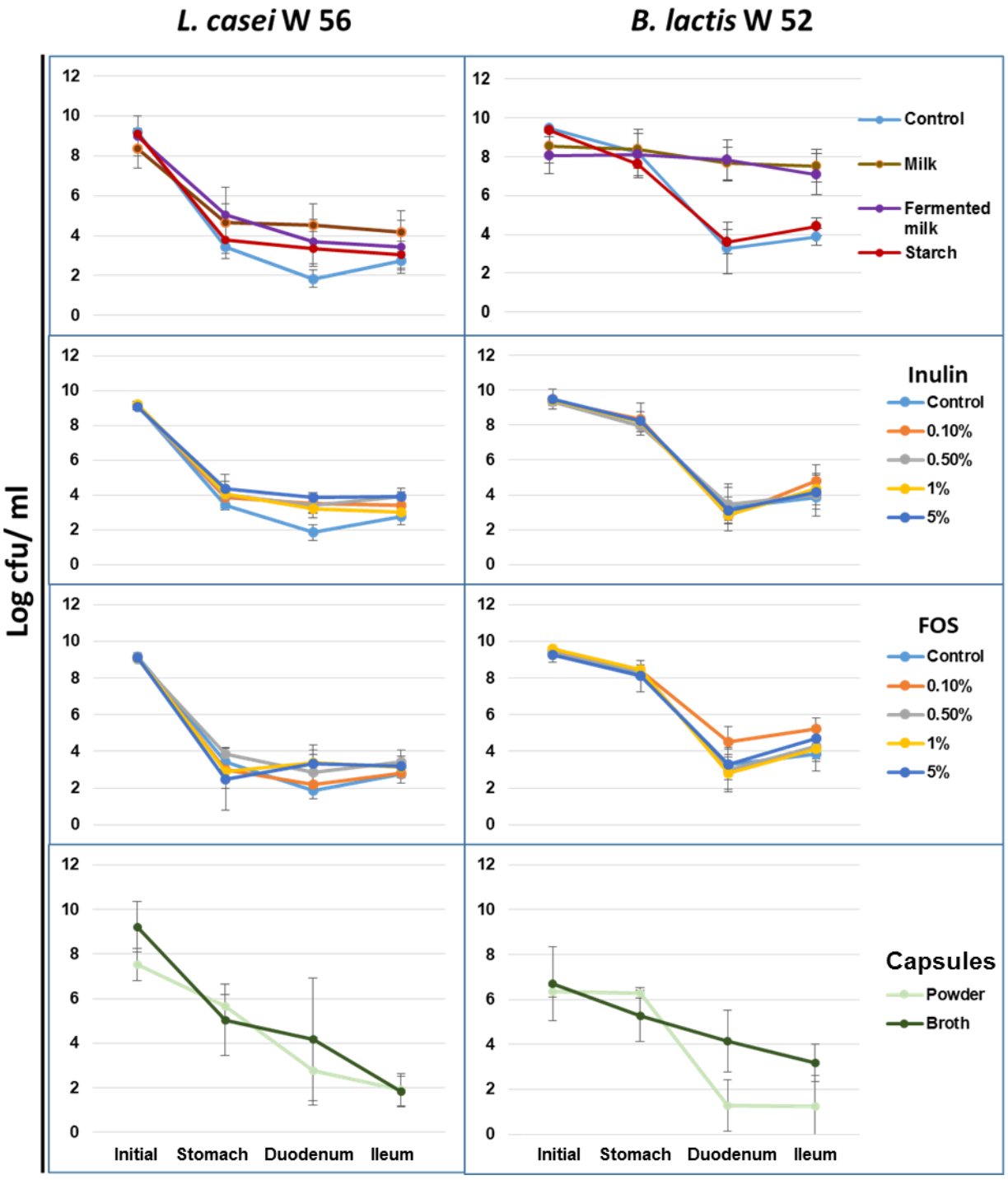

Figure 3. Survival during in vitro digestion of L. casei W56 and B. lactis W52 in presence and absence of food matrices, inulin, FOS and in capsules). Errors bars correspond to standard deviation. The counts are expressed per ml of initial solution. The dilution created by the addition of juices during the experiment were not compensated for.

