

Biorelevant assessment of commercial probiotics using porcine gastrointestinal fluids

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Abstract

Aims Porcine gastrointestinal fluids offer biorelevant alternatives to human fluids for assessing probiotic viability due to their similar pH, enzymes, and bile salts. Despite extensive research on synthetic fluids, porcine fluids remain underexplored. This study evaluated commercial probiotic survival in porcine gastric fluid (PGF) and porcine intestinal fluid (PIF), bridging *in vitro* simulations with human and veterinary applications.

Methods and results Representative doses of commercial probiotic products and 10^6 – 10^8 colony forming units (CFU)/ml suspensions of commercial cells were incubated at 37°C in PGF (pH 3.38 ± 0.03) and PIF (pH 6.41 ± 0.01) obtained from healthy pigs from an abattoir. Viability was quantified through serial dilution and enumeration of CFU at different time points. Significant differences were observed between the commercial products and the free cells. The commercial products and cells showed significant decreases in viability in PGF after 180 min. Differences in cell viability were also observed among the products. However, the commercial probiotic products retained their viability during 240 min of simulated small-intestinal tolerance in PIF. A 1–1.5 log reduction in viable count was noted for the commercial cells in PIF during the first 30 min after which viability was maintained.

Conclusion This study established porcine gastrointestinal fluids as a biorelevant alternative to synthetic simulations and human-derived fluids, which involve greater ethical complexity for probiotic viability assessment. The findings highlight the importance of developing strategies to enhance probiotic resilience during gastric transit, the main barrier to oral survival, ensuring their functional delivery to the intestinal tract, where their benefits can be maximized.

Impact statement

This study advances probiotic research by establishing porcine gastrointestinal fluids as a more ethically acceptable and physiologically relevant medium that more accurately reflect human digestive conditions than conventional synthetic fluids, enabling a more realistic *in vitro* assessment of probiotic survival and improving the relevance of current quality control models. The findings identify the gastric environment as the principal barrier to probiotic viability, highlighting the need for formulation strategies that enhance resistance to gastric transit. Overall, this work informs the rational development and quality control of probiotic products, with important implications for both human and veterinary health.

Keywords probiotics, biorelevant media, oral viability, porcine gastrointestinal fluid, quality control

Introduction

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health effect on the host” (Hill et al. 2014). They are widely incorporated into functional foods and dietary supplements, with reported benefits, including immune modulation, improvement of some gastrointestinal disorders, reduction in the risk of certain cancers, and other diseases (Sanchez et al. 2017, Slizewska et al. 2020, Xia et al. 2021, Du et al. 2022, Chavez-Iniguez et al. 2023, Goodoory et al. 2023). How-

ever, inconsistencies in clinical outcomes, including limited or no benefit compared to placebo, have been reported and highlight the need for more reliable methods to assess their efficacy *in vitro* (King et al. 2019, Limketkai et al. 2020, Chavez-Iniguez et al. 2023).

Any probiotic product must first and foremost contain viable bacteria in adequate amounts to elicit the required health effect (FAO/WHO 2001). As a general rule, it is suggested that the microorganisms must be present at a concentration of 10^6 colony forming units (CFU)/ml or greater and consumed at levels of 10^8 – 10^9 CFU/day to achieve therapeutic benefits (Minelli and Benini 2008).

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Table 1 Description of products.

Product	Dosage form	Matrix type	Number of probiotic species	Other ingredients	CFU/dose
A	Liquid	Dairy-based	3	Yogurt, skimmed milk, sucrose, dextrose, milk mineral concentrate, vitamins (B6, D)	1×10^{10} CFU
B	Liquid	Non-dairy based	4	Barley, vitamin C, potassium sorbate, trisodium citrate	1×10^{10} CFU
C	Solid (powder)	Non-dairy based	8	Maltose, silicone dioxide (anti-caking agent)	4.5×10^{11} CFU
D	Liquid	Dairy-based	1	Skimmed milk solids	6.5×10^9 CFU

The viable probiotic microorganisms that are consumed must also survive the hostile conditions of the gastrointestinal tract to reach the lower intestine in adequate numbers (Minelli and Benini 2008, Fredua-Agyeman et al. 2024).

