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ORIGINAL ARTICLE

Microencapsulation increases survival of the probiotic *Lactobacillus plantarum* IS-10506, but not *Enterococcus faecium* IS-27526 in a dynamic, computer-controlled *in vitro* model of the upper gastrointestinal tract

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Keywords
Dadih, *Enterococcus faecium*, *in vitro* model of the upper gastrointestinal tract, *Lactobacillus plantarum*, microencapsulation, probiotic, survival.

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Abstract

Aim: To test the effect of microencapsulation on the survival of two probiotic strains isolated from Dadih, Indonesian fermented buffalo milk, in a dynamic, computer-controlled *in vitro* model of the upper gastrointestinal (GI) tract (TIM-1), simulating human adults.

Methods and Results: Free or microencapsulated probiotics, *Lactobacillus plantarum* IS-10506 or *Enterococcus faecium* IS-27526, resuspended in milk were studied for survival in the complete TIM-1 system (stomach + small intestine) or in the gastric compartment of TIM-1 only. Hourly samples collected after the ileal-caecal valve or after the pylorus were plated on MRS agar (for *Lactobacillus*) or S&B agar (for *Enterococcus*). Survival of the free cells after transit through the complete TIM-1 system was on average for the *E. faecium* and *L. plantarum* 15·0 and 18·5% respectively. Survival of the microencapsulated *E. faecium* and *L. plantarum* was 15·7 and 84·5% respectively. The free cells were further assessed in only the gastric compartment of TIM-1. *E. faecium* and *L. plantarum* showed an average survival of 39 and 32%, respectively, after gastric passage.

Conclusion: There is similar sensitivity to gastric acid as well as survival after complete upper GI tract transit of free cells, but microencapsulation only protected *L. plantarum*.

Significance and Impact of Study: Survival of microencapsulated *L. plantarum* IS-10506 is increased compared to free cells in a validated *in vitro* model of the upper GI tract. It increases its use as an ingredient of functional foods.

Introduction

Probiotics are "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host"(FAO, 2001, Hill *et al.* 2014).

Lactobacillus plantarum IS-10506 and *Enterococcus faecium* IS-27526 are novel probiotics isolated from a yogurt-like product, Dadih, an Indonesian traditional buffalo milk of West Sumatra (Akuzawa and Surono 2002). Both strains have been shown *in vitro* to have properties required for probiotics, such as acid and bile tolerance, adhesion to epithelium cells, and competitiveness against pathogens (Surono 2003; Collado *et al.* 2007a, 2007b). *In vivo* they have shown antimutagenicity (Surono *et al.* 2009), and inhibition against coliforms in animal models (Surono *et al.* 2010) and in human studies have demonstrated enhancement of humoural immune response, and nutrient absorption in several studies (Surono *et al.* 2011, 2014).

There is consensus that probiotics preferably should also be alive in the gastrointestinal tract to perform their function(s), although dead micro-organisms and cell

fragments also show some activities (Adams 2010; Lau 2014). The classical probiotics of the genera *Lactobacillus*, *Bifidobacterium* and *Enterococcus* show interesting probiotic activities, but their survival during passage through the upper GI tract is generally low, in the order of 1–15%, with some strains performing even much poorer. Encapsulation is a means of protecting the cells against the hostile environment of the upper GI tract (Strasser *et al.* 2009; Khosravi Zanjani *et al.* 2014). Cells of the probiotics were encapsulated with 4·75% (w/v) sodium alginate and 5·5% (w/v) calcium chloride. The added benefit of microencapsulation is that survival is increased during storage at high humidity, such as in tropical countries like Indonesia, where part of this research was carried out with the aim to increase survival of probiotics during storage.

