Efficient Protection of Probiotics for Delivery to the Gastric Tract by Cellulose Sulphate Encapsulation

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Abstract

Gut microbiota in humans and animals play an important role in health, aiding in digestion, regulation of the immune system and protection against pathogens. Changes or imbalances in the gut microbiota (dysbiosis) have been linked to a variety of local and systemic diseases, and there is growing evidence that restoring the balance of the microbiota can restore health. This can be achieved by oral delivery of members of the microbiome (including probiotics) or by fecal microbiome transplantation. In order to provide their health promoting effects, microbiota must survive (i) transport and storage (i.e. shelf life) and (ii) transit through the highly acid conditions in the stomach and bile salts in the small intestine. We have developed a cell encapsulation technology based on the natural polymer, cellulose sulphate (CS) that protects members of the microbiota from stomach acid and bile.
Keywords: probiotics, microbiome, microbiota, encapsulation, acid protection, cellulose sulphate, living cell encapsulation

Background

The human gut microbiome, comprising the total genome of gut microbiota, [1] plays a major role in facilitating host metabolism and is a major contributor to the regulation and maintenance of host physiology, immunity and the nervous system. Tiny alterations in the status and composition of the human microbiome can have tremendous effects, resulting in dysfunction of metabolic, immunological and nervous pathways, and contributing to a broad spectrum of diseases [1] [2]. A recent example specifically links a reduction in Dialister and Coproccus species that synthesize the dopamine metabolite 3,4-dihydroxyphenylacetic acid with depression [3]. If the microbiome could be brought back into balance then such diseases could potentially be treated.
One way to achieve rebalancing is fecal microbiota transplantation (FMT) and there are a number of ongoing clinical trials in this area [4]. Clostridium difficile infections are notoriously difficult to treat but FMT has shown a more than 90 per cent success rate in treating this infection [5]. Recently, a group of “super donors” whose stool is significantly enriched in certain microbial strains such as those of the Ruminococcaceae and Lachnospiraceae families, have been described and they result in significantly more successful FMT outcomes [6] [7]. Unfortunately, to date the main ways to efficiently deliver bacteria to the gut is by endoscopic delivery, naso-intestinal tube delivery or retention enemas [8]. Since the success of FMT is often associated with repeated application of the fecal matter, these current delivery methods are a barrier to widespread use of FMT, except in critical cases [9]. Oral delivery of microbiota has been hampered by the highly acidic stomach conditions, followed by exposure to bile [10] encountered during ingestion coupled with the necessity for
release in the intestine \[^{[11]}\]. There are some bacteria that show a high degree of acid resistance over the stomach residence time such as L. reuteri \[^{[12]}\], however most microbiota are sensitive to pH2 and it has been shown that pH is the major driver of microbial diversity in FMT \[^{[13]}\]. Although acid coatings have been developed for drugs, these are generally not compatible with the growth and survival of living organisms like probiotics and other microbiota. Further, studies that have shown that extremely high numbers of at least one hundred million \((10^8)\) viable probiotic bacteria must repeatedly reach the intestine for health benefits to be achieved for the patient \[^{[14]}\] suggest that bacteria-compatible acid protective coatings must be extremely effective in order to be able to deliver therapeutically relevant doses of microbiota or probiotics. Moreover, the requirement for continued maintained therapeutic levels of microbiota requires regular bacterial consumption, as has been demonstrated in dose-response
studies. In such studies, probiotics like Lactobacillus rhamnosus GG only transiently colonize the gastro-intestinal tract, for example fifteen days after terminating the administration of L. rhamnosus GG in adults, the probiotic bacterium could only be recovered from stool samples of 27% of the volunteers [15].

A major challenge to experimentally determining the best protection method for orally delivered microbiota is the correct choice of artificial gastric juice. The makeup of gastric juice varies between individuals and according to the type and amount of food ingested [16] and the presence of milk components enhance the survival of bifidobacterial in simulated gastric juice [17]. Studies using artificial gastric juice containing lipids (L+AGJ) such as NGYC medium show a reduction in free L. acidophilus of between 3.5 and 5.5 logs [18] at pH2 over three hours, whereas use of a non-lipid containing artificial gastric juice (AGJ) results in a reproducible reduction of 6 [19] to 6.5
logs [10]. Other bacteria are even more sensitive and reduction
in viability of 8.5 logs for L. casei and of more than 11 logs for
B. bifidum have been cited after 2 hours exposure to pH2 in
AGJ [20]. Perhaps even more importantly is proteolysis of
bacteria by pepsin in the stomach [21]. Thus, the makeup of the
artificial gastric juice used for testing survival of encapsulated
bacteria has a huge effect.

