Pediatric probiotic culture survival study in acidic pH using an in vitro model

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SUMMARY
Lactobacillus and Bifidobacterium are two genera of bacteria in probiotics that are known to possess significant immunomodulatory health-promoting properties. Probiotics are allowed to be used in foods and vitamins by the Food and Drug Administration (FDA) with little regulation as long as there are no claims to treat any disorder or condition. Regulatory agencies throughout the word classify and define probiotics differently with an uncertainty on the efficacy. The viability of bacterial strains influences probiotic stability and properties can be influenced by manufacturing and storage processes. It is imperative to also consider the viability of the probiotics after consumption. The aim of this study was to investigate the survival of the strains in the commercial probiotic Lovebug in acidic conditions modeling the human upper gastrointestinal (GI) tract in vitro. To test the ability of probiotics strains in the Lovebug probiotic to survive under acidic conditions, we incubated the probiotics in degassed acidified 0.8% sodium chloride at various pH levels for 2 h and measured the resulting colony forming units. Our study observed an overall survival of approximately 20–40 % after being incubated for 2 hours at pH 2–4. This supports that the bacterial genera of Lactobacillus and Bifidobacterium in the probiotic Lovebug would likely survive at a high enough rate in the human upper GI tract to provide benefit to the pediatric population.

INTRODUCTION
Previous studies have reported that gut microbiota play a role in preventing pathogen colonization, stimulating the production of gastrointestinal (GI) hormones, regulating brain behavior through production of neuroactive substances, and shaping our immune system (1-3). During neonatal and childhood development, different sites in the human body get colonized by microbial communities and the community composition varies at different sites, as well as in a healthy versus diseased state. The gut microbiome plays an important role in influencing human health as well as disease development starting in utero and extending into adolescence (4). It is mainly body habitats (i.e., skin, mouth, and gut) that determine the community composition (5). The microbiome in the human gut is influenced by evolutionary selection forces acting both at microbial cell and at host level. Microbial diversity is based on gut colonization ecological selection pressure, which is alike for mutualist as well as pathogens (6). Throughout the human body several genera and species from the following bacterial phyla predominate the microbiome composition: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (7, 8). The normal intestinal microbiota of various mammals consists of Bifidobacteria and Lactobacilli, and these genera are the best characterized and most widely used in commercialized probiotics (9). Bifidobacteria and Lactobacilli are non-sporing, gram-positive, lactic acid-producing bacteria (LAPB). Bifidobacteria are important producers of short-chain fatty acids (SCFA), whereas Lactobacilli have limited biosynthetic abilities and ferment refined sugars, generating lactic acid as the major end product (10, 11). Despite the fact that Lactobacilli and Bifidobacteria have some common properties they belong to two taxonomically distinct groups: the genus Lactobacillus belongs to phylum Firmicutes and the genus Bifidobacterium to phylum Actinobacteria (12).

Firmicutes and Bacteroidetes usually dominate the adult intestinal microbiota, whereas Actinobacteria, Proteobacteria, and Verrucomicrobia are considerably less abundant (9, 13). Studies have shown that the most common species found in healthy infants are Bifidobacterium infantis and Bifidobacterium breve (14). Even though Lactobacilli and Bifidobacteria are less dominant in adulthood, they remain stable elements of the normal intestinal microbiota and play an important role in diseases such as inflammatory bowel disease, irritable bowel syndrome, obesity, and allergic disorders (15). The role probiotics play in the GI tract is suggested to enhance intestinal barrier functions, stimulate immunity, and modulate inflammatory diseases (9). Probiotics are known to have bactericidal effects on pathogenic bacteria by restoring gut homeostasis and inhibiting pathogen and toxin adhesion to the intestinal epithelium (3).

