# Targeted delivery of probiotics to enhance gastrointestinal stability and intestinal colonisation

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**Abstract**

The aim of this work was to assess the viability of some commercial probiotics after exposure to gastric acid and the possibility of modifying these formulations for delivery into the distal parts of the intestines. Gastrointestinal tolerance testing was conducted for three commercial probiotics and an in-house freeze-dried *Lactobacillus acidophilus* strain. The contents of the commercial products and the in-house freeze-dried strain were then loaded into capsules for site-specific delivery into the colon using the Phloral® coating technology; the viability upon release was then ascertained. An assessment of the potential of these products to adhere to intestinal cells was also conducted. The results showed that all the commercial products contained the minimum number of probiotic strains as indicated on their respective packages. When gastric acid tolerance tests were performed on these products, all the commercial probiotics and the prepared freeze-dried strain demonstrated over $10^6$ CFU reductions within 5 minutes. When these were encapsulated for site-specific delivery into the distal parts of the gut, viabilities of approximately 90% were obtained after these capsules had been initially deposited in gastric acid for 2 hours. An evaluation of the ability of the probiotic formulations to adhere to intestinal cells demonstrated adhesion in the range 64-76% for the products evaluated. The need to target the delivery of probiotics into the intestines has been demonstrated here as this offers a greater potential for colonisation of the intestines once the harshness of the stomach has been overcome.

**Keywords**: Probiotics; Gastric acid tolerance tests; Phloral® coating technology; Intestinal colonisation.

# 1 Introduction

In recent years, there has been an increased interest in the use of probiotics with several probiotic products on the market with various delivery mechanisms. The concept of probiotics emerged from the early 20th century when the Russian immunologist Elie Metchnikoff observed that Bulgarian peasant farmers had long life-spans. He suggested this was due to the consumption of large quantities of fermented milk, rich in lactobacilli. Metchnikoff suggested that pathogens present in the intestine released toxins which were poisonous to the body and the consumption of fermented milk helped alleviate the effects of these pathogens and their toxins (Fuller, 1991, Vasiljevic and Shah, 2008, Dixon, 2002). The Food and Agriculture Organisation of the United Nations, and World Health Organisation jointly describe probiotics as live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002).

For an organism to be considered as a probiotic, it must have been documented to have a health benefit (Sanders, 2009). Several health claims have been attributed to probiotics, some of these health claims are towards gastrointestinal health whereas others are intended for systemic benefits contributing towards overall general wellbeing. Gastrointestinal health claims attributed to probiotics include the alleviation of lactose intolerance, prevention of antibiotic-associated diarrhoea, and management of inflammatory bowel disease (Tung et al., 2009, Vasiljevic and Shah, 2008, Gismondo et al., 1999, Guslandi et al., 2003, Mimura et al., 2004). Probiotics have also been reported to have roles in immune stimulation and modulation as well as in the management of obesity (Morita et al., 2002, Kato et al., 1999). Some probiotics have been reported to have anti-carcinogenic and cholesterol-lowering properties (Couteau et al., 2001, Tabuchi et al., 2003).

The recent increased interest in probiotics also stems from the greater awareness of the human microbiome and its potential applications. An increased realisation of the need for alternatives to antibiotics has partly contributed to this. Even though antibiotics have been around for over 50 years, hospital infection rates are not declining and multi-drug resistant bacteria continue to emerge creating a major public health problem as a result (Broeckx et al., 2016, Teughels et al., 2011).

Ideally, a probiotic must have viable organisms at the point of consumption, minimum numbers of $10^6$, $10^7$, and $10^8$ CFU/g at the point of consumption have been reported (Shah, 2007, Douglas and Sanders, 2008, Krishnakumar and Gordon, 2001). The FAO/WHO, however, recommends that minimum viable numbers of each probiotic strain in a product at the end of the product's shelf-life should be indicated on the product label. Probiotic formulations, upon consumption, must withstand gastrointestinal transit and colonise the intestines for benefits to be observed (FAO/WHO, 2002, Vasiljevic and Shah, 2008, Sanders, 2009). Dairy products supplemented with probiotics are a natural means of probiotics administration, however, for the purposes of prevention or treatment of diseases, specifically targeted applications, formulations, devices, or carriers with a slow release of probiotics might be needed (Meurman and Stamatova, 2007). There have been reports of commercial products exhibiting significant drops in viability after exposure to gastric fluids; other products have been reported with probiotic populations that do not correspond to numbers indicated on products (Hoa, 2000, Hamilton-Miller et al., 2007, Fredua-Agyeman and Gaisford, 2015, Temmerman et al., 2003, Huff, 2004, de Vos et al., 2010, Masco et al., 2005, Charteris et al., 1998, Caillard and Lapointe, 2017).

