Journal of Student Research (2017) Volume 6, Issue 2: pp, 14-20
Research Article

Phenotypic Effects of Probiotics on *Xenopus Laevis* Development

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Probiotics supplementation, such as *Lactobacillus acidophilus* and *Bifidobacteria*, has become increasingly popular as a means to naturally treat minor ailments and attain overall better health. This research sought to determine whether probiotics *L. acidophilus* and *Bifidobacteria* as well as a combination (*Lactobacilli* and *Bifidobacteria*) had an impact on the development of *Xenopus laevis* (African clawed frog) embryos. We used several control and experimental groups per probiotic species. For experimental groups, each probiotic species (or combination) was isolated and dissolved in dechlorinated distilled water to create a 0.0025% and 0.025% solution. Embryos were measured/observed to determine presence of abnormalities; collect intraocular, gut, and head measurements; and examine behavior. After organogenesis, we euthanized embryos to examine the presence of specific species of bacteria using EnteroPluri tubes. All groups showed similar mortality, morphology, and activity ($P > 0.05$), except for those treated with *Bifidobacteria* ($P \leq 0.05$), and in all instances, activity and stage of development were positively correlated ($r = 0.38$ to 0.69). Gut bacterial composition was similar between *Bifidobacteria* and control groups, but gut compositions were different among *L. acidophilus* and combination (*Lactobacilli and Bifidobacteria)* groups. All data combine to indicate that *Bifidobacteria* should be limited or avoided as it contributes to smaller overall embryo size and higher activity levels, while *L. acidophilus* consumed at a 0.0025% concentration (recommended dosage) is the best option as a probiotic supplement as it provides the benefits associated with this probiotic without adversely impacting the embryo. We suggest additional research to examine the positive influence of *L. acidophilus*, as these data suggest its usefulness as a probiotic.

Keywords: Probiotics; African clawed frog; Xenopus laevis; Lactobacillus acidophilus; Lactobacilli; Bifidobacterium

Introduction

The human gut is not only a breeding ground for bacteria but also plays a role in the maintenance of overall health. Different strains of healthy bacteria, termed "probiotics," inhabit the gut in enormous numbers, establishing a symbiotic relationship with the host. In particular, bacteria from the genera *Lactobacillus* and *Bifidobacterium*, among others, stand out as important contributors to the microbial activity in the intestines [1]. The World Health Organization (WHO) formally defined probiotics in 2010 as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1]. Although probiotics are healthy and safe to consume, studies are still examining the magnitude of the effects that these microorganisms have on specific ailments. Doctors cannot yet recommend the consumption of a particular probiotic as a treatment or preventative measure for a specific illness due to the lack of data on its effects on particular ailments. Therefore, a host of studies recently examined such in order to establish a more reliable basis for making specific recommendations.

Most probiotics reside in the gastrointestinal (GI) tract, so their presence has a direct impact on gut health. As such, studies extensively examined their effects on GI disorders with the most conclusive results [2]. A number of probiotics, but most potently a mix of *Lactobacillus* and *Bifidobacterium* strains, could drastically reverse irritable bowel syndrome (IBS) symptoms [3], and inflammatory bowel disease (IBD) [4]. Probiotic consumption could shift the gut microbiota imbalance towards the healthy bacteria's side, and eliminate the production of an excess of pro-inflammatory molecules. Further, probiotic supplementation can initiate the remission of ulcerative colitis; however, the same is not true of Crohn's disease, a similar disorder [5].

In addition to direct impact to gut health, probiotic consumption may have a positive effect on both mental and neural health due to the gut-brain axis. The gut-brain axis provides a connection between gut bacteria and both the activity and health of the brain. Anatomically, this axis consists of between 200 and 600 million neurons that connect to the GI tract [6]. Data indicate that gut microbiota affect the central nervous system (CNS) development [6] as well as behavior in animals [7]. Specifically, it plays a major role in the development of the enteric nervous system (ENS) [7], thus, linked to the GI tract function. The interconnection between gut bacteria and the ENS allow germ-free mice exposed to normal gut microbiota to normalize density and activity of enteric neurons [7].

