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# Assessing gastric viability of probiotics: real testing in real human gastric fluid

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**Abstract**

***Background:*** It is believed that the harsh conditions of the upper gastrointestinal tract, such as gastric fluid acidity, may affect the viability of ingested probiotics. Thus far, this notion has been verified in *vitro* by viability testing in simulated gastric fluid.

***Objective:*** In this study, the survival of 8 probiotic bacteria was investigated in real human gastric fluid to determine the response of the bacteria in the actual biological medium.

***Methods:*** Gastric tolerance of the different probiotic bacteria was determined by inoculation of the bacteria in human gastric fluid, sampling at 30 min, 60 min, 120 min, 180 min, serial dilution and spread plating. Tolerance was also determined in traditionally simulated fluids at pH of 2.2 ± 0.1 and 2.8 ± 0.1, mimicking the pH of the human gastric fluid.

***Results:*** All the probiotic bacteria tested except for one strain, which showed less than 1 log CFU/mL loss in viability in the two fluids, were susceptible to the gastric fluids. The results showed significant ($p < 0.05$) strain-specific differences in the sensitivities of the bacteria in the gastric fluids. Some species were more sensitive to the real human gastric fluid than the simulated fluid. However, overall, the simulated gastric fluid did not significantly differ ($p > 0.05$) and hence provided a comparable environment to the actual human fluid at a similar pH.

***Conclusion:*** More than 80% of the tested probiotic strains were susceptible to real human gastric fluids. The results demonstrated strain differences in the susceptibility of different probiotic bacteria to gastric fluid. Also noteworthy are the differences in the behaviour of some of the probiotic bacteria in the real fluid against the simulated fluid. The result highlights the importance of using biorelevant test systems in viability assays.

**Keywords:** Probiotic; lactic acid bacteria; human gastric fluid; gastric tolerance

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## INTRODUCTION

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [1,2]. Probiotics are usually members of the lactic acid bacteria and bifidobacteria. Probiotics are obtained by isolation; some are available commercially for inclusion in food or other matrices and/or formulated into a dosage form mainly for oral administration or other applications. The amount of viable probiotics needed to obtain a clinical effect is generally quoted as $10^6$ - $10^8$ CFU/mL in the lower gastrointestinal tract [3,4]. Probiotic products are often required to contain greater than $10^8$ CFU/mL due to likely viability losses that may occur after ingestion. For instance, Health Canada and the Italian Ministry of Health require a minimum of $10^9$ CFU/mL viable cells per serving for probiotics [1]. The Food and Agriculture Organisation of the United Nations (FAO) and the World Health Organisation (WHO) established a guideline for the evaluation of probiotics in food. The guidelines require that tests for establishing the health benefits of probiotics should first involve the use of an appropriate *in vitro* study before undertaking in vivo investigations [2,5]. The *in vitro* tests are to predict their

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ability to exert a function since the health benefits are derived from the growth and activity in the human body. *In vitro*, tests such as acid and bile tolerance, antimicrobial production and adherence ability to human intestinal cells are required to be performed depending on the anticipated health benefit. *In vitro* acid and bile tolerance studies of newly isolated or commercial lactic acid bacteria are consequently routinely performed when establishing the health benefits of nearly all potential probiotics [6-10].

These *in vitro* acid and bile tolerance tests are to mimic the harsh conditions of the upper gastrointestinal tract, such as the acid of the stomach, the presence of bile salts and digestive enzymes, as well as factors affecting residence or transit, which may affect the viability of ingested bacteria. The resistance of the probiotics during the *in vitro* gastrointestinal tolerance predicts that the ingested probiotics will be able to reach the lower gut in quantity sufficient to produce the intended health benefits of probiotics, such as improved gut health and enhancement of the immune system [1,2]. However, routinely, these *in vitro* gastrointestinal tolerance tests are conducted using either buffer or sodium chloride solutions adjusted to pH 1.2 – 4.0 and higher pH with or without bile salts [7,9,11,12] to simulate the gastric fluid and intestinal fluid. Pepsin is sometimes added to the saline solution to make it more biorelevant [7]. Also, growth media, adjusted to similar pH, have been used to simulate gastric fluid [13]. The probiotics are incubated in the simulated fluids, sampled and enumerated periodically over an average of 3 hours [3]. Cells that maintain viability during the tolerance study are often favoured. Techniques such as microencapsulation and coating mechanisms such as enteric coating, Phloral®, coating and many others are investigated to improve the chance of survival of those with poor tolerance but with other suitable properties [14-19].