Formulating probiotics can be challenging since product viability must be maintained during formulation and after consumption by consumers. Formulating products that can withstand the harshness of the gastrointestinal tract and target them to the intestines is currently receiving great interest as are formulation processes that are not deleterious to organisms (Kailasapthy, 2002, Mortazavian et al., 2007). The aim of this work was, therefore, to assess the viability of some commercial probiotics after exposure to gastric acid and the possibility of modifying these formulations. The modification approach used here was the Phloral® coating technology to target probiotics to the colon. Phloral® consists of a blend of bacteria-activated (resistant starch) and pH-activated (Eudragit® S) components. The independent triggers of a bacterially-triggered component within a pH-responsive polymer are effective, complementary, and act as failsafe mechanisms for each other in drug delivery (Ibekwe et al., 2008, McConnell et al., 2008, D'Haens et al., 2017). An assessment of the potential of these products to adhere to intestinal cells was also conducted.

## 2 Materials and methods

### *2.1 Materials*

Pepsin, trehalose, xylitol, sucrose, and triton X-100 were purchased from Sigma, UK. Dulbecco's Modified Eagle Medium, heat-inactivated foetal bovine serum, phosphate-buffered saline (PBS), 1% non-essential amino acid, and trypsin-ethylene diamine tetraacetic acid were from Gibco, UK. de Man, Rogosa and Sharpe (MRS) agar and broth were from Oxoid, UK. Hydrochloric acid was purchased from VWR, UK. Sodium chloride was purchased from Fisher Scientific, UK.

Three commercial probiotic products were bought from a health food shop and analysed. The choice of probiotics was limited to products that contain only lactobacilli strains; these are represented here as Products A, B, and C and their composition is indicated in Table 1. These probiotics were selected because they contain strains common to most probiotic products, and so the results have wide applicability. An in-house probiotic was also prepared by freeze-drying *Lactobacillus acidophilus* LA 5.

Table 1: Composition of probiotic products used

| **Product** | **Formulation** | **Composition** | **Stated minimum content per capsule (x $10^8$ CFU)** |
|---|---|---|---|
| Product A | Encapsulated dried powder | *Lactobacillus acidophilus* PXN 35<br>*Lactobacillus plantarum* PXN 47 | 5 |
| Product B | Encapsulated dried powder | *Lactobacillus plantarum* LP 299v | 200 |
| Product C | Encapsulated dried powder | *Lactobacillus plantarum* CECT 7527<br>*Lactobacillus plantarum* CECT 7528<br>*Lactobacillus plantarum* CECT | 12 |

## *2.2 Preparation of freeze-dried probiotic*

An in-house freeze-dried probiotic formulation was prepared for comparison with the commercial products. *Lactobacillus acidophilus* LA5 was used as the model probiotic strain with 10% sucrose or 10% trehalose used as protectants.

### 2.2.1 Cultivation of microorganism and preparation of bacterial culture

*Lactobacillus acidophilus* was initially grown on MRS (de Man, Rogosa, and Sharpe) agar and incubated under anaerobic conditions for 48 hours at 37 ºC. A few colonies were taken and used to inoculate 7 mL of MRS broth to create a starter culture and incubated for 24 hours. 99 mL of fresh MRS broth was inoculated with 1 mL of starter culture to create a 1: 100 dilution and this was incubated for 24 hours. The culture was then mixed using a magnetic stirrer and 30 mL each was dispensed into 3 falcon tubes (Tubes A, B, and C). Centrifugation was done at 9500 rpm and 4 ºC for 10 minutes to harvest the cells after which supernatant was removed carefully by suction. The cells were washed with PBS and centrifuged at 9500 rpm and 4 ºC for 10 minutes. The supernatant was removed by suction and the washing process repeated. The cells in tube A were resuspended in ¼ Ringer's solution only and tubes B and C resuspended in 10% sucrose and 10% trehalose in ¼ Ringer's solution respectively. The bacterial cultures were mixed continuously using a magnetic stirrer and 2 mL aliquoted into sterile 5 mL glass vials for freeze-drying.

