Comparative survival of commercial probiotic formulations: Tests in biorelevant gastric fluids and real-time measurements using microcalorimetry

*M. Fredua-Agyeman, S. Gaisford**

UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London, WC1N 1AX

Corresponding author: Simon Gaisford

Tel: +44 (0) 20 7753 5863

Fax: +44 (0) 20 7753 5942

Email: s.gaisford@ucl.ac.uk

Abstract

The large number of probiotic products now available makes the decision about which product to choose difficult both for the consumer and for the specialist providing dietary/nutritional advice. Data on the viability of the bacteria in these products, in the *in-vivo* situation, are therefore important. This study was designed to explore the comparative health and survival of probiotic species in various commercial formulations, using more realistic test systems that might allow further understanding of factors that must be controlled to give the greatest chance of delivery of live healthy bacteria to the lower gut.

A total of eight commercially available probiotic preparations were selected for enumeration tests and *in-vitro* gastric tolerance tests. Tolerance assays were conducted in porcine gastric fluid (PGF) fed and fasted state (pH 3.4 ± 0.04), simulated gastric fluid (SGF, pH adjusted to 1.2 and 3.4) and fasted state simulated gastric fluid (FaSSGF, pH adjusted to 1.6 and 3.4). Isothermal microcalorimetry was also used to measure real-time growth of probiotics after exposure to simulated gastric fluid.

Results from the enumeration tests indicated that recovery of viable organisms per dose is the same as or better than the stated label claims for liquid-based formulations but lower than the stated claim for freeze-dried products. Results from the *in-vitro* tolerance tests overall suggest that the PGF provided a harsher environment than the simulated systems at similar pH. In general, liquid-based products tested tended to give superior results in terms of survival compared with the freeze-dried products tested. Results from tests in the fed state in PGF suggested that food greatly affects viability. Microcalorimetric data showed that for some products probiotic species were able to grow following exposure to gastric fluid, suggesting that viable bacteria reach the gut *in-vivo*.

Introduction

Probiotics have become a huge commercial success story in recent years; in 2006 it was estimated that the UK population spent £189 million on probiotics (Food Commission 2006) and the global market for these products is forecast to be worth US$32.6 billion in 2014 (Cook et al. 2012). At the same time, the number of clinical trials investigating the use of probiotics has also seen a huge increase; a simple search on PubMed shows that between 1992 and 2002, a total of 113 clinical trials on probiotics were published, yet from 2002 to 2012, this number had increased ten-fold to 1041 (PubMed 2013). The large numbers of probiotic products available makes the decision about which product to choose ever more difficult both for the consumer and for the specialist providing dietary/nutritional advice. Data on the health and survival of the bacteria in these products, measured in experiments that most closely mimic the *in vivo* situation are therefore important.

The term probiotic encompasses all *'live microorganisms that when administered in adequate quantities confer a health benefit on the host'*; this definition, agreed by the FAO and WHO (FAO/WHO 2001; 2002), assumes viability of the bacterial content of each probiotic product; i.e. that an appropriate number of bacteria survive transit through the gastrointestinal (GI) tract and are fully able to grow and colonise in the colon on arrival. Although there is no global agreement on the minimum number of bacteria per gram or millilitre of product necessary for functionality, it is generally accepted that at the point of consumption, probiotic products should have a minimum concentration of $>1 \times 10^6$ colony forming units (CFU)/mL or gram and that a total of some 10^8 to 10^9 probiotic microorganisms should be consumed daily if therapeutic effects are to be realised (Minelli and Benini 2008). Again, these definitions assume that these are live, healthy bacteria at the point of consumption. The concentration of live, healthy bacteria in the colon that are necessary to confer therapeutic benefits is unknown yet is clearly important.

The health and survival of bacteria in probiotic products in the range of environments they will encounter in use (during manufacture, storage, transit through the stomach and arrival at the small intestine) is critical in ensuring potential effectiveness. Of these, transit through the gut potentially induces the greatest loss in viable organisms. The main physical and chemical barriers of the GI tract include pH, volume, composition and buffer capacity of the gastric juice, transit time and the presence of foods. The dose and formulation of the probiotic can be optimised to mitigate the effects of some or all of these factors.

Gastric juice is a crucial barrier to most pathogens; it displays large variability in characteristics under different conditions, for example during the fed and fasted states (Evans et al. 1988; Fordtran and Walsh 1973). Under fasted conditions the pH in a healthy human stomach is acidic, generally ranging between 1 and 3 (Dressman et al. 1990; Kalantzi et al. 2006). After food, the stomach environment varies considerably over the course of gastric residence of the meal (Kalantzi et al. 2006), the pH climbing briefly to a median peak value of 6.7 before declining back to the fasted state value within two hours or less (Dressman et al. 1990). The stomach also displays large variability in emptying times between fed and fasted states (and within these states there are also large inter-individual variations); in general, emptying times in the fasted state are considerably quicker (mean ±SD of half gastric emptying time ($t_{1/2}$) 80.5 ± 22.1 minutes) than those in the fed state (median time of $t_{1/2}$ 127 minutes (25–75% percentiles: 112.0–168.3 minutes) (Hellmig et al. 2006)) and in general water-based liquids traverse faster than non-water-based particularly in the fasted state; non-nutrient liquids do not normally interrupt the migrating myoelectric complex and are typically emptied in an exponential pattern (Steingoetter et al. 2006). Studies have shown the half-time

for saline emptying from the human stomach to be approximately 12 minutes (Granger 1985), and the half-time for emptying water to be in the region of 15-16 minutes (Steingoetter et al. 2006). There is also variability in complexity of chemical constituents such as bile salts and proteolytic enzymes and in the volume of the gastric contents; the capacity of the stomach has been noted to be 1.5 L (Aulton, 2013) but can contain up to 63 mL of free fluid in the fasted state (McConnell et al. 2008).