We have developed a novel encapsulation method based on a
simple extrusion technique using a modified form of cellulose
sulphate that has not been used for bacterial encapsulation
before [22]. In contrast to previous methods of encapsulation,
the bacteria can be encapsulated at low density and expanded
post encapsulation before being freeze dried and stored for
long periods without cooling.
Methods

Bacteria growth and encapsulation

Lactobacilli acidophilus, Lactobacilli johnsonii and Bifidobacterium longum were grown in De Man, Rogosa and Sharpe (MRS) medium. The bacteria were grown overnight, pelleted and washed and resuspended in 10ml or 20ml cellulose sulphate at a concentration of 2x10^6 cells/ml. The solution was put into a syringe and attached to a custom-built cell encapsulation machine which creates droplets of equal size. The droplets fall into a second solution, polyDADMAC, which is in excess and causes gelation of the droplets from outside the bead. After 2 minutes, the gelation was stopped by washing the capsules five times in excess volume of PBS. Typically, 30,000 capsules are produced per run at lab scale using this protocol.
Viability in acid and testing

Artificial gastric juice (AGJ) was produced by mixing HCl (pH 2), pepsin (10 g/L), NaCl (2.79 g/L), KCl (8.74 g/L), CaCl₂ (0.24 g/L), glucose (77 g/L), glucosamine (33 g/L), lysozyme (1.52 g/L). Control gastric juice (CGJ) had the same composition as AGJ, except that the HCl, pepsin and lysozyme were not added.

Decapsulation

Capsules can be de-encapsulated (decapsulation) using a proprietary solution that allows a cell-friendly dissociation of the capsule membrane and releases the cells alive into any media of choice for further culture or processes such as cell counting. For decapsulation, 50 capsules were incubated with gentle agitation in 8mls of decapsulation solution for 30 minutes at 37°C x 50rpm.
**Cellulase digestion assay**

A range of different cellulase enzymes concentrations (10, 5, 1, 0.5, 0.1, 0.05, 0.01 U/ml) were tested using cellulase from *Trichoderma reeseei* since it contains three enzyme components and play the role in the overall conversion process of cellulose to glucose.

Ten empty capsules were placed in each well of a 24-well plate. 2ml of cellulase solution was added to 10 capsules for each sample, with each well receiving a different dose of cellulose (10, 5, 1, 0.5, 0.1, 0.05, 0.01 and 0.0 U/ml) in sodium acetate buffer solution. The plates were incubated at 37°C and examined every 30 mins for first 3 hours and then after 8 hours incubation as well as overnight incubation.

**Dilution plating**

The released bacteria were diluted in 10 fold dilution steps in MRS medium before being plated out on MRS agar plates.
Testing of encapsulated bacteria in mice

A genetically modified strain of E. coli K12 MG1655 kindly provided by Mark Tangney and colleagues [23] was used that colonises the mouse gastrointestinal (GI) tract to high levels [24]. It carries the luxCDABE operon so that it constitutively auto-bioluminesces in the absence of exogenous substrate [25]. Two groups of male nude mice (Charles River/ Nu-FOXn1\textsuperscript{nu}) received two different concentrations (2.7×10\textsuperscript{9} CFU (dose 1) or 5.4×10\textsuperscript{9} CFU (dose 2)) of non-encapsulated E. coli-Lux or encapsulated E. coli-Lux [26] [27] administered in 600μl of saline which was orally dosed by gavage. Fecal pellets were collected 2 hours, 4 hours and 24 hours post gavage. At 24 hours after gavage of test articles, animals were euthanized. After the necropsy, the stomach, cecum and colon were harvested. The organs and fecal pellets were subjected to bioluminescence imaging using an IVIS 200 spectrum (Perkin Elmer) imaging system. The luminescent exposure time was optimized and the samples were exposed to the emission spectrum of luciferase
for 5, 1, and 0.5 seconds. The tissue samples and feces were exposed to the emission spectrum of luciferase for 10 seconds, 1, and 2 minutes. The bioluminescence was measured with an open filter. The signal was visualized as pseudocolor images indicating light intensity (red being the most intense and blue the least intense), which are superimposed over the grayscale reference photographs. The images were analyzed by Living Image 4.4 software.

**Freeze-drying**

The capsules were washed 5 times with 50ml of fresh medium and resuspended in 20mls medium. 20ml of freezing medium was added, followed by incubation for 25 mins at RT. Every 25 mins, 20ml of the incubating medium was replaced with 20ml of fresh freezing medium and this was repeated 5 times. The medium was then removed and 1ml of freezing medium added and the capsules plus medium transferred into 2R glass vial. The vials were then capped and shock-frozen in 100% ethanol.
and dry ice. They can be stored at -80°C at this step. The capsules were freeze dried using a commercially available freeze drying machine. When the collecting chamber temperature of the freeze dryer reached -80°C, the vacuum pump was started and frozen vials with half-opened caps were placed into the freeze drying machine quickly, the door closed and the vacuum pump immediately re-started. Once freeze drying was completed, the freeze-dryer door was opened, the caps quickly closed and sealed with parafilm to ensure the vacuum and airtightness of vials. The freeze-dried vials were stored at room temperature.

Results

To evaluate the use of this new cellulose sulphate based delivery method, five different strains of probiotic bacteria (Lactobacillus acidophilus, L. johnsonii, L. casei, L. casei shirota and Bifidobacterium longum) were encapsulated in cellulose sulphate and survived the encapsulation process with
good viability (60-70% for L. acidophilus and L. johnsonii, 90-100% for L. casei and B. longum). Good viability was also observed for other strains of probiotic bacteria and yeasts (data not shown), indicating that the cellulose sulphate is not toxic for all strains of bacteria and yeast analysed so far. Each cellulose sulphate bead has a diameter of 0.7 mm and contains on average approximately 10 million L. casei, or 0.5 million L. acidophilus and B. longum when full (after growth of bacteria within the capsule). The encapsulation process is such that beads of a defined and reproducible size can be produced with either increased or decreased diameter as might be needed.