The gastrointestinal environment presents multiple physiological barriers to probiotic survival. The complex composition of the gastric juice, including hydrochloric acid, bile salts, pepsin, lipase, other digestive enzymes, and electrolytes, plays a critical role in the survival of probiotics (Mudie et al. 2010, Litou et al. 2020). Hydrochloric acid, its main component, imposes severe bactericidal stress by disrupting cellular pH, membranes, and metabolism, while pepsin enhances this effect through pH-dependent proteolysis (optimal at pH 2.5–3.5) (Rotimi et al. 1990, Zhu et al. 2006, Schwarz et al. 2022, Wang et al. 2023). The bile-rich environment of the small intestine imposes significant antimicrobial stress, and together with gastric conditions, represents a major barrier to probiotic survival after ingestion (Lindahl et al. 1997, Sanchez et al. 2007, Amund 2016, de Waal et al. 2023).

Despite this, probiotic survival is commonly assessed using simplified *in vitro* models that inadequately reflect the *in vivo* gastrointestinal conditions. Conventional simulated gastric and intestinal fluids are typically based on buffered saline or acidified media, which lack the complexity of human gastrointestinal fluids (Caillard and Lapointe 2017, Fredua-Agyeman et al. 2020, Liu et al. 2022, Goderska and Agudo Pena 2024). This limitation reduces the predictive accuracy of such models and may contribute to discrepancies between *in vitro* findings and clinical outcomes. There is therefore a critical need for more physiologically relevant *in vitro* systems to improve the reliability of probiotic evaluation. Porcine gastric fluid (PGF) is believed to be more representative of human gastric fluid (Fredua-Agyeman and Gaisford 2015, Henze et al. 2021, Rivera et al. 2023). Like humans, pigs are omnivores and possess a comparable digestive system with similar stomach, small intestine, and microbiome characteristics (Gonzalez et al. 2015, Henze et al. 2021). Although pigs have prolonged gastric emptying, the small intestinal transit conditions, gastrointestinal pH, pressure, and other properties have been shown to be similar to those in humans (Henze et al. 2020, 2021). These physiological similarities position porcine gastrointestinal fluids as a biorelevant surrogate that bridges the gap between artificial fluids and human fluids, offering a practical solution to the methodological limitations currently facing probiotic research and product development. PGF was previously demonstrated to be more harmful to commercial probiotic products than simulated gastric fluid (SGF) at a similar pH (Fredua-Agyeman and Gaisford 2015). The findings validated PGF as a biorelevant testing medium for the gastric phase of probiotic transit. However, while the gastric phase has been ex-

plored, there remains a significant gap in understanding probiotic survival under biorelevant intestinal conditions. Given that the small intestine is a critical barrier to microbial survival and a key site influencing probiotic functionality, this gap limits the comprehensive evaluation of probiotic performance. Therefore, this study extends previous porcine gastric fluid research (Fredua-Agyeman, 2015) by evaluating probiotic survival also in porcine intestinal fluid (PIF). By incorporating biorelevant intestinal conditions, this work provides a more comprehensive and physiologically relevant assessment of probiotic viability, with important implications for formulation and quality control.

Materials and methods

Probiotic products

Commercial products A, B, C, and D were obtained from manufacturers or distributors or purchased from local pharmacies or supermarkets (Table 1). These products were selected from brands previously evaluated in our earlier study (Fredua-Agyeman, 2015) based on predefined criteria including commercial availability, variation in formulation type and differences in strain composition. They were stored according to the label instructions and were used before the expiry date.

Commercial probiotic cells

The commercial probiotic species used were *Lactobacillus acidophilus* LA-5[®] and *Bifidobacterium lactis* BB-12[®]. Both were obtained in powdered form from Chr. Hansen's Culture Collection (Hørsholm, Denmark). They were rehydrated with de Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, UK). The broth suspensions obtained were sub-cultured on MRS agar supplemented with 0.05% w/v L-cysteine hydrochloride to obtain pure colonies of the cells. The pure colonies obtained were used to make a lawn of the bacteria by spreading the colonies on fresh agar plates. Thick suspensions of the cells in MRS broth supplemented with 0.05% w/v L-cysteine hydrochloride and 15% v/v glycerol were made from the lawn and this was stored at -80°C and used as the parent culture. To prevent batch-to-batch variability, cells were maintained as frozen stocks from the parent culture (Dodoo et al. 2025). The cells were grown at 37°C in MRS broth supplemented with 0.05% w/v L-cysteine hydrochloride. At the stationary phases of growth, the cultures were harvested by centrifugation (Heraeus Stratosbiofuge, Thermo Scientific, UK) at 3500 *g* for 10 min at 4°C and the pellets obtained were washed twice with phosphate