The human stomach is known to have an ionic strength of 0.1 ± 0.025 mM and concentrations of potassium, sodium, chloride, calcium, at 13.4 ± 3.0 mM, 68 ± 29 mM, 102 ± 28 mM, and 0.6 ± 0.2 mM respectively (Lindahl et al. 1997). Its bile salt concentration is approximately 0.06 mM in the fed (Rhodes 1969) and 0.2 ± 0.2 mM in the fasted state (Lindahl et al. 1997). To date, various media have been developed to simulate the fasted gastric content (British Pharmacopoeia 2012; FDA 2000; United States Pharmacopoeia 2000). Often, these media try to mimic the salt concentration of the gastric juice through the addition of up to 0.5% w/v of sodium chloride to HCl (Charteris et al. 1998). Surface tension and bile content is also simulated through the addition of bile salt, pepsin and/or surfactants to HCl (Aburub et al. 2008; Vertzoni et al. 2005). Addition of these has changed the solubility and dissolution of drugs and has often been recommended in dissolution fluids (Mudie et al. 2010; Nicolaides et al 1999).

Gastric tolerance assays for potential new probiotic formulations (Chandramouli et al. 2004; Cook et al. 2011; Cui et al. 2000; Ding et al. 2009; Mokarram et al. 2009) and commercial probiotic products (Sahadeva et al. 2011) or organisms (Jensen et al. 2012) routinely use either buffer or saline solutions adjusted to pH 1.2 to 4, or growth media, adjusted to these same pH's, to simulate the gastric juice. These simulated fluids often lack the complexity of real gastric fluid and gastric volumes are often not accounted for or ignored.

Gastric tolerance assays frequently use the plate count technique to assess the survival of bacteria after exposure. While this is a useful and widely accepted technique it does have inherent problems; it is extremely labour intensive and time-consuming and can result in overestimation or underestimation of numbers of viable organisms. For example, stress during plating can cause underestimation of numbers; organisms may be unevenly distributed on the plate and may occur in chains and/or clumps. The surface of an agar plate may also be a difficult place for some organisms to start growth after being removed from their natural milieu (Lydell et al. 2002), while some organisms may be difficult to grow on a plate relative to a liquid medium. For those organisms that thrive on agar, plate counting is still not a suitable model for assessment of *in-vivo* growth, because the plate is designed to provide a nutrient-rich environment to support growth to enable visual counting. By so doing it can skew results by allowing any surviving bacteria to grow and recover in an environment that does not mimic the continuing harsh *in vivo* situation where, in the case of probiotics, any surviving bacteria would go on to face transit through the small intestine.

An alternative, real-time measurement of viability would be a significant improvement for assessing likely *in-vivo* efficacy of probiotic products. One option is isothermal microcalorimetry (measurement of the rate of heat production, or power, as a function of time at constant temperature, O'Neill and Gaisford, 2011). As microcalorimeters are able to measure heat production rates of less than a microwatt they can measure the metabolism and growth of relatively small numbers of bacteria (as low as 10^4 - 10^5 CFU/mL depending on species, Braissant et al. 2009). Because dissimilar bacterial species utilise nutrients via different metabolic pathways, the power-time curve is indicative of species and can be used for bacterial identification (Boling et al, 1973). An additional benefit is that there is no

requirement for optical clarity in the growth medium and so growth rates can be determined directly in opaque fluids (Said et al, 2014).

This study was designed to explore the comparative health and survival of probiotic species in various commercial formulations, using an experimental approach to mimic oral delivery. It set out to investigate the concentrations of bacteria present in selected products at the point of consumption and to measure survival, using plate-count techniques, following exposure to three different gastric fluids to explore the importance of using bio-relevant test systems. Lastly it investigated the use of microcalorimetry to measure real-time survival of probiotics following exposure to simulated gastric fluid. The overall objectives were to develop more realistic test systems, to understand further the factors that must be controlled in delivering live healthy species to the lower gut and to recommend to consumers best practice in taking probiotic supplements.

Materials and methods

A total of eight commercially available probiotic preparations (Table 1) were selected for study. All were purchased from local supermarkets or pharmacies or obtained from the manufacturers or distributors. All were stored according to label and all were used for study before their expiry dates.