For instance, a 6 log CFU/mL reduction in viable cells was obtained when *L. plantarum* was exposed for 120 min to simulated gastric fluid, whereas microencapsulated cells decreased by 2.9 CFU/mL in the simulated fluid under similar conditions. This indicated better tolerance and enhanced survival of the microencapsulated microorganism than free probiotic bacteria [19]. Gastric fluid is composed of swallowed saliva, hydrochloric acid, bicarbonate, bile salts, pepsin, phospholipids, lipids, lipase, potassium, sodium, chloride and calcium a with characteristic pH, buffer capacity, osmolality, surface tension and viscosity which could have a major influence on the survival capacity of probiotics [20,21]. The simulated fluids used routinely in the *in vitro* tests often lack this complexity. They only mimic the salt concentration of the gastric fluid through the addition of sodium chloride and the hydrogen ion concentration by the addition of HCl. This work aimed at exploring the survival of some lactic acid strains with established probiotic potential in real human gastric fluid. This was to determine the response of the cells in the real biological fluid and to ascertain if the routinely used simulated fluid was representative of the real fluid in terms of the sensitivities of the bacteria to the fluid. To the best of our knowledge, no prior study appears to have examined this topic.

## MATERIALS AND METHODS

### Microbiological media and chemicals

De man rogosa sharpe (MRS) broth (2276280) and agar (2465177) were from Oxoid Ltd, Basingstoke, UK. L-cysteine hydrochloride (22063/1a) was from Surechem Products Ltd., UK. HCl (37%) was purchased from Sigma-Aldrich, UK. NaCl and phosphate-buffered saline (PBS) tablets were from Fisher Scientific, USA. NaOH (10222/4) was obtained from Park Scientific, UK.

### Probiotic strains

Eight (8) lactic acid strains were used for the study. Four (4) of the strains, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus* and *Enterococcus faecium*, which are component strains of the commercial product, Symprove™ with previously demonstrated probiotic properties were obtained from the manufacturer, Symprove™ Ltd, UK. Four local strains, which included *Lactobacillus fermentum* FSI3-D, *L. fermentum* FSI3-LBC, *L. plantarum* FSC3-LBC and *Lactobacillus salivarus* FSDI-D, were isolated from faecal samples in Ghana and previously demonstrated to have potential probiotic properties [9]. The strains were previously isolated on de Man Rogosa Sharpe (MRS) agar supplemented with 0.05% w/v L-cysteine hydrochloride and 0.002% w/v of bromophenol blue and incubated anaerobically at 37 ºC for 48 hours [9]. Pure colonies obtained were maintained in MRS broth or agar supplemented with 0.05% L-cysteine hydrochloride. Identification of isolates was done with Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) using the MALDI Biotyper® and validated with 16S rRNA gene sequence analysis [9].

### Collection of human gastric fluids

Seven volunteers were recruited for the study. The volunteers were Ghanaians of normal body weight who were between the ages of 20 - 40 years. The volunteers had or were enrolled in tertiary-level education. Only samples from volunteers who did not have any upper gastrointestinal disease that was discovered during the examination were used for the study. Samples contaminated with intestinal content were also discarded. Gastric aspirates were obtained from the seven (7) healthy volunteers who had fasted for at least 12 hours prior to the procedure. The sample collection was carried out at the endoscopy unit of Korle Bu Teaching Hospital, Accra, using an upper gastrointestinal endoscopy. The inclusion criteria for the study were healthy adults who were 18 years and above without any chronic disease and who signed a consent form to participate after it had been explained to them. Persons with gastrointestinal disorders and on medications for acid blockade, as well as those on antibiotics, were excluded.