The addition of probiotics to foods for infants and children for the treatment of acute gastroenteritis and the prevention of antibiotic-associated diarrhea and gastroenteritis is supported by the American Academy of Pediatrics (16). Antibiotic use has been linked to disruption of the gut microbiota (dysbiosis), even leading to low species diversity and taxonomic richness (17–19). Antimicrobial agents can cause a reduction in microbial diversity of intestinal and oral microbiota; subsequently, complete recovery of the initial bacterial community composition is rarely achieved (20, 21). Antimicrobial treatment has additional risks associated with it, including the selection of antibiotic-resistant strains of bacteria and the development of Clostridium difficile associated diarrhea (22). Due to dysbiosis with antibiotic treatment, the presence and expression of microbial genes
are altered (23). The impact on microbial diversity has a
detrimental impact and may lead to decreases in SCFA
production, vitamin production, and glycolysis, which may
impact protection against pathogens (24, 25). Compared to
antibiotics that can result in dysbiosis, probiotics are defined
as live microorganisms that, in adequate amounts, provide
health benefits to the host: supporting a healthy digestive
tract and a healthy immune system (26, 27). Normal intestinal
microbiota of various mammals contains *Bifidobacteria*
and *Lactobacillus*, which are well-characterized species and
widely used in commercialized probiotics (9). For example,
*Lactobacillus rhamnosus* GG (LGG; ATCC 53103) has shown
to be effective in controlling erythromycin-induced diarrhea
when administered in yogurt (28).

It is critical that probiotics are manufactured in a
reproducible manner, not only in terms of delivery but
protection technologies, too (29). The Food and Drug
Association (FDA) and European Food Safety Authority
(EFSA) have not approved probiotics for use in health claims.
As the probiotic market is increasing across the world, there
is a need further investigation for probiotic efficacy (3). There
are risks of using probiotics without consulting a physician
since there is no clinical evidence showing benefits in
immunocompromised patients with gut issues (30). Serious
infections with probiotic strains of *Lactobacillus* are very
uncommon, though *Lactobacillus rhamnosus* bacteremia
(presence in the blood) is an emerging clinical entity (30, 31).
To be effective the probiotics strains should be able survive
gastrointestinal digestive process. Probiotic study in healthy
children has demonstrated presence and survival of *L. casei*
for up to 3 days after consumption thus proving resistance
from gastric juices, hydrolytic enzymes and bile salts (32).
Administration of probiotics (non-living, usually fibrous,
compounds intended to "feed" the microbiota) can enhance
beneficial effects by enhancing metabolic activity and growth
of administered probiotics as well as the endogenous gut
*Bifidobacteria* and *Lactobacilli* (33).

In our study, the viability and survival of the pediatric
Lovebug probiotics with rich bacterial diversity containing
LGG, *Bifidobacterium infantis*, *Bifidobacterium lactis*,
*Lactobacillus reuteri*, *Bifidobacterium longum*, *Lactobacillus
casei*, *Lactobacillus gasseri*, and *Lactobacillus paracasei*
was investigated by mimicking the acid pH condition of
the human upper GI tract. The study was initiated from a pediatric
public health perspective to confirm the viability and survival
through gut-like conditions to confer the benefits in toddlers
as recommended by the manufacturers. Our study supported
our hypothesis of survival with lower viability of the strains at
acidic pH 2 than at pH 3-4.

**RESULTS**

To test the ability of probiotics strains in the Lovebug
probiotic to survive under acidic conditions, we incubated
the probiotics in degassed acidified 0.8% sodium chloride
at various pH levels for 2 hours and measured the resulting
Colony Forming Units (CFU) per 100 ml. These conditions
simulated the transit time of food and probiotics through the
infant gut. Sodium chloride was selected to prevent cell lysis,
while the pH was to cover the acidic range of the stomach (pH
2–4) and a neutral pH of 7 was the control.