Enumeration of viable organisms in probiotic products

De Man Rogosa Sharpe (MRS) agar (Oxoid) containing 0.05% w/v L-cysteine hydrochloride "MRSc" was used as the growth medium. The content of a capsule or sachet of the freeze-dried products were dispersed in 10 mL sterile phosphate buffered saline (PBS) (pH 7.4). The mixture was then vortexed to homogeneity, serially diluted in PBS and spread-plated on MRSc agar. Liquid products were serially diluted and spread-plated on MRSc agar. All plates were then incubated at 37 $^{\circ}$C in anaerobic jars (Oxoid) using the Anaerogen GasPak System (Oxoid). Colonies were counted after incubation for 48 hours.

The actual contents of a dose of these products were calculated using the formula:

Actual content (CFU/dose) = (CFU/mL) * dose

For a solid (dried) product, content per capsule or sachet = (CFU/mL) * 10 mL, for a liquid product, content per dose = (CFU/mL) * recommended volume

All enumeration experiments were performed in triplicate and CFU values were expressed as log values.

Preparation of gastric fluids

Three types of gastric fluid were used for study; porcine gastric fluid (PGF), simulated gastric fluid (SGF) and fasted state simulated gastric fluid (FaSSGF).

PGF was obtained from an abattoir from a freely fed, adult animal. Juices were dispensed into multiple aliquots and frozen at -80 $^{\circ}$C. Prior to use, they were thawed at room temperature, and then centrifuged at 10000rpm (Mini spin, Eppendorf, Germany) for 10 minutes at 25 $^{\circ}$C. The supernatant obtained was sterilized by filtration using a membrane filter of 0.22 μm pore size.

SGF was prepared by adding HCl (Sigma Aldrich, 37%) to a solution of NaCl (2 g/L, AnalaR BDH 58.44g/mol) and adjusting the pH (pHenomenal®, UK) to either 1.2 ± 0.1 or 3.4 ± 0.1.

FaSSGF was prepared according to the composition presented by Vertzoni et al (2005) with SIF® powder (Biorelevant Ltd). NaCl (2 g) was dissolved in purified water (1 L) and the pH adjusted to either 1.6 ± 0.1 or 3.4 ± 0.1 as appropriate with HCl. A total of 0.06 g of SIF® powder was added to the prepared HCl/NaCl solution to a volume of 1L to give a final composition of sodium taurocholate 80 µM; lecithin 20 µM; pepsin 0.1 mg/ml; sodium chloride 34.2mM. All artificial juices were used within 48 hours of preparation.

Measurement of surface tension, osmolality, pH and buffer capacity of the gastric fluids

The surface tensions of the gastric fluids were measured using the du Noüy-Paddy method (Delta-8 automatic multichannel microtensiometer, Kibron Inc., Finland). The temperature of the instrument was between 27.7-27.9 °C. Measurements were made on 50 µL/wells of 8 samples of each fluid in 96-well plates. Calibration was performed with deionized water prior to the experiments.

Osmolality of the fluids was measured using the freezing point depression technique with a digital micro-osmometer, (Hermann Reobling Messtechnik, Germany) on 100 µL samples. Calibration was undertaken with deionized water and measurements made in triplicate.

The buffer capacities of the fluids were measured by adding NaOH standard solution (0.1 M) to each fluid (3 mL) and noting the volume of NaOH needed to change the pH by 0.5 units.

Buffer capacities were determined using the equation;

$$\beta \ (mmol/L/\Delta pH) = \Delta AB/\Delta pH$$

where ΔAB is the small increment in mol/L of the amount of acid or base added to produce a pH change of ΔpH in the buffer. This equation can be rewritten as;

$$\beta \ (mmol/L/\Delta pH) = \Delta AB/\Delta pH = ((M_a \ x \ V_a)/\Delta pH) \ x \ 1000/V_b$$

where M_a is the molarity of the acid, V_a is the volume of acid in mL, V_b is the volume of buffer in mL, ΔpH is the change in pH. The equation is multiplied by 1000 to express the volume in litres.

Measurement of the in-vitro gastric tolerance of probiotics in the fasted state

For these tests it was assumed that the fasted stomach has a fluid capacity of 60 mL (McConnell 2008). The probiotics were mixed with SGF or FaSSGF (60 mL) to simulate *in-vivo* conditions upon ingestion. Doses of commercial products were as follows:

- Symprove (60 mL)
- Actimel and Yakult (entire bottle, 100 and 65 mL respectively)
- Align, Bio-kult, Biobalance Support, Probio 7 and VSL#3 (entire content of capsule/sachet).

For the bio-relevant test, probiotics were mixed with PGF in a ratio representative of the artificial fluids to the probiotics (1.5 mL PGF was mixed with: Align 4.4 mg; Biobalance support 7.5 mg; Bio-kult 4.4 mg; Probio7 11 mg; VSL#3 111.7 mg; Actimel 2.5 mL; Symprove 1.5 mL or Yakult 1.63 mL) and the mixture was vortexed at maximum speed for 10 seconds.

All samples were incubated at 37 $^{\circ}$C and aliquots of 50 µL were withdrawn at 5, 10, 20, and 30 minutes, serially diluted in PBS and plated on MRSc agar for the determination of viable counts.