### 2.2.2 Freeze-drying

Freeze-drying was done with VirTis-Advantage freeze-dryer (UK). The samples were initially submerged under dry ice for about 60 minutes. Once frozen, the vials were transferred onto pre-cooled shelves in the freeze-dryer. The freezing and condenser temperatures were -40 $^{o}$C and -60 $^{o}$C respectively. The primary drying step was at -20 $^{o}$C for 48 hours. The vacuum pressure was maintained below 200 mBar.

Secondary drying was done at 20 $^{o}$C for 3 hrs. Enumeration after freeze-drying was conducted and percentage recovery obtained.

### *2.3 Evaluating viability of probiotic products*

The contents of one capsule of each commercial probiotic were suspended in 5 mL of PBS and vortexed for about 10 seconds and allowed to stand for 10 minutes and vortexed once more for homogeneity. These were then serially diluted and plated onto MRS agar. The agar plates were incubated at 37 $^{o}$C under anaerobic conditions for 48 hours after which colonies were counted. 100 mg was used for the prepared freeze-dried formulation.

### *2.4 Evaluating the in vitro adhesion of probiotics to intestinal cells*

The evaluation done here was similar to work carried out by Forestier et al. (2001). The growth medium used was Dulbecco's Modified Eagle Medium supplemented with 10% of heat-inactivated foetal bovine serum and 1% non-essential amino acid. The prepared medium was used to routinely grow Caco-2 cells in cell culture flasks with surface area 75cm$^2$ or to seed cells in a well-plate. All cells were incubated at 37 $^{o}$C in a humidified atmosphere of 5% $CO_2$.

Caco–2 monolayers were seeded at a concentration of 2 x $10^5$ cells per well in 12-well plates and incubated at 37 $^{o}$C with humidified atmosphere of 5% $CO_2$. Caco–2 cells were used in the late post-confluence stage with passage numbers between 39 and 54. Prior to adhesion testing, Caco–2 monolayers were washed twice with 1 mL PBS; 1 mL of the cell culture growth medium was then added to each well. The contents of one capsule of each commercial probiotic and 100 mg of the freeze-dried *L. acidophilus* strain were suspended in 5 mL of PBS. These were serially diluted such that the estimated bacterial numbers did not exceed the Caco-2 cell numbers. 1mL of each test sample was then added to 1mL of growth medium in each well and plates incubated for an hour. The monolayers were then washed three times with 1 mL PBS to remove any non-adhered bacterial cells. The cells were lysed by addition of 1 mL 0.1% triton X-100 solution and the number of viable adhering bacteria determined by plating out serial dilutions onto MRS agar. The number of adhered

cells was then expressed as a percentage of the initial number. An estimate of the number of Caco–2 cells in the wells was made each time adhesion tests were performed. This was to ensure the available Caco-2 cells were not less in number than the bacterial cells being added. The Caco-2 cells used for these tests were between $2.9 \times 10^5$ – $1 \times 10^6$ cells per well.

### *2.5 Evaluating tolerance to gastric fluids*

#### 2.5.1 Preparation of Simulated Gastric fluid (SGF)

100 mL of sodium chloride and hydrochloric acid (HCl) mixture was first prepared by dissolving 0.2 g of sodium chloride in 90 mL purified water. The pH was adjusted to 1.2 with HCl and volume made up to 100 mL. 0.32 g of pepsin was then added to 50 ml of the sodium chloride and HCl solution and stirred until complete dissolution. The volume was adjusted to 100 mL afterwards. The solution was filter-sterilised and used within 48 hours of preparation.

#### 2.5.2 Tolerance testing

The contents of one capsule of each product were deposited into the SGF for 2 hours. All the media were pre-warmed to 37 $^{o}$C prior to use and bacterial enumeration was conducted at set times. To ensure the process was carried out at 37 $^{o}$C with 50 rpm paddle movement as observed in USP dissolution testing, a hot plate magnetic stirrer was used with temperature and speed set to 37 $^{o}$C and 50 rpm respectively. 50 mL of gastric media in sterile duran bottles were used. Aliquots were taken at set times and enumeration of probiotic species conducted using MRS agar plates. The plates were incubated for 48 hours afterwards under anaerobic conditions. 100 mg was used for the prepared freeze-dried formulation.

