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Survival of a probiotic-containing product using capsule-within-capsule technology in an *in vitro* model of the stomach and small intestine (TIM-1)

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RESEARCH ARTICLE

Abstract

The aim of the research was to compare the survival of a blend of five probiotic strains (2 bifidobacteria and 3 lactobacilli) in a capsule within capsule (Duocap®) containing Ahiflower® oil, as compared to the strains in the powder (with or without Ahiflower oil), or the strains when present in the inner capsule only. This was tested in a validated, dynamic *in vitro* model of the stomach and small intestine (TIM-1), simulating human adults. Experiments were performed both in the gastric compartment of the model, as well as in the complete system (stomach + small intestine). Survival of the strains after transit through the gastric compartment in the Duocap capsule was higher by about a factor of 1.5 compared to the other 3 variables. In these gastric experiments, the Ahiflower oil did not seem to have an additional benefit, in the sense that it did not increase survival over the strains alone. After transit through the complete gastrointestinal tract survival was approximately 2-fold higher for the strains within the Duocap capsule, compared to the strains within the inner capsule or the powder. In these experiments, Ahiflower oil did have an additional benefit. The survival of the strains in the combination of powder with Ahiflower oil showed a similar survival as that of the Duocap, although in the first few hours of the experiments survival of both species lagged behind, and only caught up at the end of the test. In conclusion, the developed capsule-in-capsule technology increased the amount of viable cells in the upper gastrointestinal tract, mainly due to the presence of the polyunsaturated fatty acids contained in the outer capsule, which particularly protected the blend of probiotics in the small intestine.

Keywords: probiotic, survival, capsule, *Lactobacillus*, *Bifidobacterium*

1. Introduction

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2001; Hill *et al.*, 2014). Nowadays, besides probiotic bacteria in dairy products, a high diversity of probiotic containing dietary supplements is offered to the consumer, such as tablets, capsules and sachets. Survival of probiotics during transit through the gastrointestinal (GI) tract is strain dependent (Campana *et al.*, 2017; Marteau *et al.*, 1997). Survival of probiotics within a food matrix is usually higher than that when the strains are ingested with a glass of water in a tablet or capsule (Eiberger *et al.*, 2011). This is due to the buffering capacity of the meal, which results in a higher pH in the gastric compartment, and thus less exposure to high concentrations of gastric acid. Protection against the effects of bile in the small intestine would also be beneficial. Therefore, technologies to improve survival of probiotics in tablets or capsules are being sought for. This may be through use of an enteric coating around a tablet that prevents the gastric acid from interacting with the bacteria (Venema *et al.*, 2019), microencapsulating the bacteria (Surono *et al.*, 2018), or combining probiotics with prebiotics, where the idea is that the bacteria are provided with a 'lunchbox' that would help them survive the harsh conditions of the upper GI tract (Martinez *et al.*, 2011).

However, this lunchbox phenomenon does not always work and sometimes even leads to lower survival (Venema, 2015).

Duocap® is a patented capsule-in-capsule delivery system that is developed for combination or dual release products. The oral dosage delivery system in the current experiments involved inserting a smaller probiotic-filled capsule into a larger oil-filled capsule. The outer capsule contained Ahiflower® oil (containing omega-3, omega-6 and omega-9 unsaturated fatty acids). The inner capsule contained a blend of 5 probiotic strains. Both the inner and outer capsule were made of hypromellose.

The use of a validated, dynamic, computer-controlled *in vitro* model (TIM-1) to screen for survival of a number of different products or variables under standardised conditions in the upper gastrointestinal (GI) tract has been reported. Survival of various probiotic strains have been evaluated in this system, ranging from strains belonging to lactic acid bacteria and bifidobacteria (Marteau *et al.*, 1997; Martinez *et al.*, 2011; Venema *et al.*, 2019), and bacilli (Hatanaka *et al.*, 2012; Keller *et al.*, 2017) to yeasts (Blanquet-Diot *et al.*, 2012). This model is highly validated and predictive for what happens with food (component)s, including probiotics, in the upper GI tract (Marteau *et al.*, 1997; Minekus, 2015; Minekus *et al.*, 1995). Such predictive *in vitro* models are a helpful tool in the development and screening of new formulations containing probiotic bacteria, and they allow for mechanistic evaluation of the formulations, e.g. by the ability to study effects in the gastric compartment separately from those in the complete model. This has for instance triggered the development of an enteric coated tablet (Venema *et al.*, 2019), due to low survival of the probiotics in the stomach.