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On the day of the procedure, the volunteers were asked to sit and relax. They were briefed about the procedure, after which a 10% Xylocaine pump spray, a local anaesthetic, was sprayed into their throats, which they were asked to hold for a minute before swallowing. The volunteers were made to lie down on their left side and bite on a plastic mouthguard. An endoscopic tube was inserted through their mouth, down their throat, and into their stomach. Prior to insertion, 2% Xylocaine gel was applied to the tube for anaesthetic purposes. Gastric aspirates were collected by suction into a dry sterile trap inserted at the exhaust portion of the endoscopic tube during endoscopic examination. Collected aspirates, which measured between 15 mL and 40 mL, were stored in sterile 50 mL Falcon tubes, preserved with ice in a thermostat container, and transported over approximately 1 hour to the Microbiology laboratory at the School of Pharmacy, University of Ghana, Legon. They were examined, filtered with a 0.22 □m micropore filter, aliquoted into 1.0 mL portions into sterile cryovials, and stored at -80 ºC. Before use, they were thawed in a water bath with the temperature set at 40 ºC for 3 min and maintained at 37 ºC in an incubator.

**Determination of pH and buffer capacity of gastric fluids**

Simulated gastric fluid was prepared by dissolving 0.2 g of NaCl in 100 mL of purified water and adjusting pH to 2.2 ± 0.1 or 2.8 ± 0.1 with HCl. The pH and buffer capacity of the gastric fluids were determined using a calibrated pH meter (pHep®, HANNA, Instruments). The buffer capacities of the real and simulated gastric fluids were compared at the two different pHs [22]. The buffer capacities of the fluids were measured by adding NaOH standard solution (0.1 M) to 3 mL of each fluid and noting the volume.

Buffer capacities were determined using the equation:

$$\beta \text{ (mmol/l/}\Delta\text{pH)} = \Delta AB / \Delta pH$$

Where ΔAB is the small increment in mol/l of the amount of acid or base added to produce a pH change in the buffer. This equation can be rewritten as:

$$\beta \text{ (mmol/l/}\Delta\text{pH)} = \Delta AB/ \Delta pH = (M_a \times V_a)/ \Delta pH \text{ x } 1000/ V_b$$

Where $M_a$ is the molarity of the acid, Va is the volume of acid in mL, $V_b$ is the volume of buffer in mL, and ΔpH is the change in pH. The equation was multiplied by 1000 to express the volume in litres.

**Gastric tolerances**

Tolerance of the lactic acid strains to the gastric fluids was performed by inoculating 100 µL of a culture of each strain in 1 mL of respective gastric fluid. The cells were incubated at 37 ºC, and samples taken at 30 min, 60 min, 120 min, and 180 min serially diluted in PBS (pH 7.4) and spread-plated on MRS agar supplemented with 0.05% w/v L-cysteine hydrochloride for the determination of viable counts.

**Statistical analysis**

Experiments were performed in triplicates. Results of the gastric tolerance assay (viable cell count, log CFU/mL) were expressed as mean ± standard deviation. Statistical analysis was performed in Origin Pro Version 8.6 (Microcal Software Inc.). The significance of difference was evaluated with a t-test or analysis of variance (ANOVA). P values less than 0.05 were regarded as significant differences between means.

## RESULTS

Two batches of gastric fluid from two of the volunteers were used for the study. The pH of the two batches of gastric fluid collected from the volunteers were 2.2 ± 0.1 and 2.8 ± 0.1. The buffer capacities of real human gastric fluid (HGF) and simulated gastric fluid (SGF) at pH 2.8 were 12 mmol/l/ΔpH and 8.08 mmol/l/ΔpH respectively. The buffer capacities of HGF and SGF at pH 2.2 were 4 mmol/l/ΔpH and 32.36 mmol/l/ΔpH, respectively. When the results for buffer capacities were compared to pH, a lower buffer capacity for increasing pH was noted for SGF and vice versa for HGF. The human gastric fluid (HGF) with pH 2.2 ± 0.1 was used for the tolerance assessment of the commercial strains, including *L. acidophilus, L. plantarum*, *L. rhamnosus* and *E. faecium*. The HGF with pH 2.8 ± 0.1 was used for the tolerance assessment of the locally isolated strains.