### *2.6 Encapsulation of probiotics for site-specific delivery*

Capsules were coated using Phloral® – a coating technology developed at University College London that targets the release of drugs to the colon. The contents of one capsule of each commercial probiotic and 100 mg of the in-house freeze-dried *L. acidophilus* were then transferred into empty size 0 capsules (Qualicaps® USA) and coated using the Phloral® coating technology as reported by Ibekwe et al. (2008). These were then analysed using a modified dissolution testing method mimicking standard USP dissolution testing.

Capsules were immersed in three consecutive media i.e., 0.1M HCl (2 hours), phosphate buffer at pH 6.8 (2 hours), and phosphate buffer at pH 7.4 (1 hour). All the media were pre-warmed to 37 $^{o}$C prior to use. Bacterial enumeration was conducted at hourly intervals in all media. To ensure the process was carried out at 37 $^{o}$C with 50 rpm paddle movement as observed in USP dissolution testing; a hot plate magnetic stirrer was used with temperature and speed set to 37 $^{o}$C and 50 rpm respectively. 50 mL media in sterile duran bottles were used and all capsules were completely immersed with the aid of sinkers.

## 3 Results and Discussion

### *3.1 Freeze-drying Lactobacillus acidophilus LA5*

The freeze-dried formulations with the protectants yielded recoveries over 90%; a complete loss of viability was observed when no protectant was added (Table 2).

Table 2: Percentage recovery after freeze-drying *L. acidophilus* LA5 using sugars as protectants

| **Sample** | **Percentage Recovery (%)** |
|---|---|
| Control | 0 |
| 10% Sucrose | 92.94 ± 1.08 |
| 10% Trehalose | 94.22 ± 2.67 |

Freeze-drying is a procedure based on sublimation under high vacuum, this occurs in three phases, i.e., freezing, primary drying, and secondary drying. During freeze-drying, the formation of intracellular ice crystals and macromolecule denaturation results in the loss of viability; hence, to preserve cell viability, protectants are added (Santivarangkna et al., 2007, Zayed and Roos, 2004, De Giulio et al., 2005). There are numerous protectants used to improve cell viability during such procedures. Sugars have been reported to be beneficial to most lactobacilli during drying and upon storage; 10% sugar concentrations have been used with high recoveries after freeze-drying (Costa et al., 2000, Zhao and Zhang, 2005). In this study, 10% trehalose or 10% sucrose was, therefore, used as protectants during freeze-drying. Other additives like non-skimmed fat milk, polyols, polymers, amino acids have also been used (Efiuvwevwere et al., 1999, Costa et al., 2000). There is no single additive that fits all organisms, therefore, as best practice, a variety of excipients are explored during freeze-drying and the best excipient with the most improved viability chosen. Viabilities of 93% and 94% were obtained when 10% sucrose and 10% trehalose were used respectively as compared to a complete loss of viability when no protectant was included in freeze-drying *L. acidophilus*. De Giulio et al. (2005) also observed viabilities over 90% after freeze-drying some lactobacilli; they, however, used 32% sucrose and trehalose. The concentration of sugar used in freeze drying is

important; when Zayed and Roos (2004) used 4% sucrose and 4% trehalose as protectants, they could recover only 13% and 34% viabilities respectively after freeze-drying. They needed a combination of both amounts in addition to 18% skimmed milk to obtain 83-85% survival rates.

### *3.2 Evaluating viability of product content*

An evaluation of the content in each of the products analysed is shown in Figure 1. All the products met the minimum indicated content on their packages, with Product A having more than 10 times the microorganisms as stated on product package.