After encapsulation at fairly low bacterial density (2x10^6 cells/ml), the bacteria increased in number within the bead within hours. The number of viable L. casei were determined in the capsules immediately after encapsulation using an indirect metabolic activity assay and set as 100% (Fig. 1A column 1).
medium the metabolic activity in the capsules had increased by 80% (Fig. 1A column 2). This was also visually evident when comparing the capsules immediately after encapsulation (Fig. 1B left hand image) with the capsules 24 hours later (Fig. 1B right hand image).

To evaluate whether the capsules could provide an effective protection against stomach acid, artificial gastric juice (AGJ) supplemented with pepsin and lysozyme (AGJ+P) was used. Exposure times up to 4 hours at pH2 [28] were tested, the mean fasting retention time in the stomach.

After exposure of encapsulated L. casei for 3 hours to AGJ+P at pH2.0, microscopic analysis clearly showed that the capsules remained intact with no deformation (Fig. 1C) even at high magnifications (Fig. 1D). Capsules containing L. casei were recovered after 3 hours exposure to AGJ+P at pH2.0 and the capsules dissolved using a proprietary solution that releases the bacteria alive. After dilution in MRS medium and plating out on MRS agar plates, the growth of encapsulated
bacteria exposed to AGJ+P at pH 2.0 for 3 hours (Fig. 1E) is no
different to the growth of encapsulated bacteria cultured in
MRS throughout and not exposed to AGJ+P or acid (Fig. 1F).

In a quantitative evaluation, free, non-encapsulated (▲ green
lines) or encapsulated (■ red lines) L. acidophilus (Fig. 2A), L.
johnsonii (Fig. 2B), Bifidobacterium longum (Fig. 2C) and L.
casei shirota (Fig. 2D) were exposed to AGJ+P at pH 2 for 2
hours and the viability after exposure plotted as a percentage of
the initial viability. The viability of the bacteria in AGJ without
pepsin or acid was also measured (◆ blue lines). The results
showed that all four strains of encapsulated probiotic bacteria
(red lines) survived AGJ+P at pH 2 better than non-
encapsulated bacteria (green lines), where viability was
reduced to undetectable levels after 30 minutes for all four
bacteria (Fig. 2A, B, C and D).
In a second set of experiments, encapsulated (■ red lines) or non-encapsulated (▲ green lines) L. casei (Fig. 3A) or Saccharomyces boulardi (Fig. 3B) were exposed to AGJ+P at pH2 for four hours, followed by exposure for 1 hour to bile. The viability of the free, non-encapsulated bacteria or yeast in AGJ without pepsin or acid was also measured (● orange lines), as was the viability of encapsulated bacteria or yeast exposed to AGJ at pH 7.0 (◆ blue lines) and showed no changes in viability. The viability of non-encapsulated L. casei was reduced ~8 logs within 1 hours exposure to AGJ+P (A. ▲ green line 1 hour point) whereas encapsulated L. casei exposed to AGJ+P at pH 2 for 4 hours, followed by 1 hour bile exposure showed no significant effect (A. ■ red line 5 and 6 hours points).

Similarly the viability of non-encapsulated S. boulardi was reduced ~5 logs within 1 hours exposure to AGJ+P (B. ▲ green line 1 hour point) whereas encapsulated S. boulardi exposed to AGJ+P at pH 2 for 4 hours, followed by 1 hour bile exposure showed no significant effect (B. ■ red line 5 and 6 hours points).
points). In both cases the addition of bile juice to the encapsulated microbiota caused a transient reduction in cell number followed by recovery within the next hour.

The release of encapsulated bacteria after transit through the stomach and intestine is a result of a combination of the presence of low amounts of active cellulase produced by commensal bacillus species in human gastrointestinal tract [29, 30], and peristaltic movement causing breakage or bursting of the capsules. In this respect, it is important to note that the robustness of the capsules can be increased or decreased by modifying the encapsulation parameters.

To demonstrate release under these conditions in vitro, capsules were incubated at room temperature with gentle shaking in various concentrations of cellulose chosen to reflect those produced by commensal bacillus species in the human gastrointestinal tract [29, 30]. Fig. 4 shows visually the effects of
overnight incubation and shaking without cellulase (Control), and with increasing amounts of cellulase (1U/ml, 5U/ml and 10U/ml). Incubation with 10 U/ml cellulase and overnight shaking caused the capsules to visually disintegrate (Fig. 4).

Table 1 shows the results of the complete experiment in which cellulase concentrations between 0.01U/ml and 10U/ml were tested with or without touch and after incubation for between 1 hour and overnight. Cellulase concentrations of 0.05 U/ml were sufficient to cause capsule disruption (+) on touch after 8 hours (Table 1), whilst even concentrations as low as 0.01 U/ml caused capsule disruption (+) on touch after overnight incubation.

To demonstrate release of bacteria from the capsules by growth through the pore structure intact capsules were incubated in MRS medium after encapsulation. A sample was taken from the incubating medium for agar plating to check release of bacteria from the capsules into the incubating
medium. The results showed bacteria were slowly released from the capsules (Table 2).