The aim of the current experiments was to evaluate survival of a blend of two probiotic lactobacilli in combination with three bifidobacteria, and evaluate the benefit of the Duocap technology, compared to the individual components (inner capsule, and Ahiflower oil).

2. Materials and methods

Probiotic powder and capsules

Probiotic powder, Ahiflower oil, and Duocap and inner capsules with the probiotics inside were provided by Nouri Life (Conyers, GA, USA). The powder contained blend of 2 bifidobacterial strains (*Bifidobacterium lactis* UM-B1, *Bifidobacterium longum* UM-B2) and 3 lactobacilli (*Lactobacillus acidophilus* UM-L1, *Lactobacillus plantarum* UM-L2, *Lactobacillus brevis* UM-L3). Characteristics about the cfu content of the powder are provided in Table 1. The powder contained bifidobacteria and lactobacilli in a ratio of approximately 30%:70% (Table 1).

TNO *in vitro* model of the stomach and small intestine (TIM-1)

Supplementary Figure S1 shows a schematic of the *in vitro* model, which has been described extensively before, e.g. (Hatanaka *et al.*, 2012; Surono *et al.*, 2018; Venema *et al.*, 2019). The model was set-up and run according to the validated protocol for survival of probiotics (Marteau *et al.*, 1997). Briefly, the model comprises four connected glass compartments, that represent the stomach, duodenum, jejunum and ileum. Each compartment contains a flexible silicone inner wall. The space between the inner and outer walls is filled with water of 37 °C, which is also used to create peristalsis, by periodically applying pressure on the water, which squeezes the flexible inner walls allowing mixing and movement of the chyme through the system. In each compartment the pH is measured continuously and regulated by 'secretion' of hydrochloric acid in the gastric compartment and sodium bicarbonate in the three intestinal compartments. The set-points of pH, gastric emptying and intestinal transit time are controlled by a computer and in the current experiments simulated the average physiological conditions as found in the human gastrointestinal tract for adults (Supplementary Figure S2). The gastric emptying, intestinal residence time and

Table 1. Initial cell count (cfu/g) as determined by microbiological cell count in the probiotic powder, and average cumulative survival (after 3 h for gastric and 6 h for complete TIM-1 runs) of the combined *Lactobacillus* and the combined *Bifidobacterium* strains.

	Product	Lactobacilli	Bifidobacteria	Total count
	Probiotic powder	2.04×10^{10}	4.53×10^{10}	6.57×10^{10}
TIM-experiment (gastric; after 3 h)	Probiotic powder	9.62×10^{8}	7.53×10^{9}	8.50×10^{9}
	Probiotic powder + Ahiflower oil	1.08×10^{9}	8.08×10^{9}	9.17×10^{9}
	Inner capsule	1.03×10^{9}	7.82×10^{9}	8.85×10^{9}
	Duocap capsule	1.36×10^{9}	1.08×10^{10}	1.22×10^{10}
TIM-experiment (complete model; after 6 h)	Probiotic powder	2.10×10^{8}	4.93×10^{8}	7.04×10^{8}
	Probiotic powder + Ahiflower oil	4.14×10^{8}	9.33×10^{8}	1.35×10^{9}
	Inner capsule	2.28×10^{8}	4.14×10^{8}	6.43×10^{8}
	Duocap capsule	3.97×10^{8}	9.63×10^{8}	1.36×10^{9}