There is a possible link between gut bacteria content and depression, where probiotic consumption helps improve depression scores [8]. This may relate to how the gut-brain axis affects immune system activation, stimulation of the vagus nerve, and synthesis of metabolites [9]. Both *Lactobacillus* and *Bifidobacterium* genera have been shown to possess the ability to restore the hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine component of the body that, when experiencing interference, can lead to mood disorder development [10]. For example, *Lactobacillus rhamnosus* directly affects neurochemistry via the vagus nerve [11], while *Bifidobacterium longum* reverses anxiety and stabilizes neurotrophic factors in mice suffering from infectious colitis [12]. The CNS and gut microbiota are directly related, making further research in this realm promising.

Data support probiotic supplementation for use in GI tract ailments, but lack of substantial data precludes their use on diseases outside of the GI tract [2]. Some studies have set out to discover the effects of probiotics on a developing embryo or fetus when the pregnant mother uses them as a supplement. Dotterude et al. (2010) found that probiotic supplementation during the perinatal period lowers the incidence of atopic dermatitis by 40% in children until they reach age two [14]. Ent et al. (2014) concluded that infant eczema symptoms can be avoided or lessened by a year-long perinatal maternal

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probiotic supplementation program [15]. However, Brede et al. (2015) found that maternal probiotic supplementation administered directly after birth in breast-feeding mothers does not provide a measureable positive effect in infants [16]. No studies examine the effects of maternal probiotic supplementation on human embryos, and few studies examine the impacts to embryos of model organisms [17].

Studies that examine embryonic development may provide evidence to recommend probiotics to pregnant mothers. Knowledge in this area is crucial, as the presence of a teratogen during prenatal development can alter vitality of the embryo. Therefore, this research determines whether certain species of probiotics, as well as a combination of probiotics, have an impact on the development of *Xenopus laevis* embryos, providing possible implications for human development. *X. laevis*, the African clawed frog, is a model organism widely used to answer fundamental questions in biology and medicine [18], largely because it is easily manipulated in the lab and relatively inexpensive. *X. laevis* is a bioindicator—an organism that, by virtue of its existence, can provide insight on ecological health (whether good or bad) given exposure to its environment. Applications for *X. laevis* as a model system are vast, including nervous system development [19, 20]. Thus, *X. laevis* serves as a model system in this project to obtain data on whether probiotics (*L. acidophilus* and *Bifidobacteria* as well as a combination [*Lactobacilli* and *Bifidobacteria*]) affect morphological development, activity, size, and gut microbiota of the embryos. We expect that there will be a positive effect on morphological growth, activity, and size in growing embryos exposed to probiotics, and species richness of gut microbiota will be higher in developing embryos exposed to probiotics.

Experimental Procedures

Experimental Design-- *Xenopus laevis* frogs were bred by injecting the female with human chorionic gonadotropin (hCG) and then placing a male and female together in the same tank. All frogs were maintained in tanks with dechlorinated water maintained at 20 ◦C and light/dark patterns consistent with normal day and night hours. Within 24 hours of when the female dropped her eggs, approximately 140 unhatched eggs were collected. Each of 14 glass dishes were filled with 200 mL of the respective solution, and 10 unhatched eggs were randomly placed in each dish. Two dishes served as controls, while the remaining 12 dishes served as experimental groups (see experimental groups below).

Different brands of probiotics were selected for their high content of the desired probiotic species. Spring Valley Acidophilus Probiotic, containing active *Lactobacillus acidophilus* cultures, and Equaline 4x Probiotic, containing four species of active *Bifidobacteria* cultures (*B. bifidum, B. infantis, B. lactis,* and *B. longum*), were selected to create *Lactobacillus acidophilus* and *Bifidobacteria* experimental groups, respectively. Nature's Way Fortify Daily, fortified with *Lactobacilli* and *Bifidobacteria* cultures, was selected to create the combination experimental group.