buffered saline (PBS). The washed pellets were resuspended in 15% v/v glycerol in $\frac{1}{4}$ strength Ringer's solution, aliquoted into sterile cryovials, and frozen over liquid nitrogen (Fredua-Agyeman 2024, Doodoo et al. 2025). The samples were stored in liquid nitrogen until required. Prior to use, the samples were removed from the storage liquid nitrogen container and thawed at 40°C in a water bath for 3 min.

Porcine gastric and intestinal fluids

PGF and PIF were obtained from the stomach and duodenum of an adult pig, *fed ad libitum*, and obtained from an abattoir. Ethical approval was not required for this study, as the samples were obtained from an animal slaughtered for commercial food production, with no animals sacrificed specifically for the research. The pH of the juices was determined using a calibrated pH meter (pHennomenal®, UK). Samples were dispensed into multiple aliquots and frozen at -80°C. Prior to use, the samples were thawed at room temperature and centrifuged at 10 000 rpm (Mini spin, Eppendorf, Germany) for 10 min at 25°C. The supernatant obtained was sterilized by filtration using a membrane filter with a 0.22 µm pore size.

This approach ensured consistency across experiments; however, the use of a single animal source may limit generalizability due to potential biological variability.

Gastric and intestinal tolerance test

Gastric and intestinal tolerance tests were performed by mixing representative doses of commercial products with PGF (1.5 ml) or PIF (Fredua-Agyeman and Gaisford 2015). The mixture was vortexed for 10 s and incubated at 37°C under static conditions. For the gastric tolerance, aliquots of 50 µl were withdrawn at 5, 10, 20, 30, 90, 120, and 180 min, serially diluted in PBS, and plated on MRS agar supplemented with 0.05% L-cysteine hydrochloride. The plates were incubated anaerobically at 37°C in anaerobic jars (Oxoid) using the Anaerogen GasPak System (Oxoid) (Fredua-Agyeman and Gaisford 2015). Viable counts were expressed as CFU/ml. For the intestinal tolerance test, aliquots of 50 µl were withdrawn at 30, 60, 90, 120, and 240 min, serially diluted in PBS, plated on MRS agar supplemented with 0.05% L-cysteine hydrochloride and incubated anaerobically at 37°C. The incubation times were selected to reflect physiologically relevant gastrointestinal transit under fed conditions with 180 min gastric exposure representing the upper range of reported gastric residence times and 240 min representing small intestinal residence times (Vinarov et al. 2021).

The gastric and intestinal tolerance of free cells of the commercial probiotic strains were determined by inoculating 10^6 – 10^8 CFU/ml of cell suspensions of *L. acidophilus* LA-5® and *B. lactis* BB-12® in PGF or PIF. The cultures were incubated at 37°C, and aliquots were withdrawn, serially diluted, and plated as described previously. The experiments were performed in triplicate.

Statistical analysis

Results are expressed as the mean ± standard deviation of triplicate results. Statistical analysis was performed using OriginPro 10.0 (OriginLab Corporation). Analysis of variance (ANOVA) with Tukey's *post-hoc* analysis was used for the comparison of means.