Measurement of the in-vitro gastric tolerance of probiotics in the fed state in PGF

For these tests it was assumed that the fed stomach had a fluid capacity of 1.5 L (Aulton 2013). A representative dose of the probiotics was mixed with PGF in a ratio depictive of *in-vivo* situation upon ingestion. The total volume of porcine fluid used was 1.5 mL. Thus 60 µL, 100 µL and 65 µL of Symprove, Actimel, Yakult and 0.174 mg of Align, 0.300 mg of Biobalance Support, 0.171 mg of Bio-kult, 0.439 mg of Probio 7 and 4.45 mg of VSL#3 were weighed and all probiotics were added to PGF in a 1.8 mL micro tube. The mixture was vortexed at maximum speed for 10 seconds and incubated at 37 $^{\circ}$C. Aliquots of 50 µL were withdrawn at 5, 10, 20, 30, 90, 120 and 180 minutes, serially diluted in PBS and plated on MRSc agar plates for the determination of viable counts.

Microcalorimetric gastric tolerance assay

Probiotics were exposed to PGF mimicking the fasted state for 30 minutes prior to testing. Inoculua (30 µL) were added to MRSc (2.97 mL, pre-warmed to 37 $^{\circ}$C). Experiments were performed in a 2277 Thermal Activity Monitor (TAM, TA Instruments Ltd) operated at 37 $^{\circ}$C. Samples were accurately pipetted into glass ampoules (3 mL). Ampoules were sealed with a crimped aluminium cap, a rubber disc ensuring an air-tight enclosure. Ampoules were placed into the loading position of the TAM and allowed to reach thermal equilibrium (30 minutes) before being lowered into the measuring position. Data were recorded (1 point every 10 seconds) with the dedicated software package Digitam 4.1. An ampoule containing buffer (3 mL) was used as a reference. The amplifier setting was 1000 µW and the instrument was calibrated with the electrical substitution method prior to first use.

Results

Results from the enumeration tests of viable organisms per dose are shown in Figure 1. They indicate that for the liquid-based products recovery of viable organisms per dose is the same as or better than the stated label claims. For the freeze-dried products the results show that viable content was generally lower than the stated claim.

The characteristics of the simulated gastric fluids and PGF are given in Table 2 and compared with published results for human gastric fluid. These results highlight the considerable differences between human gastric fluid, PGF and the two simulated fluids and show that PGF more closely resembles the osmolality, buffer capacity and surface tension of human gastric fluid than simulated fluids.

Results from the *in-vitro* tolerance tests in the fasted state at similar pH are given in Figure 2 for all test systems at pH 3.4. The results highlighted important differences between PGF, SGF and FaSSGF, particularly evident for some products. In all systems, all probiotics with the exception of one liquid-based product, showed a decline in viability during the first five minutes, probably as a result of acid shock. The results from tolerance testing at the lower pH for SGF (1.2) and FaSSGF (1.6) are given in Figure 3, where similar trends are seen.

Results from the *in-vitro* tolerance test in PGF in the fed state are presented in Figure 4. In general, the liquid-based products retained viability better than the freeze-dried products (the exception being VSL#3). The water-based product exhibited the best tolerance to PGF relative to the other products in the fed state.

Results from the microcalorimetric gastric tolerance assay are given in Figures 5 and 6. Figure 5 shows the characteristic calorimetric output plotted as power versus time from the different probiotics, while Figure 6 represents growth curves (plotted as cumulative heat versus time, discussed below) because this provides a better correlation with conventional optical density data (Braissant et al, 2011).

Discussion

The results are important for several reasons: they suggest that for many commercially available probiotics, claimed content and actual viability may not always be the same; they suggest that current simulated fluids used for gastric tolerance testing show a considerable difference from human gastric fluid, highlighting the importance of using bio-relevant testing systems; and they also suggest that real-time estimation of bacterial viability and growth is possible using microcalorimetry and that this technique may give further insight into comparative gastric survival.

Results from the enumeration tests showed that the claimed content and actual viable cells in terms of CFUs per dose differ in many of the commercial products tested and that in particular, the freeze dried preparations tested had a lower viable content than stated claims. While freeze-drying is a commonly used method to preserve bacterial strains, its effectiveness is species-dependent and failures in viability have been reported (Stoianova and Arkad'eva, 2000). It has been shown that it is the dehydration stage that results in loss of viability (Harrison and Pelczar, 1963). Further, control of the rehydration stage is critical to maximise recovery, but this will vary from consumer to consumer. Ultimately, the potential efficacy of the products could be affected; amongst all the products tested, only the water-based product showed recovery close to 100% in respect of the ratio of claimed to actual content.

Overall our findings suggest that the PGF provided a harsher environment than the simulated systems at similar pH, possibly as a result of the enzymatic and digestive substance composition of the gastric fluid. Even when the simulated systems were used at a pH more consistent with the human stomach, survival was better than in PGF, again illustrating the harsher environment of PGF.