Probiotic survival was determined by counting the colonies
after incubation. Two different colony morphologies were
observed on the plates, as expected, from a mixed culture
sample of *Bifidobacteria* and *Lactobacillus* species (Figure
1). Dilution plating on blood agar plates from 10⁻¹ through 10⁻⁴
had too many colonies to count or referred to as too numerous
to count (TNTC), but colony counts could be obtained on the
more dilute plates at pH 7, 4, and 3 (Table 1). At pH 7, 4, and
3, the colony counts were taken from 10⁻⁵–10⁻⁶-fold dilution
plates. Plates incubated at pH 2 had no live colonies after the
2 hr incubation on the 10⁻⁵ and 10⁻⁶ dilution plates. However,
the colonies could be counted on the 10⁻³ and 10⁻⁴ dilution plates
at pH 2 (Table 1). It was determined that the number of viable cells in the probiotic was 4.7x10⁶ CFU per dose,
based on growth after incubation in pH 7 for 0 h, which was
more than the manufacturer’s count of 1.5x10⁵ CFU per dose.
However, these figures are within the same order of magnitude
and demonstrates that the manufacturer’s claim that there are
at least 1.5x10⁶ CFU per dose is accurate. Approximately
20–40% of the initial bacteria strains in the probiotic survived
the 2-hr incubation at pH 7, 4, and 3 (Figure 2). We found
the pH of the environment had a substantial influence on the
survival rate of the bacteria in the probiotic, as can be seen
by the less than 10% survival when incubated in pH of 2 for 2
hours (Figure 2).

**DISCUSSION**

Our study demonstrated probiotic survival was 20–40%
at pH 3-4. Interestingly, incubating the probiotic at pH 7 also
seemed to impact the survival of the bacteria strains with
a little over 20% survival. Other studies have also reported
to be around 20–40% for selected strains of *Bifidobacteria*
and *Lactobacilli* (33). A pH 7 also seemed to impact the
survival strains, which could be attributed to the fact that
most *Lactobacillus* strains are acidophilic or aciduric in nature
(34). *Bifidobacteria* sampling from the cecum in humans have
shown that, when probiotics are given in fermented milk, they
had a survival of 23.5% ±10.4% of the administered dose
(35). While other studies have shown that *B. bifidum* and *L.

Figure 1: Representative FAA Plate. Colonies observed after
Lovebug Toddler Probiotic was treated with 0.9% sodium chloride
spread plated and incubated for 48 hrs at 37°C under anaerobic
conditions are shown.
acidophilus delivery to the cecum was 30% and 10% of the administered dose, respectively (36). This study supports our hypothesis that the viability of the bacteria strains is lower in acidic environments with a pH 2 than at neutral pH 7. The beneficial effects of probiotics in influencing intestinal ecosystems support their survival capabilities in the gut (1). Our results, along with the customer reviews for lovebug, also support the hypothesis that Lactobacillus and Bifidobacterial strains survived the gut environment after ingestion. The details on the species-level survival of LGG, B. infantis, B. lactis, L. reuteri, B. longum, L. casei, L. gasseri, and L. paracasei were beyond the scope of this study. The study demonstrates that bacteria in the Lovebug probiotic survive in acidic conditions like the gut environment, which could account for Lovebug probiotics’ positive customer reviews about its desired effect (Appendix 1). While our experiment does show some of the bacteria survive acidic conditions, there is a reduction in percent survival that supports our hypothesis. The bacteria survival at pH 7 was also low, and this could be attributed to acidophilic nature of the bacteria instead of being neutrophilic. Static experiments have shown that Bifidobacterium spp. and L. acidophilus are more acid-resistant than are L. bulgaricus and S. thermophilus (37). This could be attributed to the fact that Lactobacillus strains, which constitute the majority of strains in the probiotic, are acidophilic or aciduric in nature and prefer acidic environment (34). Our study demonstrated total survival rates comparable with what has been observed previously. The experiment could be repeated at pH 5 and 6 to further support the observation that pH impacts bacterial survival. Additional time intervals could be added to the experiment to generate a more fine-scale timeline of survivability for the strains in this toddler probiotic. The main obstacle to survival of the strains is gastric acidity. Viability depended on the pH, length of the exposure to acid, and bacteria species and strain. Probiotic survival in the small intestine is impacted by presence of bile salts, which are known to cause cell lysis (33). However, for the purpose of this study we focused on pH. Additionally, testing different concentrations of bile acid could be done to assess the impact of bile acids on infant probiotics.