Survival in the upper gastrointestinal (GI) tract is difficult to establish in humans. Validated and predictive *in vitro* models that closely mimic the dynamic changes in physiological parameters in the GI tract are an excellent alternative to study survival of probiotics. Some of these dynamic changing conditions, such as gastric acidity and concentrations of bile along the length of the small intestine, greatly influence probiotic survival. The TNO computer-controlled, dynamic *in vitro* gastrointestinal model of the stomach and small intestine (TIM-1) is such a validated and predictive model which simulates to a high degree the successive dynamic processes in the stomach and small intestine (Minekus *et al.* 1995; Minekus 1998). This model was validated with intubated human volunteers and was shown to closely predict survival of microbial strains (both from yogurt as well as probiotics added to the yogurt) (Marteau *et al.* 1997). It has been used previously to study survival in the upper GI tract of probiotics of various genera, including yeasts (Blanquet *et al.* 2003, 2004; Maathuis *et al.* 2009; Martinez *et al.* 2011; Hatanaka *et al.* 2012).

The aim of this study was to evaluate the effect of microencapsulation on survival of the two probiotic strains during transit through the upper GI tract. To this end, free and encapsulated cells of each strain were added to TIM-1 in a milk matrix and samples were collected at the end of the system to determine viable counts. Moreover, to study sensitivity to gastric acid, similar experiments were done, but then only in the gastric compartment of TIM-1.

Materials and methods

Bacterial strains and microencapsulation

Lactobacillus plantarum IS-10506 and *E. faecium* IS-27526 were isolated from Dadih, and were identified by 16SrRNA gene sequencing as *L. plantarum* (Gene Bank accession no. DQ860148) and as *E. faecium* (no. EF068251) (Collado *et al.* 2007b). They were cultivated in de Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK) in a fermenter SP30 L (BIOTRON), at pH 6·0. The cells were harvested by centrifugation at 3000 × ***g*** for 15 min at 4°C using a cold centrifuge (Thermo Scientific, Sorvall RC 3BP+, Germany), and were washed once with sterile deionized water, then resuspended in UHT milk to form a cell paste. Two hundred millilitres of *L. plantarum* cells solution was atomized by using a fluid nozzle in side-spray position using a peristaltic pump applying a spraying air pressure and sprayed onto 2 kg of powdered cellulose carrier material Flocel pH 101 (Gujarat, India) mixed with 2 kg of skim milk powder (NZMP, New Zealand). The inlet air temperature was set to 45°C which resulted in a maximal bed temperature of 37°C depending on the spraying rate. The spraying rate ranged from 5 to 10 ml min^{-1} and was adjusted so that agglomeration of particles was avoided. The powder was dried in a pilot scale fluid bed dryer (HongDau, Taiwan). Cells were encapsulated with 4·75% (w/v) sodium alginate (0·4 M), in a sterile environment. Microbeads were allowed to solidify in a 5·5% (w/v) calcium chloride (0·5 M) as described elsewhere (Prakoeswa *et al.* 2017).

In vitro model of the stomach and small intestine (TIM-1)

The TNO *in vitro* model of the stomach and small intestine, TIM-1 (Fig. S1), was used to study the survival of the probiotics. The model was setup and run as described before (Minekus *et al.* 1995), according to the validated protocol for survival of lactic acid bacteria and probiotics (Marteau *et al.* 1997). In brief, experiments were performed in duplicate under the average physiological conditions as found in the human gastrointestinal tract for adults. The gastric emptying, intestinal residence time and gastric and intestinal pH curves mimicked the situation as found in humans for intake of a glass of milk (Fig. S2; Minekus *et al.* 1995). The concentrations of electrolytes, enzymes, bile and pancreatic juice were adjusted to the average concentrations as described for healthy humans. Pancreatic output was simulated by secreting 10% pancreatin (Pancrex V, Paines and Birne, Greenford, UK) in small intestinal electrolyte solution (containing NaCl 5 g l^{-1}, KCl 0·6 g l^{-1}, $CaCl_2$ 0·22 g l^{-1}) at 0·25 ml min^{-1}. Biliary output was simulated by secreting a 4% bile (porcine bile extract, Sigma) solution at 0·5 ml min^{-1}. Prior to the experiment, the compartments were filled with start residues as described before (Minekus *et al.* 1995), except for the gastric residue, which was mixed with the 'meal'. Hollow fibre

membrane systems continuously dialysed the digested and dissolved low-molecular weight compounds from the jejunum and ileum compartments (Fig. S1-M), which simulated the absorption of nutrients in the body, which maintains the physiological concentrations of bile and electrolytes. The dialysis solution in the jejunum dialysis bottle contained 19·5 g l^{-1} porcine bile to maintain physiological amounts of bile in the system during the experiment (Marteau *et al.* 1997). In those experiments in which gastric survival was determined, the duodenal compartment was only used for neutralization of the gastric efflux, without secretion of bile and pancreatin. Experiments were performed in duplicate.