To confirm the *in vitro* observations that encapsulated bacteria are protected from acid and bile exposure and are released in the lower intestine, two different concentration of non-encapsulated E. coli-LUX or encapsulated E. coli-LUX were administrated to the mice by gavage technique. There was no lethality and no untoward observations of toxicity during the duration of the study. After 24 hours, mice were euthanized. No significant observations were recorded at necropsy. Organs and feces were collected and placed in multi-well plates (1 plate/mouse).

Fig. 5A shows the intensity of the bioluminescent signal from Colon (upper left well), Stomach (upper center well), Cecum (upper right well), Feces 2 hours post gavage (lower left well), Feces 4 hours post gavage (lower center well), Feces 24 hours post gavage (lower right well) in mice fed 2.7x10^9 CFU free E.
coli-LUX (left most plate, M1), $5.3 \times 10^9$ CFU free E. coli-LUX (second from left plate, M2), $2.7 \times 10^9$ CFU encapsulated E. coli-LUX (third from left plate, M3) and $5.3 \times 10^9$ CFU encapsulated E. coli-LUX (right most plate, M4). The intensity of bioluminescent signal was not detectable in the tissue samples collected from mice treated with non-encapsulated E. coli-LUX (top rows of two left most plates), and only in the 2 hours feces from non-encapsulated E. coli-LUX (left most well on bottom row of two left most plates). In contrast, a clear bioluminescent signal was seen in the colon of mice treated with encapsulated E. coli-LUX (top left wells of the two rightmost plates). Similarly, the collected feces after 2, 4 and 24 hours showed detectable bioluminescent signal in the mice treated with encapsulated E. coli-LUX (bottom wells of the two rightmost plates).

The bioluminescent signal was quantitated after various timepoints of exposure and the quantitative analysis is shown in Fig. 5B. The signal was detectable mostly in the colon and feces of mice treated with encapsulated E. coli-LUX. Fig. 5B
shows the similar amounts of bacteria were found to have remained in the stomach 24 hours after gavage of marked bacteria regardless of whether they were encapsulated or not (Fig. 5B), however more bacteria were found in the cecum in those mice receiving encapsulated rather than non-encapsulated bacteria and this difference was even more marked and more than 1 log higher in the large intestine (colon). Similar differences in amounts of living bacteria were also seen in fecal pellets 2 and 4 hours post-gavage as well as 24 hours after gavage (Fig. 5B). GI transit in a mouse is around 4-6 hrs [31] [32] [33]. Thus, the data suggests that not only are the encapsulated bacteria protected from acid destruction during passage through the stomach, but additionally there is release and colonization of the intestine as evidenced by the continued presence of marked bacteria in the faeces at a constant level even after 24 hours.
Discussion

Many attempts have been made at encapsulating probiotics to protect them during passage through the GI, but none of these methods have been very effective. A recent review of protection offered to probiotics by various coatings [34] reveals that encapsulation with the de facto industry standard, alginate, followed by exposure at pH 1.8 in AGJ but with pepsin (AGJ+P) still results in loss of 10 logs activity after 90 mins for L. plantarum [35], and of at least 9 logs for L. brevis after 2 hours even in the absence of pepsin (AJG) [36].

A secondary coating of chitosan has been shown to increase the acid resistance of B. breve in alginate capsules by around 4.5 logs [36] in AGJ pH2 for 2 hours, however the overall viability is still reduced by at least 4 logs. Similar results have been reported for L. casei and B. bifidum where a coating of chitosan was applied to alginate-gelatinized starch capsules and resulted in an increase in acid resistance (compared to
alginate-gelatinized starch alone) of almost 1 log. However, the overall viability after 2 hours in AGJ+P is still reduced by 4 to 5 logs [20]. Use of AGJ also resulted in a reduction of overall viability by 2.5 to 3 logs for L. acidophilus and of 3.5 to 4 logs for L. casei after 2 hours exposure of the alginate chitosan coated capsules at pH 1.55 [19].

A secondary whey protein coating has also been applied to alginate capsules and shown to increase the resistance of encapsulated L. plantarum to acid in AGJ+P by 5 to 7 logs, however overall viability is still reduced by 3 to 5 logs after 2 hours [35].

Use of poly-L-lysine (PLL) to coat the alginate encapsulated L. acidophilus or L. casei has less of a protective effect after exposure to AGJ at pH 1.55 for two hours with losses in viability of 4-5 logs and of 5-6 logs respectively [19]. In another study losses of viability of around 3 logs have been shown for
alginate capsules coated with palm oil and PLL exposed to AGJ at pH 2 for two hours for a wide variety of bacteria (L. rhamnosus, L. salivarius, L. plantarum, L. paracasei, B. longum and B. lactis), whilst L. acidophilus only showed a loss of 2 logs [37].