Our study took into consideration gastric passage time, and hence, a 2-hour incubation time was selected. In the absence of exposure to bile, our study aligns with the results of the low bile with 30–40% survival where delivery of B. bifidum and L. acidophilus to the cecum was 20% and 10%, respectively. However, it has been shown that in the presence of physiologic bile salt concentrations that can hydrolyse bile the delivery percentages were 50% and 30%, respectively.
(33, 36). Lactobacillus and Bifidobacterium long-term gut colonization potential have shown that some strains pass through, but others colonize the gut permanently. Studies have shown that strains that stably engraft in the gut exert beneficial effects on the host by increasing the efficiency of metabolic activity. Lactobacillus and Bifidobacterium species, are likely to stably colonize in the gut, based on their history. B. longum appears to be an exemplary species with long-term colonization potential. More studies are needed to select or tailor probiotic strains with long-term gut colonization ability in a rational manner however Lactobacillus species (L. rhamnosus) have proven to stably flourish in the gut (38). Both B. longum and L. rhamnosus were in the Lovebug probiotic. Our study observed the presence and viability of Lactobacillus and Bifidobacterium in the Lovebug probiotic based on two different morphologies. However, based on the colony morphology on the plate the species survival cannot be determined. Studies to identify the survival of difference species could also be carried out using 16S rRNA sequencing. Using this technique Yang et al. have demonstrated the abundances of the phyla in the gut (13).

We demonstrated that pH reduced the percentage of surviving bacteria, however future studies should address methods to increase survivability. Strain selection has generally been based on in vitro tolerance of physiologically relevant stresses (e.g., low pH, elevated osmolarity and bile) (39). Physiologically stresses like low pH, elevated osmolarity, and bile have been used in vitro to select tolerant strains. Selection of optimal culture medium and cell protectants is also crucial to ensure the efficacy of the probiotic product. Microencapsulation can protect probiotic bacteria and has been proposed to improve the stability of the strains which can adapt to the GIT conditions (40). It is critical to ensure optimal culture medium and cell protectants for the efficacy of probiotic product. Microencapsulation also protects probiotic strains and can improve the stability in the gut environment.

**MATERIAL AND METHODS**

**Probiotic**

Lovebug Toddler Probiotics for ages 12 months to 4 years containing 15 billion CFU of L. rhamnosus GG, B. infantis, B. lactis, L. reuteri, B. longum, L. casei, L. gasseri, and L. paracasei were purchased from Amazon (Appendix 1). Fastidious anaerobic agar (FAA) (Lansing, MI, USA) supplemented with 5% sheep’s blood was used for cultivation. Degassed 0.9% sodium chloride was used for resuspension of the probiotic and pH was adjusted using HCl. Tryptic soy broth (Millipore Sigma, Burlington, MA, USA) was used for the serial dilutions.

**Survival Assay and Colony Counting**

In an anaerobic chamber (AS-580, Anaerobe Systems, Morgan Hill, CA, USA) under anaerobic conditions (10% H2, 10% CO2, balanced with N2), one sachet (1.5 g) of Lovebug Toddler Probiotic was added to 100 mL of degassed acidified 0.9% sodium chloride at various pH values (2, 3, 4, 7). The suspension was incubated at 37°C for 2 hrs, and the control was at pH 7 incubated for 0 hr. Serial dilutions from 10–1 to 10–10 were made in tryptic soy broth, and 0.1 mL of each dilution was spread/ plated on FAA supplemented with 5% sheep’s blood. FAA plates were then incubated at 37°C for 48 hours until colonies were visible and conducive to counting. Plates with CFU counts between 30–300 colonies were counted. The plating was done in triplicates and averaged for the final count.

**Data Analysis**

Colony counts were used to calculate the CFU/mL present before and after treatment with acidified 0.9% sodium chloride at various pH values (2, 3, 4, 7). The recorded number of colonies was multiplied by the dilution factor and divided by the volume plated. The CFU/100 mL was calculated, then the average across a given treatment was calculated. Graph was plotted as percent survival with the Control pH 7, 0 hr as 100%.

**APPENDIX**

Source of Lovebug probiotic: www.amazon.com/dp/B01HLSK5NA/ref=nb_sb_ss_w_as-reorder-t1_ypp_rep_k0_1_7&amp&crid=1LHWA1R2UTEAT&amp&sprefix=lovebug

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**REFERENCES**


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