gastric and intestinal pH-curves mimicked the situation as found in human adults for intake of a meal (Supplementary Figure S2; Minekus *et al.*, 1995). The concentrations of electrolytes, enzymes, bile, and pancreatic juice were adjusted to the average concentrations as described for adults. Pancreatic output was simulated by secreting 10% pancreatin (Pancrex V, Paines and Birne, Greenford, UK) in small intestinal electrolyte solution. Biliary output was simulated by secreting a 2-4% bile (porcine bile extract, Sigma-Aldrich, Zwijndrecht, the Netherlands) solution at 0.5 ml/min. 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' (see below). The start residues reflect the content of the compartments after overnight fasting and are described in detail in Minekus *et al.* (1995). In brief, for the intestinal compartments, they consist of a little bit of bile, pancreatic juice and electrolytes. The gastric residue contains electrolytes at pH 2. Although normally present in the gastric compartment, we added the gastric start residue to the 'meal' before introduction in the stomach, to be able to properly set the starting pH. Hollow fibre membrane systems continuously dialyzed the digested and dissolved low-molecular weight compounds from the jejunum and ileum compartments (Supplementary Figure S1-M), which simulated absorption of nutrients in the body, and which maintained physiological concentrations of bile and electrolytes. The dialysis solution in the jejunum dialysis bottle contained 19.5 g/l 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 neutralisation of the gastric efflux, without secretion of bile and pancreatin. As a result, in the gastric experiments, during 3 h approximately 95% of the gastric contents were gradually delivered into the small intestine through the 'pyloric sphincter' (Supplementary Figure S1-B). Experiments in the complete model lasted 6 h, after which approximately 90% of the small-intestinal contents were gradually delivered into the 'large intestine' (sampling bottle) through the 'ileo-caecal sphincter' (Supplementary Figure S1-H). The test products were introduced at the start and were tested in duplicate. The following variables were tested:

- 200 mg of the probiotic powder (equal to the amount in the inner capsule);
- 200 mg probiotic powder together with 200 µl Ahiflower oil (the amount in the outer capsule);
- the inner capsule containing 200 mg probiotic powder;
- the Duocap capsule containing 200 µl Ahiflower oil and the inner capsule with 200 mg probiotic powder.

It should be noted that although the physiological parameters simulated were those of adults ingesting the capsules with a meal, the actual meal was not used in the experiments to allow visual inspection of the capsules. So, importantly, although the capsules were not taken with an actual meal, all physiological parameters in the system were set to ingestion with a meal and thus simulated the situation as if a meal was provided. We have shown before that these physiological parameters are more important in survival of the probiotics than the actual presence of a meal. Here, we meant to mechanistically study the capsule-within-capsule technology and hence decided to not actually add a meal. For the sake of simplicity however, we call it a meal in the remainder of the manuscript.

Sampling

In the gastric experiments, the gastric efflux (Supplementary Figure S1-B; the duodenal compartment was only used to neutralise the low pH in the gastric samples) was collected every hour for 3 h. In the complete TIM-1 experiments, the ileal efflux (Supplementary Figure S1-H) was collected every hour for 6 h. For each sample collected, the volume was measured, and a 1 ml sample was taken for analysis. At the end of the experiments 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 the 3 gastric efflux, 6 ileal efflux and the residue samples taken from TIM-1 and also of the initial probiotic powder (Table 1) and these were plated on Rogosa agar from Oxoid (Thermo Scientific, Badhoevedorp, the Netherlands) for the lactobacilli and on transoligosaccharide propionate (TOS) agar containing 50 mg/l lithium-mupirocin (Sigma-Aldrich) for the bifidobacteria to determine cfu's. Subsequently, the plates were incubated at 37 °C for 3-4 days under anaerobic conditions. Cumulative survival was calculated as the sum of the surviving bacteria in the different efflux samples from TIM-1 (Table 1). The agar media did not allow discrimination of the separate *Lactobacillus* and *Bifidobacterium* strains, and hence survival is reported at the genus level.

3. Results and discussion

The TNO gastro-intestinal model of the upper GI tract (TIM-1) offers the possibility to simulate very closely the pH curves and the concentrations of enzymes in the stomach and small intestine, the concentrations of bile salts in the different parts of the gut, and the kinetics of transit of food or other materials through the stomach and intestine (Marteau *et al.*, 1997; Minekus, 2015). It has been extensively validated, also with respect to probiotic survival (Marteau *et al.*, 1997). It is used to screen products for the most efficacious one, develop novel products, such as the enteric coated probiotic tablet (Venema *et al.*, 2019), study mechanistically the effect of combinations of ingredients,

such as combinations of probiotics with prebiotics (Martinez *et al.*, 2011), or determine new applications of probiotics, such as aiding in digestion of plant-proteins (Keller *et al.*, 2017). In the current study, the model was used to evaluate the benefits of the individual components of Duocap, i.e. whether the Ahiflower oil protected the cells against the stresses encountered in the upper GI tract, and if so, what the possible mechanisms would be.