Probiotic concentrations were created in an aquatic environment for developing *Xenopus laevis* (African clawed frog) embryos. The aquatic environment contained 20 mL of water fortified with the appropriate concentrations of probiotic. Probiotic concentrations were determined by using daily intakes and colony-forming units (CFUs). Two concentrations of each probiotic were utilized to create experimental groups. The first concentration mimicked the recommended daily intake for a 135-pound female based on daily dosages (or 5 to 10 billion CFUs), yielding a 0.0025% solution or an aquatic environment with 125,000 to 250,000 CFUs for *Xenopus* embryos. The second concentration increased daily intake an order of a magnitude to create a daily dosage higher than recommend daily intakes, yielding a 0.025% solution or an aquatic environment with 1.25 to 2.5 million CFUs for *Xenopus* embryos. Two independent trials were conducted simultaneously (Trial 1 and Trial 2). There were two replicates of each treatment and two replicates of each control; each replicate contained 10 tadpoles (Appendix).

Experimental Groups 1a and 1b: *Lactobacillus acidophilus at 0.0025%-- L. acidophilus* consumed at its recommended dosage was represented by dissolving Spring Valley Acidophilus Probiotic in distilled, dechlorinated water to create a 0.0025% solution (0.5 uL probiotic added to 200 mL water). The 10 tadpoles were then placed in the solution. This was repeated for the replicate experimental group (Experimental Group 1b), exposing 20 tadpoles total between the two experimental groups.

Experimental Groups 2a and 2b: *Lactobacillus acidophilus at 0.025%--L. acidophilus* consumed at a higher dosage than recommended was represented by dissolving Spring Valley Acidophilus Probiotic in distilled, dechlorinated water to create a 0.025% solution (5 uL probiotic added to 200 mL water). The 10 tadpoles were then placed in the solution. This was repeated for the replicate experimental group (Experimental Group 2b), exposing 20 tadpoles total between the two experimental groups.

Experimental Groups 3a and 3b*: Bifidobacteria at 0.0025%--B. bifidum, B. infantis, B. longum, and B. lactis* consumed at its recommended dosage was represented by dissolving Equaline 4x Probiotic in distilled, dechlorinated water to create a 0.0025% solution (0.5 uL probiotic to 200 mL water). The 10 tadpoles were then placed in the solution. This was repeated for the replicate experimental group (Experimental Group 3b), exposing 20 tadpoles total between the two experimental groups.

Experimental Groups 4a and 4b: *Bifidobacteria at 0.025%-- B. bifidum, B. infantis, B. longum, and B. lactis* consumed at a higher dosage than recommended was represented by dissolving Equaline 4x Probiotic in distilled, dechlorinated water to create a 0.025% solution (5 uL probiotic to 200 mL water). The 10 tadpoles were then placed in the solution. This was repeated for the replicate experimental group (Experimental Group 4b), exposing 20 tadpoles total between the two experimental groups.

Experimental Groups 5a and 5b: *Combo at 0.0025%--* A combination of *Lactobacilli* and *Bifidobacteria* in an 8:7 ratio, respectively, consumed at its recommended dosage was represented by dissolving Nature's Way Fortify Daily in distilled, dechlorinated water to create a 0.0025% solution (0.5 uL probiotic to 200 mL water). The 10 tadpoles were then placed in the solution. This was repeated for the replicate experimental group (Experimental Group 5b), exposing 20 tadpoles total between the two experimental groups.

Experimental Groups 6a and 6b: *Combo at 0.025%--* A combination of *Lactobacilli* and *Bifidobacteria* in an 8:7 ratio, respectively, consumed at its recommended higher dosage was represented by dissolving Nature's Way Fortify in distilled,

dechlorinated water to create a 0.025% solution (5 uL probiotic to 200 mL water). The 10 tadpoles were then placed in the solution. This was repeated for the replicate experimental group (Experimental Group 6b), exposing 20 tadpoles total between the two experimental groups.

Control Groups 0a and 0b-- Two control dishes were used. Each dish contained 10 tadpoles in 200 mL of distilled, dechlorinated water with nothing added (20 tadpoles total served as controls).