The results for the tolerance tests of the commercial strains are given in Figure 1. Significant differences in tolerance ($p < 0.05$) of the different strains were noted. Significant differences ($p < 0.05$) were observed between *L. plantarum* and *L. acidophilus; L. rhamnosus* and *L. acidophilus; E. faecium and L. rhamnosus* and *L. plantarum* and *E. faecium.* Both *L. acidophilus and E. faecium* did not show survival in human and simulated gastric fluids after 30 min. *Lactobacillus plantarum* maintained relative survival in both fluids for 120 min, although better survival was noted in SGF than in HGF, with reductions of 1.33 log CFU/mL and 2.28 log CFU/mL, respectively, after the test. *Lactobacillus rhamnosus* showed the strongest survival in the gastric fluids, relatively maintaining survival for the duration tested. It was, however, more sensitive in HGF than in SGF, showing about 2.31 log CFU/mL and 1.67 log CFU/mL reduction in viable cells, respectively, in the fluids after the test duration.

The results for the tolerance of the local strains in HGF are shown in Figure 2. Significant reductions in viable cells ($p < 0.05$) were observed. All strains lost viability by 60 min in HGF; only *L. salivarus* FSD1-D maintained survival in HGF with about 0.91 log CFU/mL reduction in viable cells, which was similarly noted in SGF. *Lactobacillus fermentum* FSI3-D and L. *plantarum* FSC3-LBC maintained some viable cells in SGF for the duration tested, showing less than 1 log CFU/mL and about 3 log CFU/mL reduction in viable cells, respectively, after 180 min. For all the studied strains, 50% maintained some viable cells in

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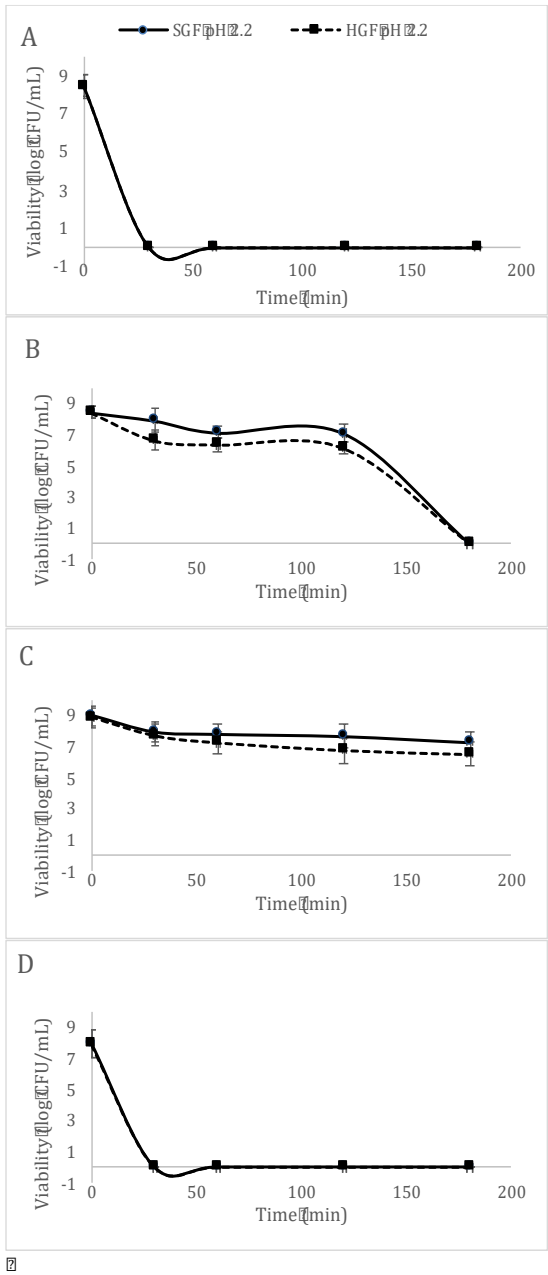