Gastric experiments

The TIM-1 system is made of glass containers with flexible silicon walls inside that carry out the peristaltics. Due to this set up, one can visually inspect the lumen of the model and observe the disintegration of the capsules. Since the size of the capsules were different (small inner and larger outer), it was easy to visually track the disintegration of each individual capsule. Visual inspection showed that the capsules disintegrated within 20 min in the gastric compartment. The outer capsule was gone within 10 min, it took another 10 min to dissolve the inner capsule with the probiotics. Cumulative survival of the combined *Lactobacillus* and combined *Bifidobacterium* strains (at the level of the genera) is shown in Figure 1A and 1B, respectively. Since all experiments started with the same amount of cfu, comparison between experiments is possible. Figure 1 shows that for both genera most viable cells exit the stomach in the first 2 h. After the first 2 h, the pH has dropped to around 2.0 (Supplementary Figure S2) and the amount of viable cells exiting the system is lower. After the 3 h experiment, the pH in the gastric compartment is 1.7 and very few viable cells are remaining in the residue (as indicated by the curves that run in a straight line from 180 min to the residue fraction). In the gastric compartment, the Duocap technology seemed to increase survival of both the lactobacilli and the bifidobacteria by about a factor of 1.5 (Figure 1; compare 'Duocap' to 'Powder'). The Ahiflower oil did not seem to have an additional benefit, in the sense that it did not increase survival (Figure 1; compare 'Duocap' to 'Powder + oil' and 'Powder').

The amount of viable cells present in the Duocap capsules was stated by the manufacturer to be 30 billion (3.0×10^{10}) at the end of shelf-life, with approximately 50% contributed by lactobacilli and the other 50% by bifidobacteria (1.5×10^{10} each). Because our test were performed before end of shelf-life and since 'over-age' is usually applied for probiotics, we tested the number of viable cells in the powder. We found 2.04×10^{10} (31%) and 4.53×10^{10} (69%) for lactobacilli and bifidobacteria, respectively (Table 1). Together with the results on cumulative viable cell counts coming from the stomach, this means that ~5.4% (average of all 4 variables) and 18.9% of lactobacilli and bifidobacteria, respectively, survived the gastric compartment.

Complete TIM-1 experiments

Figure 2 shows the cumulative survival after passage through the complete TIM-1 system (stomach + small intestine). Cumulative survival is lower than after the gastric compartment only, which is what is to be expected due to exposure to bile and pancreatic enzymes in the small intestine. Although quantitatively the cumulative number of viable cells for bifidobacteria was still higher than that of lactobacilli (as was the case in the gastric experiments), relatively to the lactobacilli, the bifidobacteria were more sensitive to bile and/or pancreatin, as their numbers dropped more in the experiment in the complete TIM-1 (compared to the gastric experiments a factor of about 12) relative to lactobacilli (compared to the gastric experiments a factor of about 3.5). Cumulative survival at the end of the small intestine was on average 1.5% of the initial dose present in the capsules for both genera.