Most recently, a study has shown that a layer-by-layer approach using chitosan, followed by alginate and repeated (LbL – (CHI/ALG)2) and even a multi-layered Chitosan capsule alone (LbL-(CHI/L100)2) can afford effective protection against pH2 over two hours with only loss of 1 log in viability in AGJ [38]. However, this study was conducted in the absence of pepsin. Thus, there is still a need to find simple methods to protect with high efficiency bacteria delivered by the oral route, such as in FMT from gastric conditions including enzymatic destruction by pepsin and lysozyme.
We have shown here, for a number of commonly used probiotic strains, the ability of cellulose sulphate encapsulation to protect from low pH in artificial gastric juice containing pepsin, followed by treatment with bile. Cellulose sulphate encapsulation offers exceptional protection (Fig. 2) also for strains thought previously to be acid resistant such as L. casei shirota and L. acidophilis \[39\] \[40\]. L. casei is afforded more than 8 logs protection by cellulose sulphate encapsulation, whilst S. Boulardii is afforded around 5 logs protection. As compared to chitosan encapsulation, cellulose sulphate encapsulation gave a 10,000 fold better protection for L.casei and a 100,000 fold better protection than alginate plus gelatinized starch after 3 hours exposure to simulated human gastric fluid \[41\]. The cellulose sulphate capsules used in this study have pores that allow larger molecules than H+ ions to enter and leave the capsules \[42\]. The internal cellulose sulphate material carries an excess of negatively charged sulphate groups and it is possible that these buffer the bacteria from the harmful effect of stomach
acid by preventing high concentrations of H\textsuperscript{+} ions from entering the capsule.

In our study, viable E. coli expressing luciferase were detectable in both the cecum and colon of mice orally gavaged with encapsulated bacteria. In contrast almost no E. coli expressing luciferase were detected in mice orally gavaged with free, non-encapsulated bacteria. The difference was especially noticeable in the colon (Fig. 4A and 4B). Further, more than 1 log more E. coli expressing luciferase were detected in mouse fecal pellets 2, 4 and 24 hours after ingestion of orally gavaged encapsulated bacteria compared to orally gavaged free, non-encapsulated bacteria (Fig. 4A and 4B), suggesting that not only had the bacteria survived the 4-6 hour transit through the gut but had been released and colonized the gastric tract as evidenced by the high levels of expression detected in the feces 24 hours after gavage.
Release is most probably a result of a combination of the low levels of cellulase found in the lower gastric tract, the peristaltic movement and the ability of bacteria to grow out of the pores of the cellulose capsules. The digestibility of cellulose and hemicellulose was previously estimated, in a group of seven women on a standardised diet, at 70% and 72% respectively [43] showing that there is extensive degradation of these polysaccharides in dietary plant cell wall material during passage through the human intestine. However, in the same study only 8% of an added refined cellulose (Solka Floc) was digested showing that the type of cellulose is apparently critical [43]. This is supported by the finding that bacteria able to grow on sources of hydrated, amorphous cellulose, such as spinach cell walls, can apparently be isolated from most individuals whereas bacteria that can degrade largely crystalline cellulose substrates, such as milled filter paper, are not always recoverable [44] [45] [46]. The bacterial strains isolated from human feces that are able to digest cellulose include
Ruminococcus sp, Clostridium sp, Eubacterium sp and Bacteroides sp recoverable [44] [45] [46] [47]. We were able to mimic this effect *in vitro* using equivalent concentration ranges of cellulase and gentle agitation overnight (Fig. 4 and Table 1). We also observed a slow release of bacteria through pores of the cellulose beads *in vitro* that presumably also occurs *in vivo* (see Table 2).

**Conclusion**

The use of FMT is complicated by the high heterogeneity of fecal samples since no two samples from different individual donors will ever be the same [48]. In this light, the ability to deliver individual or mixtures of members of the microbiome by the oral route, using cellulose sulphate capsules which protect extremely efficiently against low pH and proteolytic enzyme digestion over long periods, whilst releasing the bacteria in the lower intestine, would very much simplify such treatments and
make them more acceptable as well as more routine and less costly.

References

1. Wieërs G, Belkhir L, Enaud R et al. How Probiotics Affect the Microbiota. Front Cell Infect Microbiol. 2019;9:454.

2. Ogunrinola GA, Oyewale JO, Oshamika OO, Olasehinde GI. The Human Microbiome and Its Impacts on Health. International Journal of Microbiology. 2020;2020:1-7.

3. Valles-Colomer M, Falony G, Darzi Y et al. The neuroactive potential of the human gut microbiota in quality of life and depression. Nature Microbiology. 2019;4:623-632.

4. Lai CY, Sung J, Cheng F et al. Systematic review with meta-analysis: review of donor features, procedures and outcomes in 168 clinical studies of faecal microbiota transplantation. Alimentary Pharmacology & Therapeutics. 2019;49:354-363.
5. Quraishi MN, Widlak M, Bhala N et al. Systematic review with meta-analysis: the efficacy of faecal microbiota transplantation for the treatment of recurrent and refractory Clostridium difficile infection. Alimentary Pharmacology & Therapeutics. 2017;46:479-493.

6. Wilson BC, Vatanen T, Cutfield WS, O’Sullivan JM. The Super-Donor Phenomenon in Fecal Microbiota Transplantation. Frontiers in Cellular and Infection Microbiology. 2019;9:2.

7. Wilcox MH, McGovern BH, Hecht GA. The Efficacy and Safety of Fecal Microbiota Transplant for Recurrent Clostridium difficile Infection: Current Understanding and Gap Analysis. Open Forum Infect Dis. 2020;7:ofaa114.