Sampling

In the complete TIM-1 experiments, the ileal efflux (Fig. S1-H) was collected every hour for 6 h. The volume was measured, and a 1-ml sample was taken for analysis. In the gastric experiments, the gastric efflux (neutralized in the duodenal compartment as described above) was collected every hour for 3 h. The volume was measured, and a 1-ml sample was taken for analysis. At the end of the experiments (for both complete and gastric compartment only) the residue left in the system after the termination of the experiment was collected and analysed as well.

Analysis

Serial 10-fold dilutions were prepared of the ileal and gastric efflux and the residue samples taken from TIM-1 and of the freeze-dried powders (free and encapsulated cells) and these were plated on MRS agar plates for *L. plantarum* or S&B agar plates for *E. faecium* to determine colony-forming units (CFUs). Subsequently, the plates were incubated at 37°C for 3–4 days under anaerobic conditions. Cumulative survival as percentage of intake was calculated as the sum of the surviving bacteria in the different efflux samples from TIM-1 divided by the amount of bacteria introduced in the model with the glass of milk (determined from the CFU per g of the freeze-dried powders).

Results

Cumulative survival after transit through TIM-1 of the free (unencapsulated) cells expressed as percentage of intake was on average 15·0% for the *E. faecium* strain (Fig. 1a) and 18·5% for the *L. plantarum* strain (Fig. 1b). Microencapsulation did not change survival of *E. faecium* IS-27526 during transit through the upper GI tract (Fig. 1a), but microencapsulation did increase the survival of *L. plantarum* IS-10506 (Fig. 1b).

Based on the difference in kinetics of survival of the free and encapsulated cells of *L. plantarum* IS-10506 and *E. faecium* IS-27526, it was hypothesized that the major difference would be caused by a different sensitivity to gastric acid (*L. plantarum* IS-10506 more sensitive) and bile (*E. faecium* IS-27526 more sensitive). To test this, experiments were performed in just the gastric compartment of TIM-1, and only with the free cells. Figure 2 shows the average cumulative survival in these experiments. *E. faecium* IS-27526 showed a survival of 39% when expressed as percentage of intake, whereas *L. plantarum* IS-10506 showed a survival of 32%.

Discussion

The two strains used in this research have been shown to provide health benefits to the host, which is a prerequisite to be considered as a probiotic (FAO, 2001, Hill *et al.* 2014). The *L. plantarum* IS-10506 has been shown to enhance humoural immune response, faecal sIgA and improve zinc status of children younger than 2 years of age (Surono *et al.* 2014), and as a potential treatment in Atopic Dermatitis in children (Prakoeswa *et al.* 2017). *E. faecium* IS-27526 demonstrated significant positive effects on humoural immune response, and salivary sIgA in underweight preschool children, and on weight gain of