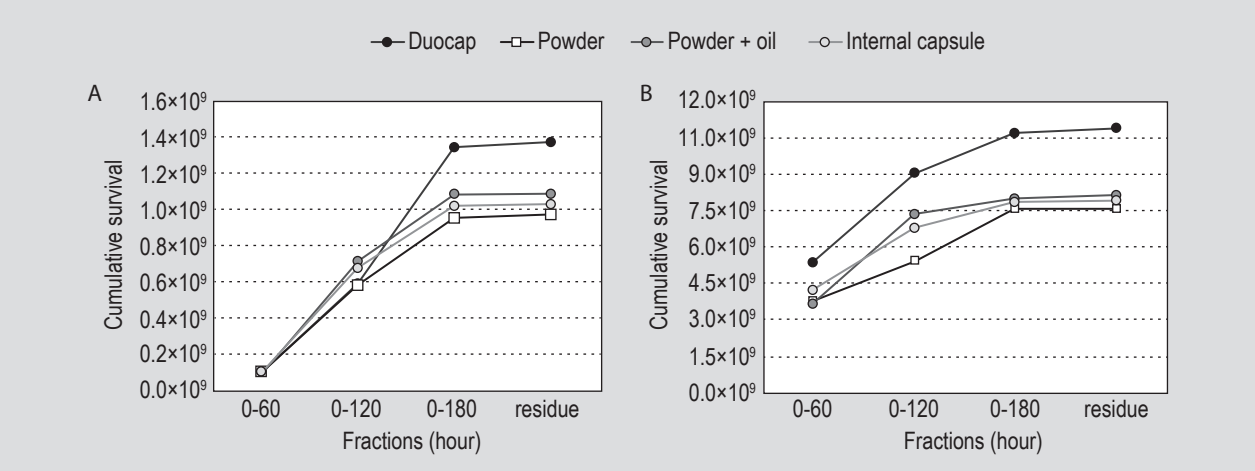

Figure 1. Cumulative survival for the two genera during transit through the gastric compartment of the TIM-1 system simulating adults. (A) Survival of the combined *Lactobacillus* strains; (B) survival of the combined *Bifidobacterium* strains.

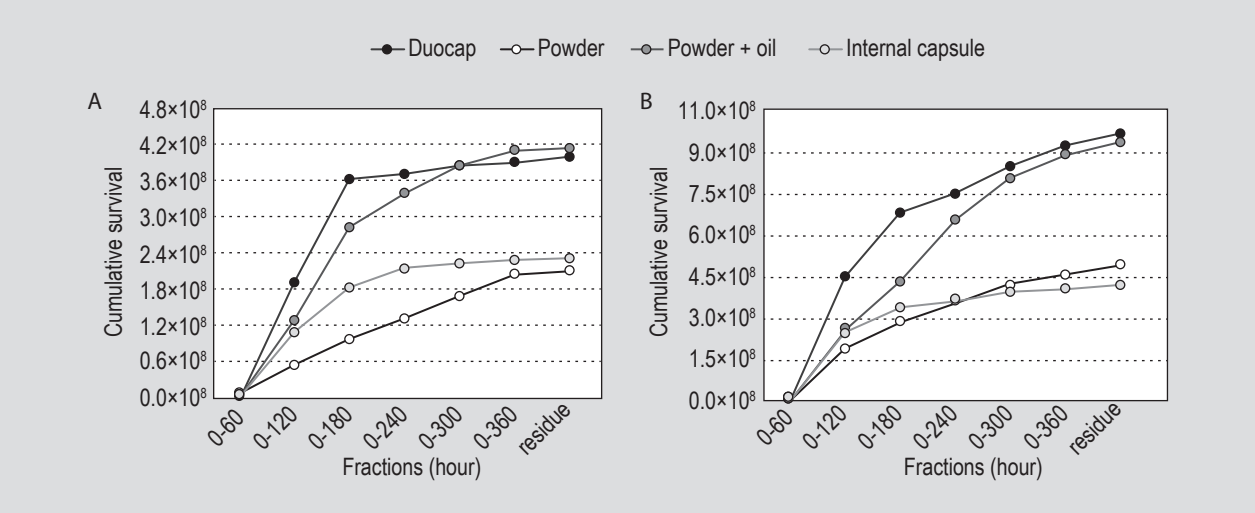

Figure 2. Cumulative survival for the two genera during transit through the complete TIM-1 system simulating adults. (A) Survival of the combined *Lactobacillus* strains; (B) survival of the combined *Bifidobacterium* strains.

From the kinetics in Figure 2 it is clear that practically no cells exited the system in the first hour. This is logical as the amount of the meal that has gone entirely through the TIM-1 system after 1 h is negligible (Supplementary Figure S2; ileal delivery curve). Most viable cells are delivered to the colon (or in this case the sampling bottle at the end of the ileum; Supplementary Figure S1-H) in the 2nd and 3rd hour. After this, most (not all) of the curves flatten out, as the time of exposure to the stressor in the GI tract (gastric acid, bile and pancreatic enzymes) leads to most cells not surviving these conditions.