8. Bhutiani N, Schucht JE, Miller KR, McClave SA. Technical Aspects of Fecal Microbial Transplantation (FMT). Current Gastroenterology Reports. 2018;20:30.
9. Khanna S, Pardi D. Fecal Microbiota Transplantation for Recurrent Clostridioides difficile infection: The COVID-19 Era. Am J Gastroenterol. 2020

10. Ding WK, Shah NP. Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria. Journal of Food Science. 2007;72:M446-450.

11. Culligan EP, Hill C, Sleator RD. Probiotics and gastrointestinal disease: successes, problems and future prospects. Gut Pathogens. 2009;1:19.

12. Su MS, Schlicht S, Gänzle MG. Contribution of glutamate decarboxylase in Lactobacillus reuteri to acid resistance and persistence in sourdough fermentation. Microbial Cell Factories. 2011;10:S8.

13. Ilhan ZE, Marcus AK, Kang D-W, Rittmann BE, Krajmalnik-Brown R. pH-Mediated Microbial and Metabolic Interactions in Fecal Enrichment Cultures. mSphere. 2017;2:e00047-17, /msph/2/3/e00047.
14. Govender M, Choonara YE, Kumar P, du Toit LC, van Vuuren S, Pillay V. A Review of the Advancements in Probiotic Delivery: Conventional vs. Non-conventional Formulations for Intestinal Flora Supplementation. AAPS PharmSciTech. 2014;15:29-43.

15. Terpou A, Papadaki A, Lappa IK, Kachrimanidou V, Bosnea LA, Kopsahelis N. Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value. Nutrients. 2019;11

16. Ulleberg EK, Comi I, Holm H, Herud EB, Jacobsen M, Vegarud GE. Human Gastrointestinal Juices Intended for Use in In Vitro Digestion Models. Food Digestion. 2011;2:52-61.

17. Ziarno M, Zaręba D. Effects of milk components and food additives on survival of three bifidobacteria strains in fermented milk under simulated gastrointestinal tract conditions. Microbial Ecology in Health & Disease. 2015;26
18. Chandramouli V, Kailasapathy K, Peiris P, Jones M. An improved method of microencapsulation and its evaluation to protect Lactobacillus spp. in simulated gastric conditions. Journal of Microbiological Methods. 2004;56:27-35.

19. Krasaekoopt W, Bhandari B, Deeth H. The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria. International Dairy Journal. 2004;14:737-743.

20. Khosravi Zanjani MA, Ghiassi Tarzi B, Sharifan A, Mohammadi N. Microencapsulation of Probiotics by Calcium Alginate-gelatinized Starch with Chitosan Coating and Evaluation of Survival in Simulated Human Gastro-intestinal Condition. Iranian journal of pharmaceutical research. 2014;13:843-852.

21. Zhu H. Bacterial killing in gastric juice - effect of pH and pepsin on Escherichia coli and Helicobacter pylori. Journal of Medical Microbiology. 2006;55:1265-1270.
22. Rokka S, Rantamäki P. Protecting probiotic bacteria by microencapsulation: challenges for industrial applications. European Food Research and Technology. 2010;231:1-12.

23. Cronin M, Stanton RM, Francis KP, Tangney M. Bacterial vectors for imaging and cancer gene therapy: a review. Cancer Gene Therapy. 2012;19:731-740.

24. Foucault M-L, Thomas L, Goussard S, Branchini BR, Grillot-Courvalin C. In vivo bioluminescence imaging for the study of intestinal colonization by Escherichia coli in mice. Applied and Environmental Microbiology. 2010;76:264-274.

25. Waidmann MS, Bleichrodt FS, Laslo T, Riedel CU. Bacterial luciferase reporters: the Swiss army knife of molecular biology. Bioengineered Bugs. 2011;2:8-16.

26. Baban CK, Cronin M, Akin AR et al. Bioluminescent bacterial imaging in vivo. Journal of Visualized Experiments. 2012e4318.
27. Cronin M, Akin AR, Collins SA et al. High Resolution In Vivo Bioluminescent Imaging for the Study of Bacterial Tumour Targeting. PLoS ONE. 2012;7:e30940.

28. Mojaverian P, Ferguson RK, Vlasses PH et al. Estimation of gastric residence time of the Heidelberg capsule in humans: effect of varying food composition. Gastroenterology. 1985;89:392-397.

29. Ariffin H, Abdullah N, Umi Kalsom MS, Shirai Y, Hassan MA. Production and characterization of cellulase by Bacillus pumilus EB3. Int J Eng Tech. 2006;3:47-53.

30. Hong HA, Khaneja R, Tam NMK et al. Bacillus subtilis isolated from the human gastrointestinal tract. Research in Microbiology. 2009;160:134-143.

31. Schwarz R, Kaspar A, Seelig J, Künnecke B. Gastrointestinal transit times in mice and humans measured with 27Al and 19F nuclear magnetic resonance. Magnetic Resonance in Medicine. 2002;48:255-261.
32. Padmanabhan P, Grosse J, Asad ABMA, Radda GK, Golay X. Gastrointestinal transit measurements in mice with 99mTc-DTPA-labeled activated charcoal using NanoSPECT-CT. EJNMMI research. 2013;3:60.