Measurements of surface tension, osmolality, pH and buffer capacity of the gastric fluids also illustrate the relevance of using a more bio-relevant fluid for gastric tolerance testing; PGF more closely resembled human gastric fluid than the other two simulated fluids at two different pH levels. Our results from the PGF system may therefore be more important in terms of relevant results for decisions about which product to use. The *in-vitro* tolerance tests in the fasted state in PGF indicate that the liquid-based products tend to give superior results in terms of survival compared with the freeze dried products. There appears to be one exception to this rule (VSL#3) which is addressed in more detail below. Further tests were undertaken with solid products that were hydrated prior to exposure to the gastric fluids (data not shown), but the results suggested that re-hydration of dried products did not necessarily alter their viability for the better. In general, the viability of products in the fasted state was improved relative to the fed state; however, results from the fed state model, based on normal gastric transit times at 90-180 minutes in the fed state, might give more relevant results for the way some probiotics are taken in real-life; many consumers choose to take them with food and many recommend that they be taken with food. Results from the *in-vitro* tolerance tests in the fed state in PGF suggest that food greatly affects viability; the different products reacted differently and of all products tested, the water-based product gave the best results.

One observation from the fasted test systems was the effect of pH shock on the probiotics from zero to five minutes. In general, liquid products fared better than solid products, probably a result of several factors, including volume and/or the effects of other excipients. For instance, one bottle of Actimel contains 100 mL of product; quite adequate to dilute the stomach acid in the fasted state. Addition of SGF (pH 1.2) to Actimel increased the pH of the final mixture to 3.30. Symprove depends on germinated barley, a prebiotic not digested by pancreatic enzymes, for protection of its bacteria against the harsh environment of the stomach. Actimel and Yakult are fermented milk products, which utilise milk proteins for protection. Dispersion of the solid products in SGF does not significantly alter pH.

An additional consideration is that the excipients in the liquid products may also aid recovery, growth and adhesion of bacteria upon arrival in the small intestine. The fat content in milk products may offer further protection during passage through the GI tract (Ranadheera et al. 2012); a small study has highlighted this by showing significant reductions of *L. casei* DN-114 001 within Actimel Fat Free (0.428 ± 0.059 $\log_{10}$ CFU/mL), after five hours of simulated gastrointestinal transit ($p<0.05$), compared with insignificant reductions for Actimel Original (Wills 2012).

One drawback of plate-counting was the inability to test bacterial viability in real-time; the technique relies on incubation of samples for 48 hours prior to counting. This means that for those products with very large numbers of bacteria in each dose, enough will survive to multiply in large numbers in this idealised environment. Isothermal microcalorimetry provides a real-time indication of growth, which may correlate better with what is happening *in-vivo* during gastric transit. Following exposure to PGF, samples were inoculated into growth medium and the growth patterns were measured. It is important to note here that the aim was to use calorimetry to determine viability after exposure to gastric acid, and so a growth medium was used to maximise growth. In vivo, bacteria would transit to the small

intestine and so be exposed to intestinal fluid in which growth would be different. The raw power output from a calorimeter is a plot of power (J/s, or W) versus time (s). As the bacteria in the sample grow and divide, the power produced will increase, resulting in a series of peaks and troughs as the organisms utilise available nutrients, Figure 5. The sequence of peaks and troughs is characteristic of species (Said et al. 2014). Conversion of the data to area under curve (cumulative heat, J) as a function of time, results in a growth curve identical in shape to that conventionally produced from optical density measurements (Braissant et al, 2011), Figure 6. The growth rate of the bacterium is reflected in the gradient of the line (higher gradient indicating faster growth). It is important to note here that the initial inoculum number of bacteria will vary between products and that the minimum concentration of bacteria necessary to produce a measureable power is ca.10^4 - 10^5 CFU/mL. The lag time before growth implies either that bacterial numbers are below this value or that the organisms experience a degree of stress when inoculated into a new medium (the species in each product may react to environmental stress differently).

The data in Figures 5 and 6 show that growth was fastest with Symprove and VSL#3. In the case of Symprove, this finding correlates with the stability data discussed earlier, the germinated barley providing both acid protection and a nutrient source. The finding in the case of VSL#3 is more surprising, given the significant loss of viable bacteria during exposure to SGF. One observation is that the growth curves of the two products were very similar; it may be the case that the majority of the species in VSL#3 are killed by the low pH in SGF, but the lactobacilli survive. Symprove contains three lactobacilli species and so the growth curves of both products are similar. The next fastest growth is seen in Actimel and Yakult, again in accordance with the viability in SGF data discussed earlier. Some growth is seen in Bio-kult and Biobalance, but not until at least 30 hours; it is likely that *in-vivo* these products would have been excreted within this time frame.

Overall our study is the first to provide comparative data on actual products in situations that most closely mimic what is happening in the human GI tract. The only similar study to our knowledge was undertaken by the FSA who published an independent assessment of probiotics in 2003 (Gibson 2003). However, in this analysis they set out to compare the viability of individual strains of bacteria rather than commercial products. Other individual studies have simply looked at one product in isolation or individual strains of bacteria in isolation or have used test systems that may have little relevance for the *in vivo* situation (Corcoran et al. 2005; Huang and Adams 2004; Iyer and Kailasapathy 2005; Oozer et al. 2006, Sabikhi et al. 2010; Sun and Griffiths 2000).