Data Sources-- Tadpoles were observed each day for 29 days, and pictures were taken daily of at least one tadpole in each dish. Morphological abnormalities and stage number were noted based upon the pictures taken. Stage number was assigned according to *eNasco X. laevis* embryological stages. Measurements were taken of intraocular distance, head length, and gut width. Activity levels were monitored by rating speed of activity on a scale of 0 to 3 (0 was stationary/inactive, 1 was slow, 2 was average, and 3 was fast). At the end of the 29 days, all numerical data were statistically analyzed.

All animals were handled according to standard care and handling protocol designed by *eNasco*, a company built around breeding, caring, and handling *X. laevis*. Welfare and health of the organisms was of upmost importance, and all rules and regulations related to Animal Care and Use Board were followed. The Northern State University (NSU) Institutional Research Board (IRB) was consulted/contacted prior to animal care and handling to ensure proper protocol was followed. Tadpoles at stage 51 (day 29) were euthanized (using *eNasco* standard care and handling protocol) and necropsied; swabs (1 to 2 swabs) of the stomach were taken and inoculated onto EnteroPluri tubes. These tubes were incubated for two days and analyzed using EnteroPluri standard color charts to identify gastrointestinal bacteria. EnteroPluri tubes test for opportunistic pathogenic bacteria only, so the goal was to determine if the positive bacteria (probiotics) would outcompete pathogenic bacteria. Data from experimental groups were compared to the control.

Data Analyses –Descriptive statistics along with comparisons of means of control to experimental groups (separate and combined) via ANOVA and post-hoc Student's t statistical tests were conducted using JMP software (SAS Institute Inc.). During statistical analysis, data for both dishes per probiotic/concentration group (i.e., experimental group) were combined (e.g., 1a and 1b), and each experimental group was compared separately to the control group. Further, correlation tests between stage and activity level among control and experimental groups were completed. p-values less than or equal to 0.05 were considered statistically significant

Results

After the 29-day trial, 13 out of the 14 dishes still contained at least one live tadpole at Stage 51. Tadpoles present in all 12 experimental dishes displayed normal morphology.

Stage of development did not differ statistically among control and experimental groups, thus there was no impact to overall embryonic growth by exposure/non-exposure to probiotic bacteria. However, specific measurement and activity data showed disparity among groups, particularly between the control and *Bifidobacteria* (0.5 uL) or 0.0025% concentration, where *Bifidobacteria* is smaller and more active. ANOVA and Student's t test indicate that *Bifidobacteria* (0.5 uL) or 0.0025% concentration was statistically different ($P \leq 0.05$) from the control when comparing all measurement data, *i.e.*, intraocular width, gut width, and head length (Figures 1-3). In all these measurements, *Bifidobacteria* was smaller than the control, particularly among the 0.5uL (or 0.0025%) experimental group. Intraocular width showed the greatest differences among measurement data between these groups with *L. acidophilus* (5 uL) or 0.025% concentration and Combo (5 uL) or 0.025% concentration statistically different than the control ($P \leq 0.05$; Figure 1), with larger than expected intraocular distances.

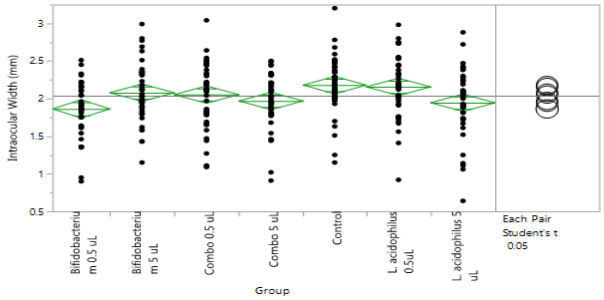

Figure 1. ANOVA (right) and Student's t (left) statistical tests comparing experimental to control groups as it relates to intraocular distance. Both tests indicate statistical difference between control and *Bifidobacertia* (0.5 uL) or 0.0025% concentration, *L. acidophilus* (5 uL) or 0.025% concentration and Combo (5 uL) or 0.025% concentration ($p \leq 0.05$). Data for each individual trial were combined in the graph for ease of understanding/overall comparison.