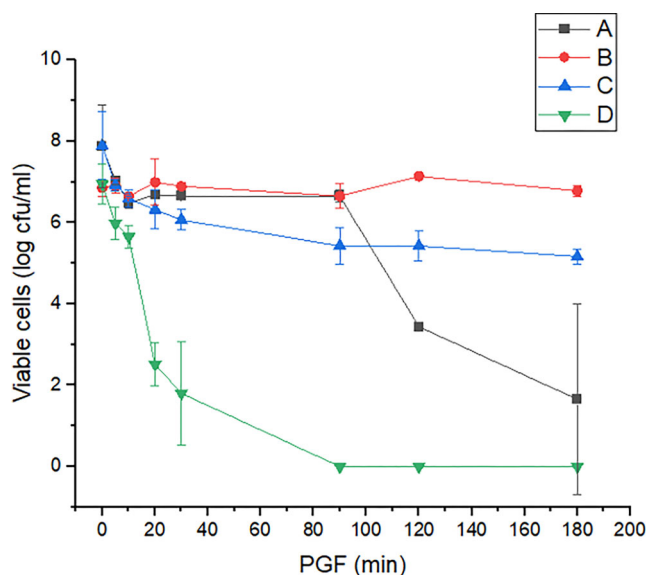


Figure 1 Gastric tolerance of commercial probiotic products, A, B, C, and D in PGF.

Due to the small sample size, formal normality testing was not conducted. *P* values were considered statistically significant at *P* < 0.05.

Results

The gastric tolerance data for the commercial probiotic products over 180 min are shown in Fig. 1. Tolerance of the commercial probiotic products to PGF showed initial non-significant reduction in viability for all products during the first 5 min of testing, demonstrating a short adaptation phase upon exposure to the acidic environment. However, differences between the products became evident thereafter, highlighting variability in formulation-dependent protection. Product C demonstrated the most rapid decline in viability, with a statistically significant decline in viability after 10 min of testing (*P* < 0.05) and an overall reduction of more than 1.5 log by the end of the test period. Product D also exhibited marked susceptibility to gastric conditions, with a significant decrease in viability (*P* < 0.05) observed after 20 min and a complete loss in viability after 90 min. In contrast, product A showed comparatively greater resistance, maintaining viability with no significant reduction for 90 min. A significant reduction in viability (*P* < 0.05) was only observed after 120 min, indicating delayed susceptibility to prolonged gastric exposure. Product B displayed the highest stability, with no statistically significant reduction in viability throughout the exposure period.

The responses of the free cells differed markedly from that of the formulated products (Fig. 2). *Lactobacillus acidophilus* exhibited an immediate loss of viability upon exposure to PGF. *Bifidobacterium lactis*, however, showed relatively greater initial tolerance, with a significant decline in viability after 90 min, demonstrating species-dependent differences in acid resistance. The simulated small-intestinal tolerance results for the commercial probiotic products and cells are presented in Figs. 3 and 4, respectively. All the probiotic products largely maintained their viability during 240 min of simulated small-intestinal tolerance in porcine

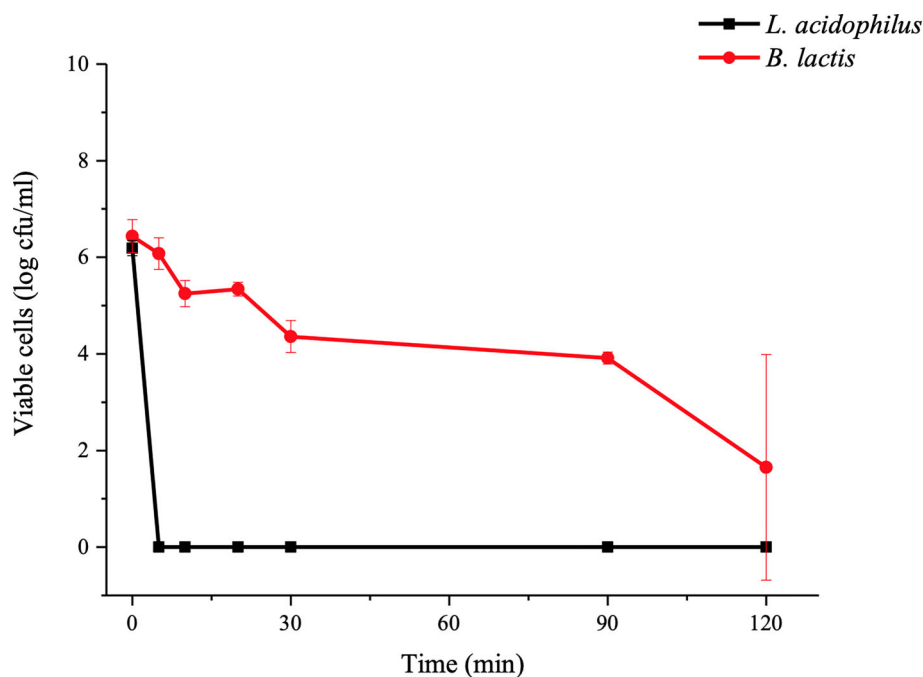


Figure 2 Gastric tolerance of free cells of *L. acidophilus* and *B. lactis* in PGF.