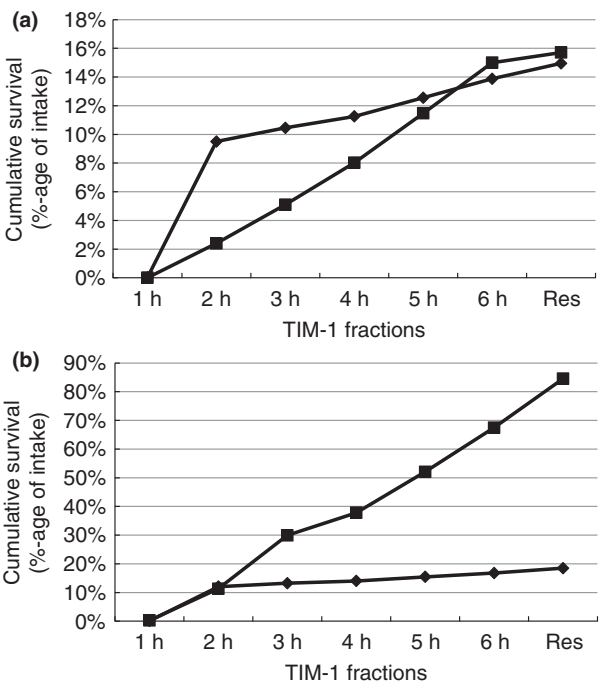

Figure 1 Cumulative survival (as percentage of intake) for the two strains in free and encapsulated forms during transit through the complete TIM-1 system. (a) *Enterococcus faecium*; (b) *Lactobacillus plantarum*; (♦) free cells; (■) encapsulated cells.

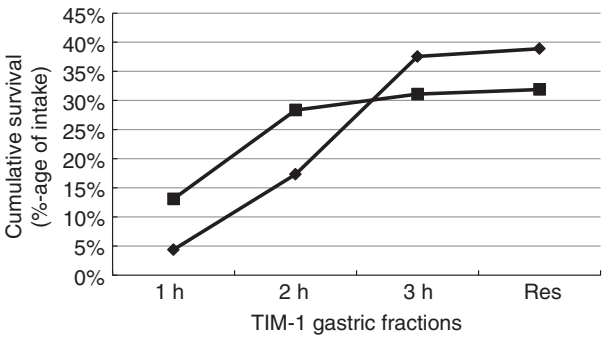

Figure 2 Cumulative survival (as percentage of intake) for the two strains as free cells during transit through gastric compartment of TIM-1. (♦)*Enterococcus faecium*; (■) *Lactobacillus plantarum.*

preschool children younger than 5 years (Surono *et al.* 2011).

The TIM-1 system has been used before to study the survival of bacterial probiotics (Marteau *et al.* 1997; Maathuis *et al.* 2009; Martinez *et al.* 2011; Hatanaka *et al.* 2012). Survival in TIM-1 was validated with human volunteers (Marteau *et al.* 1997) using a catheter that reached the terminal ileum, and through which samples were taken and plated for viable cells. In TIM-1, at the same spot in the small intestine, namely after the ileal-caecal valve (Fig. S1H) samples were taken and tested for viable cells. For several different strains this was shown to be highly similar (Marteau *et al.* 1997). The model nowadays is used to predict survival in a clinical setting, and it is also used for product development, such as the development of an enteric coating around a probiotic-containing tablet to increase survival in the stomach (Eiberger *et al.* 2011), or to test the increase in survival of probiotics in the presence of prebiotics (Martinez *et al.* 2011).

In this validated system, the goal was to assess the effect of microencapsulation with sodium alginate and calcium chloride. Encapsulation has been studied to increase survival during (i) storage, and (ii) transit through the GI tract, protecting against acid and bile stress. Alginate is a commonly used biopolymer for microencapsulating, since it is nontoxic, easy and inexpensive, and heat-resistant. Alginate in the presence of Ca^{2+} produces a particularly strong molecular framework, supporting cold-prepared, thermo-irreversible and freeze–thaw–stable microcapsules. Different alginate-to-calcium concentration form different junction zones, where the ionic strength of the medium influences the viscoelastic properties of alginate beads (Ouwerx *et al.* 1998). The excellent muco-adhesion properties typical of hydrophilic polymers such as alginate, are useful for enhancing the *in situ* delivery of bacteria along the GI tract (Wee and Gombotz 1998; Chen *et al.* 2013). Hydrophilic polymers possess charged and/or nonionic functional groups capable of forming hydrogen bonds with mucosal surfaces (Dhawan *et al.* 2004; Khutoryanskiy 2011). As examples alginate maintained viability of eight probiotic strains against heating at 65°C for 1 h (Ding and Shah 2007), and improved cell viability during storage both at refrigerated and at room temperature (De Prisco *et al.* 2015). These properties enable microencapsulated probiotics to maintain viability and to be used as a functional food ingredient in ice cream, frozen yogurt, chocolate soufflé and mayonnaise (Kebary *et al.* 1998; Khalil and Mansour 1998; Krasaekoopt *et al.* 2003; Malmo *et al.* 2013; De Prisco *et al.* 2017). Moreover, as we show here they may increase survival in the GI tract, by protecting against gastric acid.