There is a wealth of probiotics products currently on the market, including single strain and multistrain products, lyophilised capsule or powders, dairy based liquids and a water based liquid. Faced with such a huge array of products, choice of which product to use is difficult. Factors that affect choice include viability claims both about content and at the site of action. It is important that proper product quality control is conducted to ensure that the content claims of labels are actually present at the time the product reaches a patient. Further claims about gastric survival can only be upheld in test systems that more closely mimic the real-life situation, since these will provide the most relevant results for which product to choose and how best to use it. *In-vivo* the bacteria have to transit the small intestine before reaching their desired site of action. If concentrations of healthy bacteria are low after gastric transit the likelihood of any surviving to grow in the large intestine is low.

Our study has several limitations; we only looked at one factor affecting viability of the products tested and we did not test intestinal transit or the adhesive properties of the bacteria

in the colon, both crucial factors that will affect survival and functionality. In addition we did not set out to test an exhaustive list of all products available and as a result our generalisations about product groups could be erroneous. While PGF appeared to be the most bio-relevant fluid tested, the pH of this fluid was higher than that generally seen in the fasted human stomach since this was taken from a freely fed animal. In our PGF test systems volume was therefore only variable factor between the fed and fasted state. The calorimeter has limitations too; the main drawback is its lack of sensitivity since it is unable to measure numbers below 10^6 CFU/mL.

Summary

This study was designed to explore the comparative health and survival of different commercial probiotics at two stages of use; at the point of consumption and following exposure to gastric fluid models. The results show that at the point of consumption, liquid probiotic products contain close to or in excess of their claimed content and in addition, show better tolerance to gastric juice relative to many of their dried counterparts. Our real-time studies also suggest that the proportions of bacteria that survive gastric transit could be less than expected from plate-count analysis and potentially less than would be necessary for survival during the next stage of transit through the small intestine, so potentially compromising functionality. The study also highlight the importance of good administration recommendations; probiotics are likely to have greater viability if taken in the fasted state and currently only one product recommends this on its labelling.

Our recommendations from this study are that gastric tolerance testing should be conducted in models that most closely mimic the real-life situation since these will provide us with the most relevant results. Tools that allow us to investigate real-time results add to our understanding and can be of great benefit.

Acknowledgements

References

Aburub, A., Risley, D.S. and Mishra, D., 2008. A critical evaluation of fasted state simulating gastric fluid (FaSSGF) that contains sodium lauryl sulfate and proposal of a modified recipe. International Journal of Pharmaceutics 347, 16-22.

Aulton, M.E., 2013. Aulton's Pharmaceutics: The Design and Manufacture of Medicines. Churchill Livingstone, Edinburgh.

Braissant, O., Wirz, D., Göpfert, B. and Daniels, A.U., 2010. Use of isothermal microcalorimetry to monitor microbial activities. FEMS Microbiology Letters 303, 1-8

Braissant, O., Wirz, D., Göpfert, B. and Daniels, A.U., 2011. Biomedical use of isothermal microcalorimeters. Sensors 10, 9369-9383.

Boling, E.A., Blanchard, G.C. and Russell, W.J., 1973. Bacterial identification by microcalorimetry. Nature 241, 472-473.

British Pharmacopoeia. 2012. Volume V, Appendix XII B. Dissolution ANNEX: Recommendations on dissolution testing; Appendix I A. General reagents: gastric juice, artificial. http://bp2012.infostar.com.cn/Bp2012.aspx?id=902

Chandramouli, V., Kailasapathy, K., Peiris, P. and Jones, M., 2004. An improved method of microencapsulation and its evaluation to protect Lactobacillus spp. in simulated gastric conditions. Journal of Microbiological Methods 56, 27-35.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins J.K., 1998. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. Journal of Applied Microbiology 84, 759-768.

Cook, M.T.,Tzortzis,G., Charalampopoulos,D. and Khutoryanskiy, V.V., 2011. Production and evaluation of dry alginate- chitosan microcapsules as an enteric delivery vehicle for probiotic bacteria. Biomacromolecules, 12, 2834-2840.

Cook, M.T.,Tzortzis, G.,Charalampopoulos, D. and Khu Toryanskiy, V.V., 2012. Microencapsulation of probiotics for gastrointestinal delivery. Journal of Controlled Release 162, 56-67.

Corcoran, B.M., Stanton, C., Fitzgerald, G.F. and Ross, R.P., 2005. Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. Applied Environmental Microbiology 71, 3060-3067.

Cui, J.H., Goh, J.S., Kim, P.H., Choi, S.H. and Lee, B.J., 2000. Survival and stability of bifidobacteria loaded in alginate poly-l-lysine microparticles. International Journal of Pharmaceutics 210, 51-59.

Ding, W.K., and Shah, N.P., 2009. An improved method of microencapsulation of probiotic bacteria for their stability in acidic and bile conditions during storage. Journal of Food Science 74, M53-M61.

Dressman, J. B., Berardi, R. R., Dermentzoglou, L. C., Russell, T. L., Schmaltz, S. P., Barnett, J. L. and Jarvenpaa, K. M., 1990. Upper gastrointestinal (GI) pH in young, healthy men and women. Pharmaceutical Research 7 (7), 756–761.

Efentakis, M. and Dressman, J. B., 1998. Gastric juice as a dissolution medium: surface tension and pH. European Journal of Drug Metabolism and Pharmacokinetics 23, 97- 102.