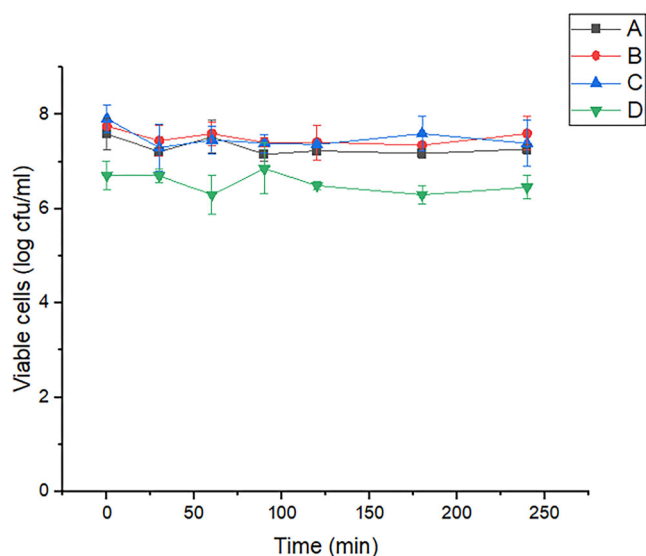


Figure 3 Small intestinal tolerance of commercial probiotic products, A, B, C, and D in porcine duodenal fluid.

duodenal fluid (Fig. 3). Products A, B, and C showed a minor, non-significant reduction in viable count <0.5 -log ($P > 0.05$) in the first 30 min before stabilizing, while product D maintained cell viability throughout the test period. In contrast, the free cells exhibited significantly greater susceptibility to intestinal conditions. Both *L. acidophilus* and *B. lactis* showed significant reductions in viability within the first 30 min, after which no further significant losses were observed (Fig. 4).

Discussion

Overall, significant differences in resilience to gastric juice were observed between the tested probiotic products and free probiotic cells. In addition, differences were noted between the products and free cells. The differences in susceptibility to gastric juice are likely to be due to differences in acid tolerance between species. This was well demonstrated when the susceptibilities of the free cells were compared. For the products, their survival in the gastric fluid may also depend on the way they are formulated, and the excipients included in the product.

Despite being a prerequisite that commercial probiotic organisms should be resistant to gastric juice (FAO/WHO 2001), some commercial strains rarely possess this property, as demonstrated in this study and in previous findings (Jensen et al. 2012, Fredua-Agyeman and Gaisford 2015, Caillard and Lapointe 2017, Vecchione et al. 2018, Reuben et al. 2020, Liu et al. 2022, Mgomi et al. 2025). For instance, Jensen et al. (2012) observed significant reductions within 90 min of a simulated gastric tolerance test at pH 3 for half of the 18 commercial and potential probiotic lactic acid bacteria strains under evaluation. Their study showed strain-specific differences in susceptibility to simulated gastric juice, with some strains showing a total loss of viability after 180 min of testing in the simulated gastric juice. Vecchione et al. (2018) also demonstrated significant reductions, including total loss of viability for seven out of ten commercial probiotic products during a 120 min simulated gastric tolerance test at pH 1.4–1.5. The commercial products they evaluated were either single strain-containing products or polymicrobial products (Vecchione et al. 2018). Similarly, Caillard, and Lapointe (2017) showed that all tested 16 probiotic strains showed high sensitivity to acidic conditions.