33. Yano JM, Yu K, Donaldson GP et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell. 2015;161:264-276.

34. Ramos PE, Cerqueira MA, Teixeira JA, Vicente AA. Physiological protection of probiotic microcapsules by coatings. Critical Reviews in Food Science and Nutrition. 2018;58:1864-1877.

35. Gbassi G, Vandamme T, Ennahar S, Marchioni E. Microencapsulation of Lactobacillus plantarum spp in an alginate matrix coated with whey proteins. International Journal of Food Microbiology. 2009;129:103-105.

36. Cook MT, Tzortzis G, Charalampopoulos D, Khutoryanskiy VV. Production and Evaluation of Dry Alginate-Chitosan
Microcapsules as an Enteric Delivery Vehicle for Probiotic Bacteria. Biomacromolecules. 2011;12:2834-2840.

37. Ding WK, Shah NP. An Improved Method of Microencapsulation of Probiotic Bacteria for Their Stability in Acidic and Bile Conditions during Storage. Journal of Food Science. 2009;74:M53-M61.

38. Anselmo AC, McHugh KJ, Webster J, Langer R, Jaklenec A. Layer-by-Layer Encapsulation of Probiotics for Delivery to the Microbiome. Advanced Materials. 2016;28:9486-9490.

39. de Vuyst L, Avonts L, Makras L. Probiotics, prebiotics and gut health. In: Remacle C, Reusens B, editors. Functional Foods, Ageing and Degenerative Disease. Woodhead Publishing; 2004. p. 416-482.

40. Shortt C. The probiotic century: historical and current perspectives. Trends in Food Science & Technology. 1999;10:411-417.
41. Salmons B, Dangerfield D, Gunzburg W. Delivery of probiotics efficiently to the intestine: the acid test. NuFFooDS Spectrum Asia. 2020 https://www.nuffoodsspectrum.asia/analysis/54/1525/delivery-of.

42. Salmons B, Gunzburg WH. Release characteristics of cellulose sulphate capsules and production of cytokines from encapsulated cells. International Journal of Pharmaceutics. 2018;548:15-22.

43. Slavin JL, Brauer PM, Marlett JA. Neutral detergent fiber, hemicellulose and cellulose digestibility in human subjects. The Journal of Nutrition. 1981;111:287-297.

44. Wedekind KJ, Mansfield HR, Montgomery L. Enumeration and isolation of cellulolytic and hemicellulolytic bacteria from human feces. Applied and Environmental Microbiology. 1988;54:1530-1535.

45. Chassard C, Delmas E, Robert C, Bernalier-Donadille A. The cellulose-degrading microbial community of the human
gut varies according to the presence or absence of methanogens: Cellulolytic microbiota and CH4 production in the human gut. FEMS Microbiology Ecology. 2010;74:205-213.

46. Robert C, Bernalier-Donadille A. The cellulolytic microflora of the human colon: evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. FEMS microbiology ecology. 2003;46:81-89.

47. Betian HG, Linehan BA, Bryant MP, Holdeman LV. Isolation of a cellulolytic Bacteroides sp. from human feces. Applied and Environmental Microbiology. 1977;33:1009-1010.

48. Culligan E, Sleator R. Advances in the Microbiome: Applications to Clostridium difficile Infection. Journal of Clinical Medicine. 2016;5:83.
**Figure Legends**

**Figure 1: Growth and survival of bacteria post encapsulation**

**A.** The growth of encapsulated L. casei determined indirectly by metabolic activity immediately after encapsulation or 24 hours after encapsulation in cellulose sulphate. **B.** Images of encapsulated L. casei immediately after encapsulation (left) and 24 hours after incubation in MRS (right). **C.** Encapsulated L. casei exposed for 3 hours to artificial gastric juice plus pepsin and lysozyme (AGJ+P) at pH2.0. **D.** Higher magnification of a cellulose sulphate capsule containing L. casei after 3 hours exposure to AGJ+P at pH2.0. **E.** MRS agar plate growth of L. casei after being recovered from capsules containing L. casei after 3 hours exposure to AGJ+P at pH2.0. **F.** Growth of L. casei on MRS agar plates after recovery from capsules containing L. casei after continuous growth in MRS media.
Figure 2: Survival of Encapsulated Bacteria after acid exposure

Timecourse of survival of encapsulated (■ - red lines) or free, non-encapsulated (▲ - green lines) Lactobacillus acidophilus (A), L. johnsonii (B), Bifidobacterium longum (C) and L. casei shirota (D) expressed as a percentage of the initial number of bacteria, after 2 hours exposure to artificial gastric juice plus pepsin and lysozyme (AGJ+P). For comparison the timecourse of survival of bacteria (◆ - blue lines) Lactobacillus acidophilus (A), L. johnsonii (B), Bifidobacterium longum (C) and L. casei shirota (D) after 2 hours exposure to artificial gastric juice without acid (AGJ) is also shown.