Evans, D.F., Pye, G., Bramley, R., Clark, A. G., Dyson, T. J. and Hardcastle, J.D., 1988. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut 29, 1035-1041.

FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. 2001. http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf

FAO/WHO Guidelines for the Evaluation of Probiotics in Food. 2002. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food. London Ontario, Canada.

FDA, Guidance for Industry. 2000. Waiver of" the in Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System"; U.S. Department of Health and Human, Food and Drug Administration (FDA), Center for Drug Evaluation and Research: Washington, DC, 1-13

Food Commission, 2006. Do we need a daily dose of bacteria? Available at http://www.foodcomm.org.uk/articles/dose_of_bacteria_06/

Fordtran, J. S. and Walsh, J. H., 1973. Gastric-acid secretion rate and buffer content of stomach after eating -results in normal subjects and in patients with duodenal-ulcer. Journal of Clinical Investigations 52, 645-657.

Harrison, A.P. and Pelczar, M.J., 1963. Damage and survival of bacteria during freeze-drying and during storage over a ten-year period. Journal of General Microbiology 30, 395-400.

Hellmig, S., Von Schoning, F., Gadow, C., Katsoulis, S., Hedderich, J., Folsch, U. R. and Stuber, E., 2006. Gastric emptying time of fluids and solids in healthy subjects determined by l 3C breath tests: influence of age, sex and body mass index. Journal of Gastroenterology and Hepatology 21, 1832-1838.

Huang, Y. and Adams, C.M., 2004. In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. International Journal of Food Microbioliology 91, 253–260.

Iyer, C. and Kailasapathy, K., 2005. Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under *in vitro* acidic and bile salt conditions and in yogurt. Journal of Food Science 70, m18–m23.

Jensen, H., Grimmer, S., Naterstad, K. and Axelsson, L. In vitro testing of commercial and potential probiotic lactic acid bacteria. International Journal of Food Microbiology, 153, 216-222.

Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B. and Reppas, C., 2006. Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. Pharmeceutical Research 23, 165– 176.

Lydell, C., Emerson, D. and Gillevet, P.M. 2002. Most probable number techniques recover much higher numbers of bacterial cells than plate counts done from salt marsh sediments. Abstracts of the General Meeting of the American Society for Microbiology, 102, 263-263.

McConnell ,L.E., Fadda, H.M. and Basit, A.W., 2008. Gut instincts: explorations in intestinal physiology and drug delivery. International Journal of Pharmaceutics 364, 213-226.

Minelli, E.B. and Benini, A., 2008. Relationship between number of bacteria and their probiotic effects. Microbiol Ecology in Health and Disease 20, 180-183.

Mokarram, R.R., Mortazavi, S.A., Najafi, M.B.H. and Shahidi, F. 2009. The influence of multi stage alginate coating on survivability of potential probiotic bacteria in simulated gastric and intestinal juice. Food Research International, 42, 1040-1045.

Mudie, D. M., Amidon, G. L. and Amidon, G. E., 2010. Physiological parameters for oral delivery and in vitro testing. Molecular Pharmaceutics 7, 1388-1405.

Nicolaides, E., Galia, E., Efthymiopoulos, C., Dressman, J.B. and Reppas, C., 1999. Forecasting the *in vivo* performance of four low solubility drugs from their in vitro dissolution data. Pharmaceutical Research 16, 1876- 1882.

O'Neill, M.A.A. and Gaisford, S., 2011. Application and use of isothermal calorimetry in pharmaceutical development. International Journal of Pharmaceutics 417, 83-93.

Oozeer, R., Leplingard, A., Mater, D.D., Mogenet , A., Michelin, R., Seksek, I., Marteau, P., Doré, J., Bresson, J.L. and Corthier, G., 2006. Survival of Lactobacillus casei in the human digestive tract after consumption of fermented milk. Applied Environmental Microbiology 72(8), 5615-7.

Ranadheera, C. S., Evans, C. A., Adams, M. C. and Baines, S. K., 2012. *In vitro* analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. Food Research International 49, 619-625.

Said, J., Dodoo, C.C., Walker, M., Parsons, D., Stapleton, P., Beezer, A.E. and Gaisford, S., 2014. An *in vitro* test of the efficacy of silver-containing wound dressings against *Staphylococcus aureus and Pseudomonas aeruginosa* in simulated wound fluid. International Journal of Pharmaceutics 462, 123-128.

Sabikhi, I., Babu, R., Thompkinson, D.K. and Kapila, S., 2010. Resistance of microencapsulated lactobacillus acidophilus la1 to processing treatments and simulated gut conditions. Food and Bioprocess Technology 3, 586–593.

Sahadeva, R.P.K., Leong, S.F., Chua, K. H., Tan, C.H., Chan, H.Y., Tong, E.V., Wong, S.Y.W. and Chan, H.K. 2011. Survival of commercial probiotic strains to pH and bile. International Food Research Journal 18(4),1515-1522.

Steingoetter, A., Fox, M., Treier ,R., Weishaupt, D., Marincek, B., Boesiger, P., Fried, M. and Schwizer, W., 2006. Effects of posture on the physiology of gastric emptying: a magnetic resonance imaging study. Scandinavian Journal of Gastroenterology 41, 1155–1164.