Evidence indicates that gastric challenges can also be overcome if non-intrinsically gastric-resistant probiotics are adequately pro-

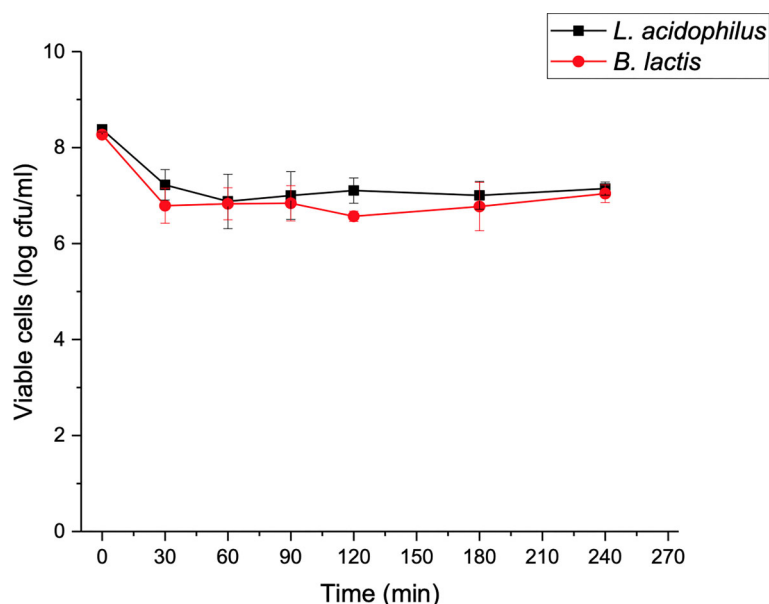


Figure 4 Small intestinal tolerance of free cells of *L. acidophilus* and *B. lactis* in porcine duodenal fluid.

vided with protection systems in a product to withstand stomach conditions (Caillard and Lapointe 2017). This can be achieved through both simple and complex formulation strategies, such as coating and microencapsulation (Caillard and Lapointe 2017, Sun et al. 2023, Fredua-Agyeman 2024) or through the effect of other food components or excipients of the probiotic product (Charalampopoulos et al. 2003, Wills 2012). To illustrate, a study by Charalampopoulos et al. (2003) revealed protective effects of barley, malt, and wheat extracts on viability of *L. plantarum*, *L. acidophilus*, and *L. reuteri* under acidic conditions. The addition of the extracts caused an increase of >0.7-log and 1.5-log cycles for *L. reuteri* and *L. acidophilus*. Approximately 3-log and 4-log cycles of improvement in viability were also observed for *L. plantarum* in the presence of barley, wheat, and malt, respectively (Charalampopoulos et al. 2003). Similarly, milk proteins and fat content in probiotic products have been demonstrated to offer protection to cells (Livney 2010, Wills 2012). (Wills 2012 demonstrated that the survival of *L. casei* DN-114 001 in Actimel® Fat-Free was significantly affected by gastrointestinal conditions, while its survival in Actimel® Original remained largely unaffected (Wills 2012). In the present study, products A and D were dairy-based products that may have benefited from the milk proteins in the products, which could protect the probiotic microorganisms. However, this protection may not have been highly efficient in Product D as significant losses were noted shortly after exposure to the gastric fluid. Product D contained non-fat milk and a single species unlike Product A which contained beside the main probiotic species, *L. casei*, contained *Streptococcus thermophilus* and *L. bulgaricus* which may have worked together to improve survival. Products B and C are also multi-strain products containing four and eight strains, respectively, which may have benefited from the combined action of the various strains. Product C had a higher overall bacterial count among the products tested, which possibly ensured that even if some strains perished in an acidic environment, others survived. Product B, a water-based product containing an extract of germinated barley, was more efficient in protecting cells

against gastric fluid. This could be due to the protective effect of barley.