From the gastric experiments (Fig. 2) it is clear that *L. plantarum* IS-10506 is rather sensitive to gastric acid, as after the second hour, there were very few viable cells coming from the system. At this point in time the gastric pH had dropped from pH 2·9 at 60 min to pH 2·0 at 120 min. For *E. faecium* IS-27526, however, the number of viable cells leaving the gastric compartment did not decline over the first 3 h. For both strains, the gastric residue contained very few viable cells. Survival after the gastric compartment was 30–40%, but survival after the passage through the complete TIM-1 system of the free cells was only 15–18%. This indicates that both the probiotic strains are also sensitive to bile. Encapsulation of the probiotics within an alginate-$CaCl_2$ matrix led to a greatly improved survival for *L. plantarum* (from 18·5 to 84·5%: Fig. 1b), whereas this had no effect for *E. faecium* (Fig. 1a). In simple *in vitro* test-tube experiments, *E. faecium* IS-27526 and *L.plantarum* IS-10506 were found to be able to tolerate pH 2 for 1 h, whereby survival dropped by 2·2–2·3 log cycles, to *c.* 1% of the original viable counts (Surono 2003). In similar test-tube experiments, *E. faecium* IS-27526 tolerates bile-salts better than *L.plantarum* IS-10506 (Surono 2003), which is in contradiction with the results presented here. One reason could be that in the test-tube experiments ox-bile was used (Surono 2003), while in the TIM-system porcine bile is used, which looks more like human bile. Moreover, the test-tube experiments simulated either the stomach (acid stress), or the small intestine (bile stress), while in the complete TIM-1 system the probiotic strains encounter

the bile stress consecutive to the acid stress. We hypothesize that this explains the lower survival of the encapsulated *E. faecium* IS-27526, which appears to be more sensitive to the two consecutive stresses, or where stress imposed by bile is somehow harsher than that encountered during acid exposure. Despite encapsulation, which protect against acid stress, free and encapsulated *E. faecium* IS-27526 cells seem to be similarly affected by and quite sensitive to bile, while *L. plantarum* IS-10506 appears to be more sensitive to acid. Future experiments will study gene expression in the probiotics in different regions of the model to see if that provides an indication as to the difference in response to encapsulation.

In conclusion, a validated, dynamic *in vitro* model of the upper GI tract, which closely mimics the situation in humans, is a valuable tool to study survival of encapsulated probiotics, and validate or reject certain hypotheses, while at the same time allowing studying underlying mechanisms. In this case, our prediction that both strains would be protected by the imposed encapsulation is refuted and new hypotheses regarding the effect of two consecutive stresses encountered by *E. faecium* IS-27526 have been defined, which will be tested in subsequent experiments using gene expression analyses.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Schematic diagram of the dynamic, multi-compartmental TNO *in vitro* model of the stomach and small intestine (TIM-1). A. stomach compartment; B. pyloric sphincter; C. duodenum compartment; D. peristaltic valve; E. jejunum compartment; F. peristaltic valve; G. ileum compartment; H. ileo-caecal sphincter; I. stomach secretion; J. duodenum secretion; K. jejunum/ileum secretion; L. prefilter; M. semi-permeable membrane; N. water absorption; P. pH electrodes; Q. level sensors; R. temperature sensor; S. pressure sensor. Reprinted from (Keller *et al.* 2017) with permission

Figure S2 Curves mimicked in TIM-1 over time, representing the gastric (♦) and ileal delivery (▲) [both expressed as percentage of the ingested meal], and the gastric pH (■).