Stoianova, L.G., and Arkad'eva, Z.A., 2000. Comparison of methods of storing lactic acid bacteria. Mikrobiologiia 69, 98-104.

Sun, W. and Griffiths, M.W., 2000. Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellanâxanthan beads. International Journal of Food Microbiology 61, 17–25.

United States Pharmacopeia USP 31, the National Formulary NF 26. 2008. The United States Pharmacopeia Convention, Inc. Rockville

Vertzoni, M., Dressman, J., Butler, J., Hempenstall, J. and Reppas, C., 2005. Simulation of fasting gastric conditions and its importance for the in vivo dissolution of lipophilic compounds. European Journal of Pharmaceutics and Biopharmaceutics 60, 413-417.

Wills R., 2012. *In vitro* assessment of the survivability of *Lactobacillus casei* DN-114 001 and *Lactobacillus casei* Shirota, within commercialised food matrices, in the upper gastrointestinal tract The Plymouth Student Scientist 5(2), 80-9.

1 **Table 1. Commercial probiotics investigated in this study**

Product	Form	Probiotic strains	Volume or weight measured per content or dose	Claimed culture concentration	Dosage/ administration instructions
Actimel	Liquid (Dairy-based)	*L. casei* DN 114 001 Also, *L. bulgaricus* and *Streptococcus thermophilus*	100 ml	10 billion per 100 ml	One bottle per day as part of breakfast
Align	Solid (Capsule)	*B. infantis* 35624	0.174 g	1 billion per capsule	One capsule per day
Biobalance support	Solid (Capsule)	*B. bifidum, L. acidophilus*, and *B. lactis*	0.300 g	12.5 billion per capsule	Capsules are to be taken once daily with liquid
Bio-kult	Solid (Capsule)	*Bacillus subtilis PXN21, Bifidobacterium spp (B.bifidum PXN23, B. breve PXN25, B. infantis PXN27), Lactobacillus spp. (L. acidophilus PXN3, L. delbrueckii spp.bulgaricus PXN39, L. casei PXN37, L. plantarum PXN47, L. rhamnosus PXN54, L. helveticus PXN45, L. salivarius PXN57), Lactococcus lactis spp. Lactis PXN63, Streptococcus thermophilus PXN66.*	0.171 g	2 billion per capsule	One or two capsules, once or twice daily. Capsule can be opened and the contents can be sprinkled on food, taken in a drink or swallowed whole
Probio 7	Solid (Capsule)	*L. casei, L. rhamnosus, L. plantarum, L. acidophilus, Streptococcus thermophilus, B. bifidum, B. breve and B. longum.*	0.439 g	10 billion per capsule	One capsule per day. Capsule should be swallowed whole
Symprove	Liquid (Non-dairy, water-	*L. rhamnosus, L. planatarum, L. acidophilus, and Enterococcus*	60mL taken from 500 mL bottle	10 billion per 50 ml	1 ml per kilogram of bodyweight as a liquid drink to

	based)	*faecium*			be taken prior to food in the morning
VSL#3	Solid (powdered sachet)	*Streptococcus thermophiles* DSM 24731, *B. breve* DSM 24732, *B. longum* DSM 24736, *B. infantis* DSM 24737, *L. acidophilus* DSM 24735, *L. plantarum* DSM 24730, *L. paracasei* DSM 24733 and *L. delbrueckii* subsp. *bulgaricus* DSM 24734.	4.447 g	450 billion per sachet	One sachet, once or twice daily. Sachets can either be sprinkled on food or reconstituted with cold water or any non-fizzy drink and consumed
Yakult	Liquid (Dairy-based)	*L. casei* Shirota	65 mL	6.5 billion per 65 mL	One or two bottles per day

2

3

Table 2. Buffer capacity, osmolality, pH and surface tension of PGF, the simulated gastric fluids and human gastric fluid

Simulated fluid	pH	Buffer capacity $(mmol.L^{-1}.\Delta pH^{-1})$	Osmolality $(mOsm.Kg^{-1})$	Surface tension $(mN.m^{-1})$
PGF**	3.381 ± 0.031	12.85 ± 0.684	255.333 ± 0.577	46.075 ± 2.552
SGF* 1.2	1.211 ± 0.022	43.333 ± 0.714	255.667 ± 0.577	74.175 ± 3.562
FaSSGF 1.6[+]	1.599 ± 0.008	41.7 ± 0.404	138.000 ± 1.000	52.038 ± 2.202
SGF* 3.4	3.363 ± 0.020	1.7 ± 0.424	82.25 ± 1.8930	73.183 ± 0.248
FaSSGF 3.4	3.372 ± 0.036	1.233 ± 0.236	83.50 ± 1.9149	65.383 ± 1.864
Human gastric fluid	1- 2.5 [a] up to 5 (fed)[b]	7-18 (fasted)[c] 14-28 (fed)[c]	559-217[c]	30-31[c] 35-45[d]

[a] Evans et al. 1988
[b] Fordtran et al. 1973
[c] Kalantzi, et al, 2006
[d] Efentakis and Dressman 1998
** PGF was taken from a freely fed animal; volume was the only variable for the fed versus fasted test system
[+]FaSSGF is made to pH 1.6 to represent the fasted state.