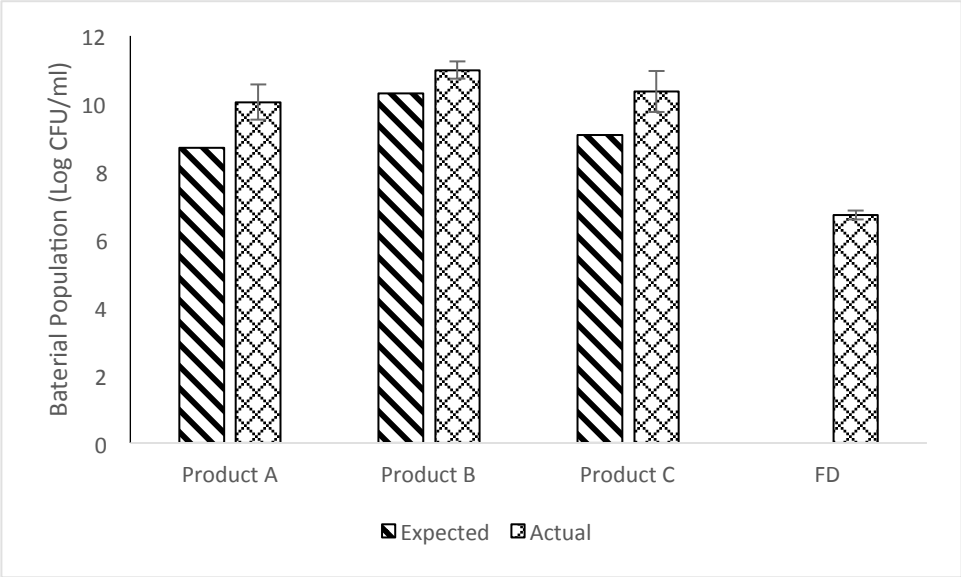


Figure 1: Bar chart illustrating the comparison between the expected and actual bacterial numbers per dose – a capsule each for the solid dose probiotics and 100 mg for freeze-dried (FD) strain – obtained after enumeration.

With several studies as indicated in the introduction reporting about the lack of viable organisms or reduced numbers than what has been indicated on product packages, this was a good indication as it confirmed the probiotics had been well preserved and from the consumer's point of view, gives confidence that the formulations purchased actually contain the stated number of bacteria. The presence of viable organisms after product formulation is key to ensuring probiotic activity.

### *3.3 In vitro adhesion of commercial probiotics to intestinal cells*

An enumeration of probiotic cells that adhered to intestinal cells for the products A, B and the freeze-dried strain showed that over 70% of the administered probiotics adhered to intestinal cells (Figure 2). Product C, however, had 64% of the administered probiotics adhering.

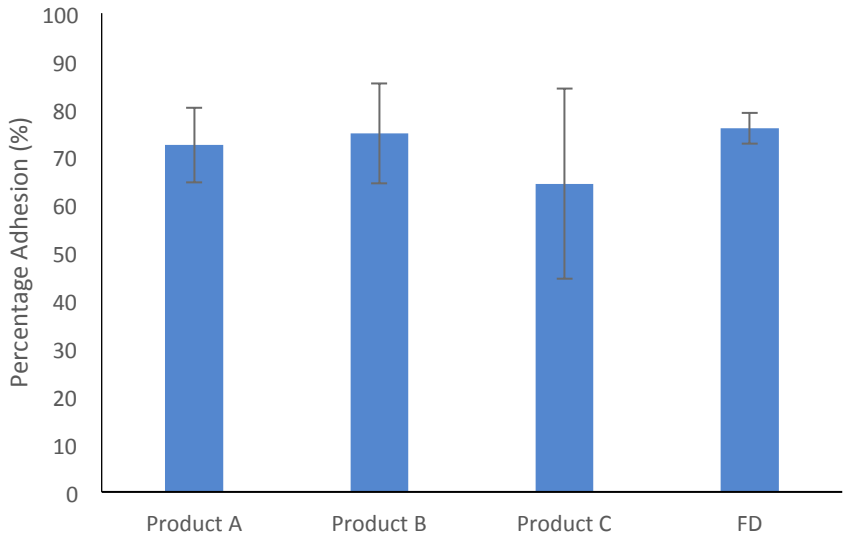


Figure 2: An illustration of the percentage of administered probiotics that adhered to intestinal cells

For probiotics delivered into the gastrointestinal tract to act, these need to colonise the intestines. Evaluation of probiotic colonisation *in vivo* is very challenging; a few studies have used the presence of probiotics in faeces as an indication of colonisation; this is, however, very speculative as faecal enumeration generally represents unadhered strains (Saxelin et al., 1993, Saxelin et al., 1995). An *in vitro* evaluation was demonstrated here whereby adhesion to Caco-2 intestinal cells was used to evaluate probiotic adhesion. This method of evaluation that ascertains probiotic organisms that adhere to intestinal cell lines as an *in vitro* model for has been reported (Govender et al., 2014, Maragkoudakis et al., 2006, Forestier et al., 2001, Gopal et al., 2001, Tuomola and Salminen, 1998). More than half the number of the probiotic organisms administered adhered to intestinal cells, Products C which exhibited the least adhesion of the products tested had 64% of administered organisms adhering to Caco-2 cells. This indicated that all the dried probiotics

evaluated had the ability to adhere to intestinal cells and could possibly colonise the intestines should these arrive there.