In the experiments with the complete model, the Ahiflower oil did seem to have an added benefit, as the cumulative survival of both genera is approaching that of the Duocap capsule itself (Figure 2; compare 'Duocap' to 'Powder + oil' and 'Powder'), although in the first few hours of the experiments survival of both species lagged behind, and only caught up at the end of the test. It is hypothesised that the oil may have shielded the probiotic cells from the bile and/or pancreatic enzymes by forming a protective film around the bacterial cells within the lumen of the GI tract. Within the capsule-in-capsule, the internal capsule does not seem to have any other function than keeping the probiotic powder separated from the oil in the Duocap capsule.

The Duocap technology protected the probiotic cells in the gastric compartment by about a factor of 1.5. This was the case for both genera. It is likely that this is caused by the release profile of the capsule, because, despite the fact that the capsules completely disintegrated within 20 min in the stomach compartment, in that time-frame, the pH has already dropped to around pH 5.0 (see Supplementary Figure S2, pH estimated after 20 min). However, the kinetics of delivery of life cells to the small intestine are not very different (Figure 1) for the 4 tested variables, and hence the exact protective nature of the Duocap technology remains to be established.

Another mechanism that cannot be explained immediately is the observed protective effect of the Ahiflower oil in the small intestine, but not in the stomach. Mixing the probiotic powder with the oil did not lead to the same effect in the gastric experiments versus experiments in the complete system. One would think that if a film of oil was established around the cells (as hypothesised above), providing protection against bile and pancreatin, that it would not matter whether that oil was first present in a capsule or added separately. One can only speculate about the mechanism, and it could be that the timing of the presence of oil during the gastric phase was important, where changes in pH change the surface properties of the bacterial cells (Larsen *et al.*, 2018), and therefore their interaction with the oil. If present from the start (powder + oil), the oil may not immediately interact with the probiotic cells if this would occur at lower pH. After release from the capsule, although at most 20 min after ingestion, the pH has dropped already by more than a unit, which could be critical in interaction of the oil with the cells. More 'oil-coated' cells would be delivered to the small intestine in that case, protecting against bile and pancreatin only under that condition. This currently is our best explanation for the observed protective effect of the Aliflower oil in the small intestine. To test this, we are currently running experiments in (the gastric compartment of) TIM-1, where the pH of the stomach is varied to see if the above hypothesis is correct.

Currently, experiments were performed with parameters simulating the intake of a meal (although the actual meal-matrix was not used). Under these circumstances, survival of both genera was in the order of 1.5% of the intake (average of the 4 variables). Using the same model, we

have observed similar survival rates before for lactic acid bacteria and bifiddobacteria (e.g. Venema *et al.*, 2019). In that study survival of probiotics in a freeze-dried powder was 5.3% for bifidobacteria and 1% for *Lactobacillus* after the gastric compartment, and 2% for bifidobacteria and 0.1% for *Lactobacillus* after the complete TIM-1 system. The physiological conditions that the cells are exposed to upon ingestion with a meal are not as drastic as taking the capsules on an empty stomach with a glass of water, in which case the gastric pH is around or below pH 2.0. It would be advisable to have a statement on the packaging 'ingest with a meal for optimal benefit', or something along those lines, to ensure maximal survival of the probiotics. Other concepts, such as combinations with prebiotics could be interesting to test in the capsule-within-capsule technology.

4. Conclusions

The Duocap technology led to an approximately 2-fold increase in viable cells in the small intestine when ingested with a meal. This fold increase seems insignificant, but may biologically just be a tipping point between an efficacious and non-functional product. Although at this stage this is purely speculative, we believe it may be important, as the increase in the amount of life microorganisms in the gut may increase the efficacy, although this obviously needs to be further tested. Particularly, the Ahiflower oil led to the increase in survival in the small intestine.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2019.0209.

Figure S1. Schematic diagram of the dynamic, multi-compartmental TNO *in vitro* model of the stomach and small intestine (TIM-1).

Figure S2. Curves mimicked in TIM-1 over time, representing the gastric and ileal delivery, and gastric pH for adults.

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Conflict of interest

CB is an employee of Nouri Life, the company that has commercialised the probiotic capsule described in this research. DK and KV have been consultants for companies in the area of gut microbiology and probiotics.

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