Figure 3: Survival of Encapsulated Bacteria and Yeast after acid exposure followed by bile

Timecourse of normalized survival of encapsulated (■ - red lines) or free, non-encapsulated (▲ - green lines) Lactobacillus casei (A), and S. boulardii (B) after 4 hours exposure to artificial
gastric juice plus pepsin and lysozyme (AGJ+P) followed by one hour exposure to artificial bile. For comparison the timecourse of survival of encapsulated (◆ - blue lines) or free, non-encapsulated (● - orange lines) Lactobacillus casei (A), and S. boulardii (B) after 4 hours exposure to artificial gastric juice at pH7.0 (AGJ) is also shown.

**Figure 4: Release of Encapsulated Bacteria *in vitro***

Capsules were incubated in three (10U/ml, 5U/ml, 1U/ml) concentrations of cellulase with gentle shaking overnight and visual disintegration of the capsules documented. The control was shaken gently overnight without the addition of cellulase.

**Figure 5: Release of encapsulated bacteria *in vivo***

**A.** Four mice were administered 2.7x10^9 CFU of free E. coli-LUX (M1) (left most six well plate), 5.3x10^9 CFU of free E. coli-LUC (M2) (six well plate second from left), 2.7x10^9 CFU of encapsulated E. coli-LUX (M3) (six well plate third from left) or
5.3x10⁹ CFU of encapsulated E.coli-LUX (M4) (rightmost six well plate) by oral gavage. 2 hour, 4 hour and 24 hours post gavage feces were harvested and 24 hours after gavage the animals were euthanized and stomach, cecum and colon harvested. These organs, as well as the feces were placed in individual wells of six well plates and exposed to the emission spectrum of luciferase for 10 seconds, 1, and 2 minutes. Here the results from 2 minutes exposure are shown. The bioluminescence was measured with an open filter. The signal was visualized as pseudocolor images indicating light intensity (red being the most intense and blue the least intense), which are superimposed over the grayscale reference photographs.

B. The bioluminescence signal from the gavage experiment described above was quantitated using Living Image 4.4 software. The signal from stomach, cecum, colon, 2 hour feces, 4 hour feces and 24 hour feces from mice administered 2.7x10⁹ CFU of free E. coli-LUX (M1) (blue bars), 5.3x10⁹ CFU of free
1. E. coli-LUC (M2) (orange bars), 2.7x10^9 CFU of encapsulated
2. E. coli-LUX (M3) (grey bars) or 5.3x10^9 CFU of encapsulated E.
3. coli-LUX (M4) (yellow bars) is shown.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

M1-free E. Coli-LUX (2.7x10^9 CFU)
M2-free E. Coli-LUX (5.3x10^9 CFU)
M3-Encapsulated E. Coli-LUX (2.7x10^9 CFU)
M4-Encapsulated E. Coli-LUX (5.3x10^9 CFU)

B

Figure 5
Table 1: Effect of various Cellulase Concentrations and Overnight Incubation with Shaking on Capsule Stability

| Incubation Time | Observation | Cellulase Concentration |
|-----------------|-------------|-------------------------|
|                 |             | 10U/ml | 1U/ml | 0.5U/ml | 0.1U/ml | 0.05U/ml | 0.01U/ml |
| 1 hour          | Debris      | +      | -     | -       | -       | -        | -        |
|                 | Burst on touch | +    | -     | -       | -       | -        | -        |
| 2 hours         | Debris      | ++     | +     | -       | -       | -        | -        |
|                 | Burst on touch | +   | -     | -       | -       | -        | -        |
| 3 hours         | Debris      | +++    | +     | -       | -       | -        | -        |
|                 | Burst on touch | +  | +     | -       | -       | -        | -        |
| 8 hours         | Debris      | ++++   | +     | -       | -       | -        | -        |
|                 | Burst on touch | + | +     | +       | +       | +        | -        |
| overnight       | Debris      | ++++   | +     | -       | -       | -        | -        |
|                 | Burst on touch | + | +     | +       | +       | +        | +        |

Debris: - no debris; + detectable debris; ++ major debris; +++ most capsules as debris; +++ all capsules as debris

Burst on touch: - no; + yes
Table 2: Release of Bacteria from Capsules by Growth through Pore Structure

| Post encapsulation period (hours) | Bacteria associated with outside of capsule (CFU/ml) | Bacteria escape from capsules (CFU/ml) |
|----------------------------------|--------------------------------------------------|--------------------------------------|
| 0                                | 0                                                | -                                    |
| 1                                | 0                                                | 0                                    |
| 2                                | 0                                                | 0                                    |
| 3                                | 0                                                | 0                                    |
| 19                               | 0                                                | 5.8x10^5                             |
| 21                               | 0                                                | 1.56x10^6                            |
| 23                               | 0                                                | 9.4x10^7                             |
| 26                               | 0                                                | 8.6x10^7                             |
| 47                               | 0                                                | 5.6x10^8                             |
| 49                               | 0                                                | 3.8x10^8                             |
| 50                               | 0                                                | 4.40x10^8                            |

After encapsulation, intact capsules were incubated in MRS medium. A sample was taken from the incubating medium for agar plating to check release of bacteria from the capsules into the incubating medium. **Bacteria associated with outside of capsules**: Bacteria count from solution used to wash capsules during the encapsulation process; **Bacteria escape from capsules**: Bacterial count from incubating capsules in MRS medium for various lengths of time post encapsulation.