The purpose of the *in vitro* intestinal tolerance assay was to observe the outcome of exposure of probiotics to biorelevant small intestinal conditions. It is believed that duodenal fluid contains digestive enzymes and significant amount of bile salts (de Waal et al. 2023). Relative to a measured pH of 3.38 ± 0.03 for the gastric fluid, the pH of the intestinal fluid was 6.41 ± 0.01 . The results showed that all tested probiotic products maintained viability in the small intestinal fluid. The free cells, however, showed a significant reduction in viability within the first 30 min but maintained viability thereafter. Previous studies that evaluated the simulated small intestinal tolerance of probiotics have largely demonstrated bacterial tolerance to the fluid (routinely with 0.1%–1% bile salt concentration) relative to simulated gastric fluid (Jensen et al. 2012, de Moraes et al. 2017, Reuben et al. 2020, Lee et al. 2024) although some susceptibility of the tested bacteria has also been reported (Vecchione et al. 2018). In the study by Jensen et al. (2012), all the tested probiotic lactic acid bacteria strains tolerated the simulated small intestinal fluid, with none of the strains showing more than a 1-log reduction in viability. Lee et al. (2024) also evaluated the gastrointestinal tolerance of potential *Lactobacillus* and *Bifidobacterium* strains in simulated gastric and intestinal fluids consisting of PBS pH adjusted to 2.5 (and supplemented with 0.3% pepsin) and 7.4 (supplemented with 1% pancreatin and 1% bile salt), respectively. While the cell viability decreased in all tested strains except for one in SGF, there was minimal decrease in cell viability after exposure to SIF.

Obtaining and using human gastrointestinal fluid for research can be challenging and ethically complex. Due to their similarities with the anatomy and physiology of the human gastrointestinal tract, pigs have frequently been suggested as alternatives in pharmacokinetic and toxicological studies (Bode et al. 2010, Henze et al. 2021). Their gastrointestinal fluids offer a practical and ethical alternative for research and can provide valuable insights that are relevant to human health. They have a gastric pH similar to that of

humans (Fordtran and Walsh 1973, Henze et al. 2019) and a small intestinal pH comparable to that of humans (Lindahl et al. 1997, Henze et al. 2021). Although the buffer capacity and osmolality of the intestine of pigs are higher than those of humans (Henze et al. 2020), the composition and characteristics of the gastrointestinal fluids, such as phospholipids, free fatty acids, free cholesterol, and surface tension, are somewhat similar to those at the lower or upper end of each other (Henze et al. 2019, 2020). The bile salt concentrations in porcine intestinal fluid are also higher than the reported concentrations in humans, with approximately 97% of conjugated bile acids (Riethorst et al. 2016, Henze et al. 2020). Using porcine gastrointestinal fluid, we determined that probiotics are susceptible to gastric fluid. Susceptibility to gastric fluid may have varied depending on the strain or formulation. While bile has been determined as an agent that causes stress in bacteria with antimicrobial potential (Flahaut et al. 1996, Sanchez et al. 2007), this study found that probiotics were more resilient in the intestinal fluid than in the gastric fluid. Bile tolerance has been identified in several *Lactobacillus* and *Bifidobacterium* probiotic strains, and some researchers have suggested the possible involvement of bile salt hydrolases, which decrease the toxicity of conjugated bile salts, as well as several other physiological mechanisms (De Smet et al. 1995, Begley et al. 2005, Sanchez et al. 2007). This may explain the less aggressive reaction to the simulated small-intestinal condition, unlike the gastric conditions.

While this study provides valuable insights into the barriers to probiotic survival after ingestion, some limitations should be considered. The number of products and strains tested was relatively small, which may limit the generalizability of the results. In addition, the variability in fluids in different animals, which is likely in nature, was not tested. The sterilization of the gastrointestinal fluids via filtration may have altered certain biological components, including enzyme activity and bile salt behaviour, which should be considered when interpreting the findings. Future research with larger samples and fluids may help to address these limitations and build on the current findings.

Conclusion

In conclusion, the data from this study indicate that although the small intestinal fluid may present some challenges to live microorganisms, the probiotic products and strains tested could withstand exposure to porcine duodenal fluid and remain viable. In contrast, gastric fluid represented the principal barrier to probiotic survival. These findings highlight the importance of enhancing probiotic resilience to gastric conditions during formulation. However, the results should be interpreted within the context of the study limitations, including the use of porcine gastrointestinal fluids and the limited number of products and strains evaluated.

Author contributions

Mansa Fredua-Agyeman (Conceptualization, Formal Analysis, Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing), Simon Gaisford (Formal Analysis, Resources, Supervision, Writing – original draft, Writing – review & editing)

Conflicts of interest

The authors have no competing interests to declare relevant to the content of this article.

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None declared.

Data availability

The authors confirm that data supporting the findings of this study are available within the article. More detailed information can be obtained from the corresponding author..

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