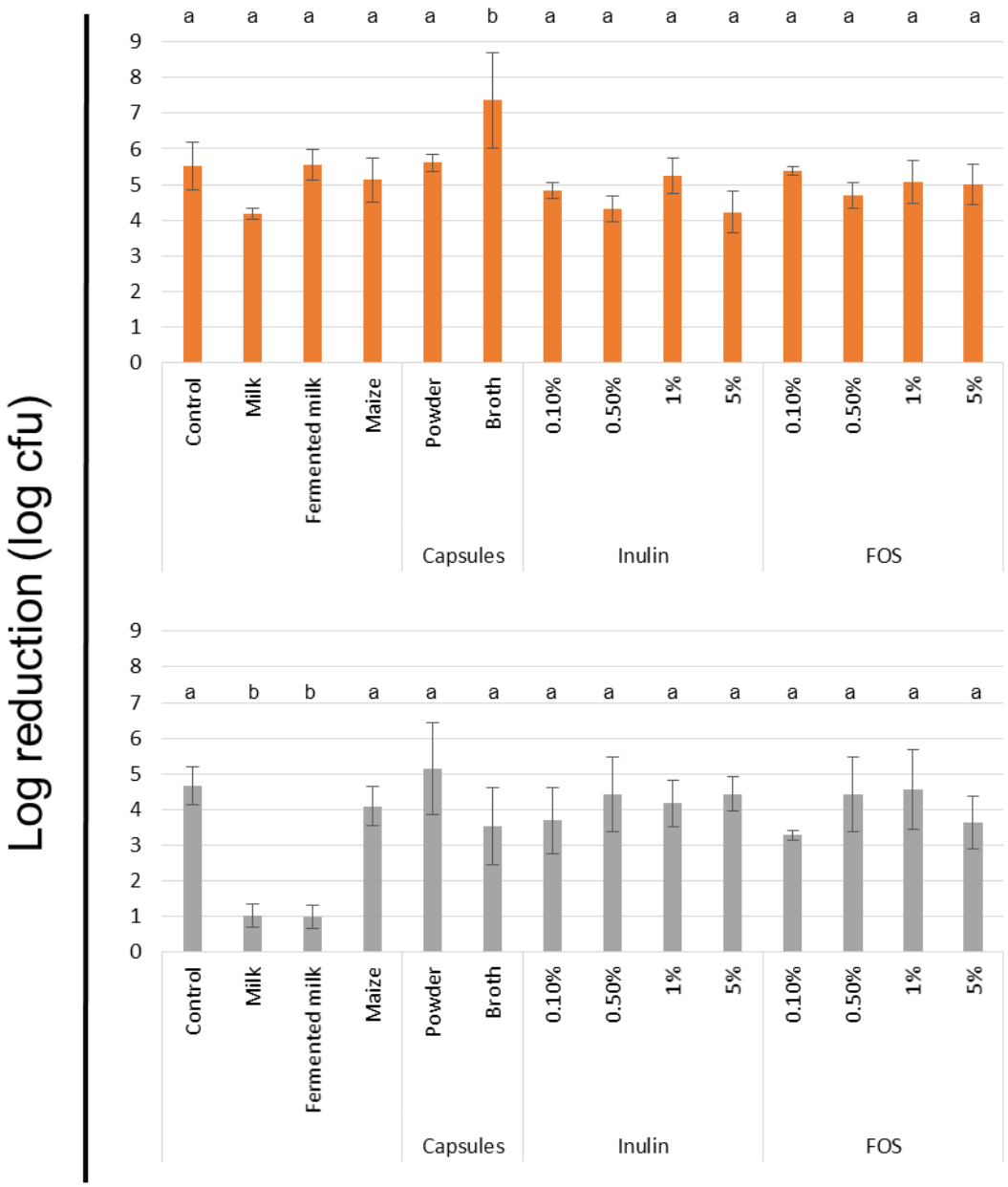

Figure 4. Total log reduction for ■ *L. casei W56 and* ■ *B. lactis W52 after challenge complete in-vitro assay. Columns note mean values and error bars standard deviations. Different letters above different columns in the same in vitro digestion part of a same graph mean significant differences between different matrices and/ or control (ANOVA and Tukey post-hoc, significance level of 0.05).*

5. Discussion

5.1 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. It has been suggested that these two probiotic species might exhibit contrasting survival in a simulated human digestive tract although it should be noted that each of these works used a different *in vitro* digestion design (Fávaro-Trindade and Grosso, 2002; Kingwatee et al., 2014; Lo Curto et al., 2011).

Here, by application of one *in vitro* digestion design for both probiotic species, we had an opportunity to verify the difference in survival. We have found that overall *B. lactis* W52 survived better the *in vitro*

digestion compared to *L. casei* W56. Nevertheless, the magnitude of the differences in survival was affected by the type of the matrix surrounding the probiotics.

We have also noticed that *L. casei* W56 and *B. lactis* W52 behaved very differently in different digestive juices. *L. casei* W56 was sensitive to stomach juice and resistant to duodenum juice, while *B. lactis* W52 on the contrary was mostly reduced in the duodenum juice. A similar behaviour of *L. casei* 01 was previously documented by Kingwatee *et al.* (2014). However, *B. lactis* Bb12 was shown to have a high resistance to bile salts in contrast to our results *B. lactis* W52 (Fávaro-Trindade and Grosso, 2002). This suggested that not only species but also specific strain type affect the survival of probiotic in the digestive tract as demonstrated previously, for example for *L. plantarum* (van Bokhorst-van deVeen et al., 2012).