### *3.4 Evaluating tolerance to gastric fluids*

When the probiotics were exposed to simulated gastric fluid as shown in Figure 3, there was a sharp drop in viability within 5 minutes for all the solid dose probiotics evaluated; this reduction was circ. over 6 Log CFU.

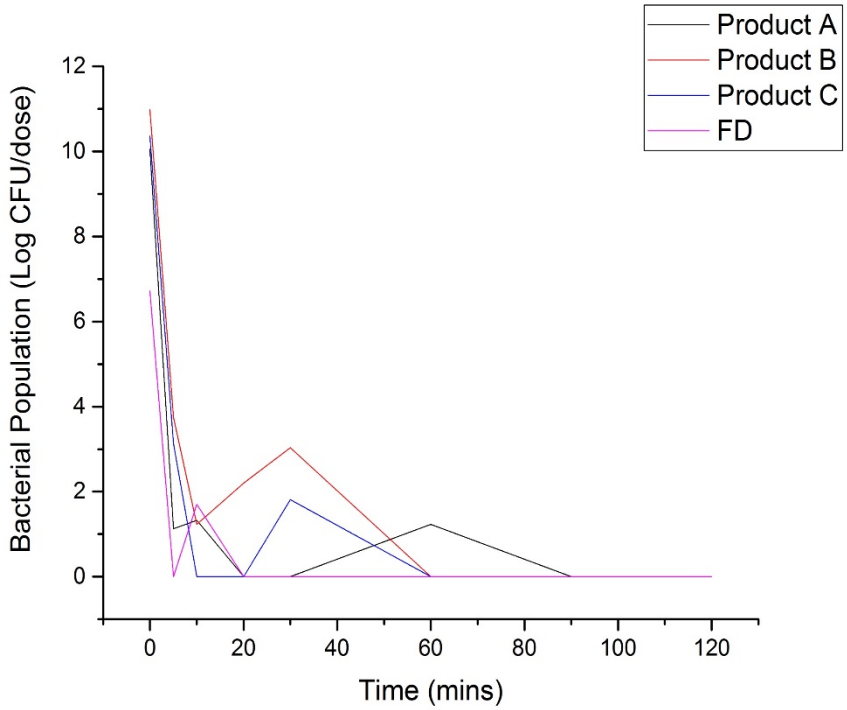


Figure 3: An illustration of probiotic population with time after exposure to fasted state simulated gastric fluid for products A-C and freeze-dried strain, FD.

Gastric fluid is one of the barriers most biological preparations need to overcome to exert their impact. Even though some reports have highlighted the harshness of this medium to probiotics, others are of the perception that probiotics are acid-producing organisms and can, therefore, withstand the acidity of the stomach (Govender et al., 2014, Sahadeva et al., 2011, Picot and Lacroix, 2004). Probiotics produce lactic acid, which is a weak acid and well tolerated by most probiotics as compared to gastric acid which is, 0.1 M HCl, a strong acid. In a study conducted to evaluate

some probiotic strains to gastric acid, only 1 of 15 probiotic strains was considered to be intrinsically resistant to gastric acid, the remaining strains were all killed (Charteris et al., 1998).

When the probiotics were exposed to SGF, there was a significant drop in viability. It was observed that all the solid probiotics were just dried probiotics with no protective mechanisms against the gastric fluid. Such formulations have a low probability of delivering viable organisms into the intestines when taken. Fredua-Agyeman and Gaisford (2015) observed similar results when they evaluated the gastric acid tolerance of some commercial solid dose formulations. It is important for probiotics to be formulated with protective mechanisms against gastric fluid otherwise most probiotic strains could be killed during transit. The outcome of this test was worrying because 2 of the products, B and C, had inscriptions on their packages indicating the tolerance of the contained probiotic strains to gastric acid, the results here, however, demonstrated otherwise.

### *3.5 Encapsulation of probiotics for site-specific delivery*

When the probiotics were encapsulated using the Phloral® coating technology, no viable bacterial enumeration was obtained until after pH 7 when the threshold of dissolution for the coating material was reached. Bacterial enumeration at the target site was over 90% for all products, except for Product A which had a percentage recovery of 88% (Figure 4).