Figure 1. Viability (log CFU/mL) of commercial probiotic strains (A) *L. acidophilus*, (B) *L. plantarum*, (C) *L. rhamnosus*, (D) *E. faecium* in simulated gastric fluid (SGF) and human gastric aspirate (HGF) pH 2.2.

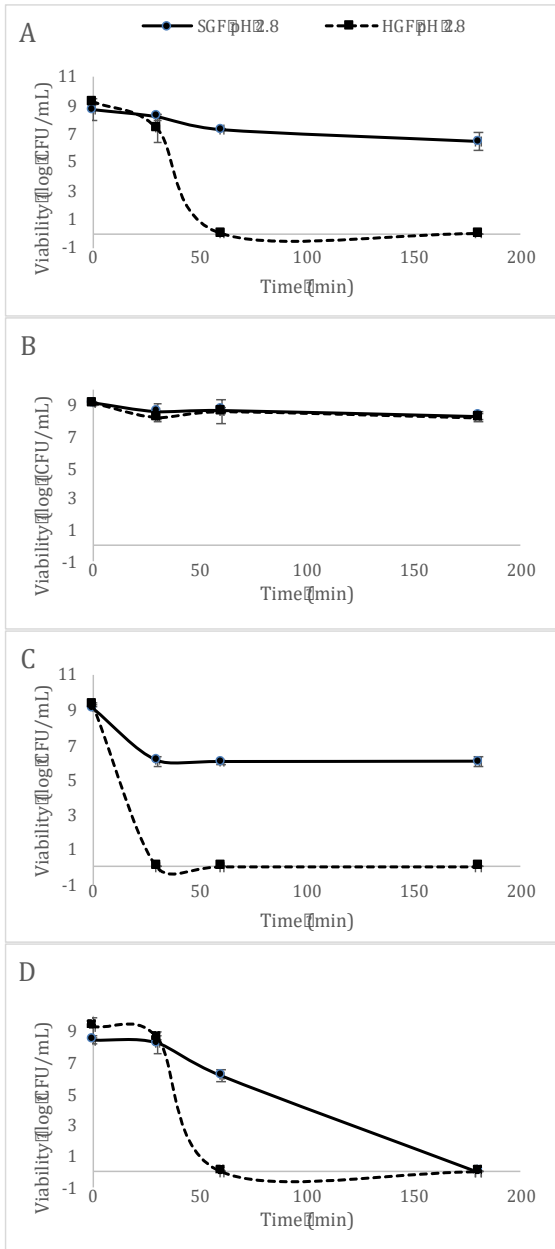


Figure 2. Viability (log CFU/mL) of isolated probiotic strains (A) *L. fermentum* FSI3-D, (B) *L. salivarus* FSD1-D, (C) *L. plantarum* FSC3-LBC, (D) *L. fermentum* FSI3-LBC in simulated gastric fluid (SGF) and human gastric aspirate (HGF) pH 2.8.

SGF, whereas 25% maintained relative viability in HGF for the duration tested.

## DISCUSSION

The main objective of using simulated fluids is to mimic the *in vivo* behaviour of materials within the respective physiologic environment. The fluids' composition and their ability to replicate the physiological condition is, therefore, very critical. Considerable knowledge exists regarding the composition of the gastric fluid in both the fasted and fed states [20-24]. However, to date, the fluid is mostly simulated with an aqueous solution of sodium chloride, pH adjusted with hydrochloric acid. This study aimed to investigate the survival of four commercial lactic acid bacteria strains and four locally isolated strains in gastric

aspirate (from fasted volunteers) and simulated gastric fluid to determine if traditionally used fluid can accurately predict survival in the real fluid at a fasted state. The results indicate that overall, the viability of the tested probiotic strains in human aspirate was not significantly different ($p > 0.05$) from the viability in the simulated artificial fluid. Viability was, however, significantly different ($p < 0.05$) amongst strains. For the commercial strains, both *L. acidophilus* and *E. faecium* lost viability within the first 30 min of testing, whilst *L. plantarum* and *L. rhamnosus* maintained relative survival for more than 90 min.