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

The presence of the food matrix, such as milk may substantially improve the survival of probiotics, several authors note the fat in the food matrix as an element which could potentially enhance probiotic survival (Govender et al., 2013; Tompkins et al., 2011). For example, Tompkins *et al.* (2011) showed that probiotics (ProtecFlor®, a commercial supplement containing 4 probiotic strains) survived better in 1% fat milk and oats compared to fruit juice and spring water. Nevertheless, this study did not focus on specific probiotic strains and did not compare efficacy of the treatment for each specific probiotic strain. In turn, 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 could be expected, since the pH of fermented milk was lower than that of fresh milk (4.3 and 6.8 respectively) indicating the production of lactic acid by fermentation. 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). This result did not support the development of acid resistance in stomach juice sensitive probiotic. In contrast development of acid adaptation was demonstrated for *L. acidophilus* LA-5 and *L. rhamnosus* GG which after exposure to low pH (3.5) prior to digestion, showed a slightly better survival in the stomach acid compared to the control (Sumeri et al., 2010).
In fermented milk, cells should reach a stationary growth phase. According to Lo Curto *et al.* (2011) probiotics challenged with digestive juices survive better when in a stationary phase compared to a 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 Lo Curto *et al.* (2011) study are differences in used probiotics strains, digestive assay design as well as the way in which cells have been grown into 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 we fermented milk overnight at 40˚C and submitted samples

to digestive assay 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. For example, it is known that the temperature of cell growth will have an effect on the expression of genes and the physiological characteristics of microorganisms (Montville and Matthews, 2005). 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 stress could be beneficial in aiding probiotic survival during passage through 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 presence of maize starch was not improved compared to control. However, high amylose maize starch was shown to enhance survival of *Bifidobacterium* Lafti™ 8B and 13B during exposure to *in vitro* as well as *in vivo* (mice) digestion (Wang et al., 1999). Interestingly both of these probiotics had an ability to use amylose from maize starch (Wang et al., 1999). 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 (Corcoran et al., 2005). 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 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.

5.3 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 (the broth method). Encapsulation of the powder might however make commercial sense, since the population of encapsulated probiotics declines during storage (Yeung et al., 2016). In this study we found that the encapsulation did not improve the overall survival of studied probiotics using either of the probiotic strains or encapsulation. These data do not generally agree well with the literature reviewed by Shiori (2017). Also, in recent published research papers, alginate-chitosan encapsulation is claimed as an effective means of protecting probiotics: nevertheless, overall results show that the level of protection is limited.

For example, Yeung *et al* (2016) encapsulated *B. infantis* UMA299 into alginate-chitosan using an injection-gelation method and observed that the encapsulation provided improved protection aginst the action of stomach juice (by 1.3 log cfu compared to control), but not to duodenum juice (Yeung et al., 2016). In the present study we also noted a significant improvement (by 3.2 log cfu) of *L. casei* W56 in stomach juice compared to the control when probiotic powder was encapsulated. Nevertheless, overall survival did not improve due to increased sensitivity of encapsulated probiotic to duodenal and ileal juice (an increase of log reduction compared to control by 1.5 and 3.2 log cfu respectively; see supplementary information). Furthermore, for encapsulates containing broth-grown *L. casei* W56, survival in stomach or any other digestive stage was not improved compared to control and total log reduction was significantly greater compared to the control.

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

5.4 The effect of the presence and concentration of prebiotics on 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 applied prebiotic concentration (Haghshenas et al., 2015; Kingwatee et al., 2014; Sanchez et al., 2014). In the present study, we did not observe an 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, was decreased by a maximum of 1.4 log cfu. Clearly this decrease was lower compared to what would be expected based on the literature (on application of prebiotic concentration ≥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 we did not see a substantial improvement of probiotic survival in the presence of prebiotics. One of the possible reasons could be mentioned above (case of starch and glucose) ability of probiotics to metabolise substances as energy source. In this study we did not focus on probiotic metabolism but only at the evaluation of different approaches for the improvement of probiotic survival. Nevertheless, obtained results and published literature data both indicate that probiotic metabolism could be one of the factors contributing to probiotic stress resistance and should therefore be a fruitful subject for further research.

6. Conclusions and recommendations for further work

We report the survivability of *L. casei* W56 and *B. lactis* W52 in the presence and absence of food matrices, prebiotics and upon encapsulation during passage through simulated 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 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 suggest 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 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 low level of 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 use of the matrix components as an energy source. Prebiotics, and maize starch, as well as metabolizable sugars may be used by probiotics as food and consequently provide energy for removal of protons from cells (as shown for glucose by Corcoran *et al.* (2005)), improving resistance to gastric acid. Although this study did not focus on the characterisation of probiotic metabolism, obtained results, highlighted that the ability to use the surrounding matrix as a food source might be of key interest if the improvement of the probiotic survivability through human digestive tract is sought.

Given the results of these studies, probiotic manufacturers could consider focusing on the development of suspension protocols for sold probiotic powders that could be applied by the consumers at home. General protocol for suspension of probiotic powder recommended by many manufacturers 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 would be of benefit to investigate how different acid sensitive probiotics may be delivered effectively to the intestines.

This research highlighted that to understand mechanisms governing probiotic survival in upper gastrointestinal tract and effectively enhance it, still more research is required. Optimisation of probiotic survival in studies investigating health benefits of probiotics may be an important factor addressing to date observed discrepancies in results of reported human trials (as noted by e.g. Kasińska and Drzewoski (2015)).

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