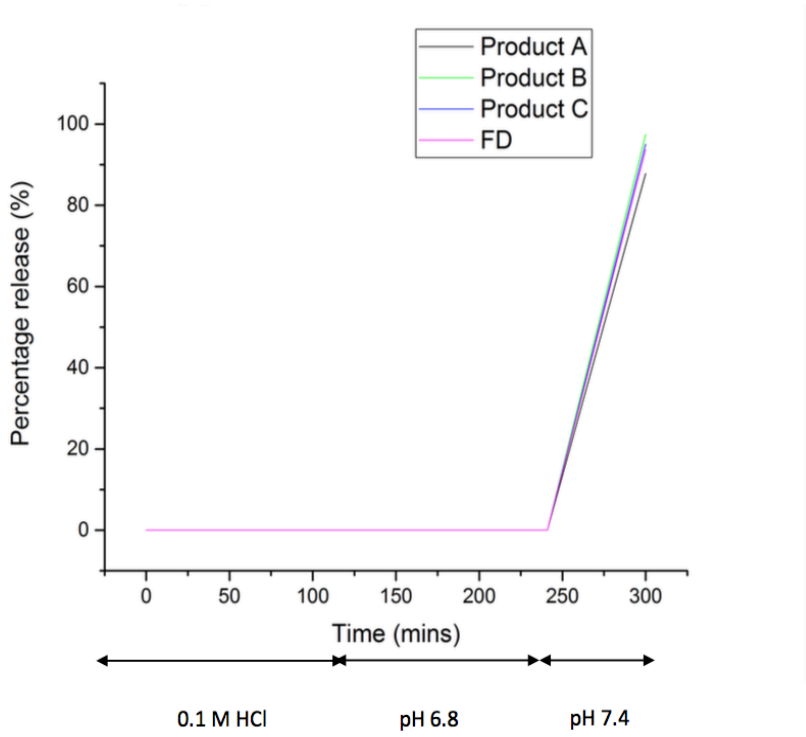


Figure 4: An illustration of percentage recovery after Phloral® coated capsules (Products A – C and the freeze-dried strain, FD) were immersed consecutively in 0.1 M HCl and pH 6.8 for 2 hours then in pH 7.4.

With the distal parts of the gut as target sites, Phloral® coating technology was used. The main components of Phloral® are Eudragit® S, which is a synthetic polymer that dissolves at pH > 7, and resistant starch, which is not digested by mammalian amylase enzymes secreted by the pancreas but by colonic bacterial enzymes; this makes the technology useful in drug targeting (Ibekwe et al., 2008, McConnell et al., 2008, D'Haens et al., 2017). Upon capsule dissolution, it was observed that the probiotics investigated all had high viabilities with the lowest recorded viability being

88%. This confirms the need for most dried probiotics to be incorporated into gastro-resistant formulations and delivered to the distal parts of the intestines. Some formulations have been reported whereby probiotics have been microencapsulated, although the reported recoveries have been low (Poelvoorde et al., 2008, Sultana et al., 2000). These formulations usually involve applying coating material directly to probiotics strains. The reduced viabilities could be because these coating materials themselves could have impacted directly on the organisms since organic solvents are sometimes used as ingredients in capsule coating (Cole et al., 2002, Huyghebaert et al., 2004). Optimisation of coating conditions may be needed to enhance product viability of these microencapsulated formulations. The need to protect probiotics was emphasised recently by Caillard and Lapointe (2017) when they evaluated some commercial probiotics and found that only the enteric-coated formulations had resistance after exposure to gastric acid at fasting and all the unprotected formulations had high drops in numbers after exposure.

## 4 Conclusion

The commercial products and freeze-dried *L. acidophilus* LA5 evaluated here all demonstrated poor tolerance to simulated gastric fluid even though some of these commercial products had inscriptions on product packs indicating tolerance of the contained probiotic strains to gastric acid. When the commercial and in- house freeze-dried probiotics were encapsulated using the Phloral® to target the delivery into lower small intestines or colon, viabilities of approximately 90% were observed after these capsules had been initially deposited in gastric acid for 2 hours. These products also demonstrated over 60% adhesion to intestinal cells *in vitro* highlighting the potential for colonisation should these overcome the harshness of the stomach. The need to target the delivery of probiotics into the intestines has been demonstrated here as this offers a greater potential for colonisation of the intestines once the harshness of the stomach has been overcome.

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