Differences in survival among Lactobacilli species and strains in acidic conditions have been reported [25,26]. It is also known that probiotic species are generally less sensitive to pH above 3 [25] and have been demonstrated to show greater sensitivities at pH 2 [27]. *Lactobacillus rhamnosus* has demonstrated resistance to pH 2.5 for more than 4 hours [28] and has shown to be more resilient relative to *L. acidophilus* and *E. faecium* in a mixed environment [29]. For this study, both *L. rhamnosus* and *L. plantarum* demonstrated survival, although relatively lower survival was recorded in HGF than in SGF for 120 min. Whilst *L. rhamnosus* maintained some viable cells, *L. plantarum* lost viability after 120 min, showing that *L. rhamnosus* was more robust than the two. For the local isolates, except for *L. salivarus* FSD1-D, none of the tested strains survived beyond 60 min in HGF. The species demonstrated differences in viability in the fluids, as previously observed. A similar rate of loss in viability was noted for the two strains of *L. fermentum* in HGF. *Lactobacillus salivarus* FSD1-D was the more resistant strain. The buffer capacity, which is the resistance to change in pH, can be important to the survival of bacteria.

The buffer capacity of the gastric fluid is contributed by the physiological pH-regulating agents that are present in the stomach as well as any food and drink that has been ingested by a person. In the fasted state, the buffer capacity is mainly regulated by the concentration of hydrochloric acid, although a potential contribution of amylase, lipase, pepsin or other protein-based components to the buffer capacity of bulk gastric contents has been asserted [22]. A linear correlation between the buffer capacity and the hydrogen ion concentration of gastric aspirate has been reported [22]. Buffer capacity tends to decrease with increasing pH of gastric aspirates. This, however, does not concur with the aspirates in the present study, which can be explained by variability within the volunteers or sample treatment. The simulated fluid demonstrated a lower buffer capacity for increasing the pH of fluid, which is consistent with previous reports [8,22]. Although a significant difference in buffer capacity between the simulated fluid and human gastric aspirate at pH 2.2 was observed, this did not result in a greater sensitivity of the strains in the simulated fluid. The presence of other substances or properties of the human gastric fluid may have also contributed to the greater sensitivity of the cells. The results demonstrate that the rate of survival was species/strain dependent. The rate of killing was, however, not particularly dependent on pH.

### Conclusion

Overall, the findings from the viability test suggest that the simulated gastric fluid provided an environment comparable to that of human gastric fluid at a similar pH. Whilst most strains showed similar trends in simulated and real fluid, this was strain-specific as significant differences were observed among some strains. This study demonstrates that the bactericidal action of gastric juice may be attributed to pH and possibly other components of the juice. It highlights the significance of using biorelevant fluids in viability assay and indicates the need to offer gastric protection to probiotics to maintain viable cells and obtain health benefits. This study has some limitations; it only considered eight (8) probiotic strains and one type of fluid used in simulating the gastric juice.

## DECLARATIONS

### Ethical consideration

Ethical approval was obtained from the institutional review board of Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Reference number NMIMR-IRB CPN 073/20-21. Informed consent was obtained from volunteers before participation.

### Consent to publish

All authors agreed on the content of the final paper.

### Funding

None

### Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

### Author contribution

MFA participated in the conceptualisation, supervision, and analysis of the data for the manuscript. MFA and NAA-A participated in the Investigation and methodology. MFA, NAA-A and SG participated in the resources, writing, review and editing.

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### Availability of data

Data for this work is available upon reasonable request from the corresponding author.

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