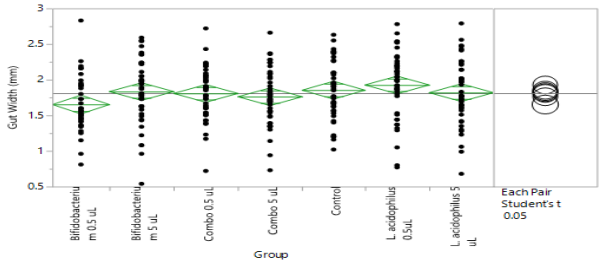

Figure 2. ANOVA (right) and Student's t (left) statistical tests comparing experimental to control groups as it relates to gut width. Both tests indicate statistical difference between control and *Bifidobacteria* (0.5 uL) or 0.0025% concentration ($p \leq 0.05$). Data for each individual trial were combined in the graph for ease of understanding/overall comparison.

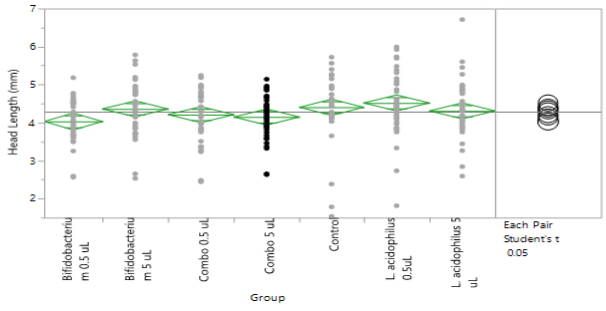

Figure 3. ANOVA (right) and Student's t (left) statistical tests comparing experimental to control groups as it relates to head length. Both tests indicate statistical difference between control and *Bifidobacteria* (0.5 uL) or 0.0025% concentration ($p \leq 0.05$). Data for each individual trial were combined in the graph for ease of understanding/overall comparison.

Activity varied among control and experimental groups, where embryos exposed to probiotics showed more or less activity depending on the probiotic. Data analyses indicate four experimental groups were statistically different from the control ($P \leq 0.05$; Figure 4). *Bifidobacteria* (0.5 uL) or 0.0025% concentration and *L. acidophilus* (5 uL) or 0.025% concentration were more active, while Combo (0.5 uL) or 0.0025% concentration and Combo (5 uL) or 0.025% concentration were less active than the control.

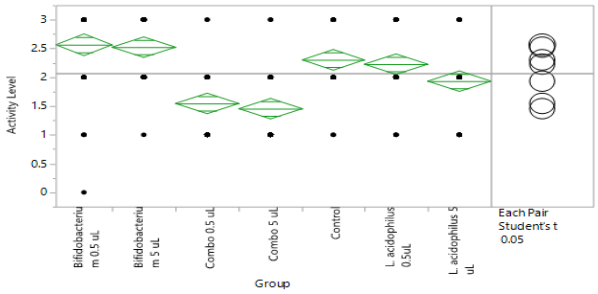

Figure 4. ANOVA (right) and Student's t (left) statistical tests comparing experimental to control groups as it relates to activity levels. Both tests indicate statistical difference between control and *Bifidobacteria* (0.5 uL) or 0.0025% concentration, *L. acidophilus* (5 uL) or 0.025% concentration, Combo (0.5 uL) or 0.0025% concentration, and Combo (5 uL) or 0.025% concentration (p-value ≤ 0.05). Data for each individual trial were combined in the graph for ease of understanding/overall comparison.

To add to activity data, both Figures 5 and 6 are pictures taken within minutes of one another on Day 17. In comparing these figures, we found no major morphological differences. However, correlation data between stage (or size) and activity range from $r = 0.38$ to $r = 0.69$ as size increases, activity increases. According to activity data, the *Bifidobacteria* at 0.0025% group of tadpoles (Figure 5) was substantially faster (i.e., more active) than Combo at 0.025% (Figure 6) on this particular day—and over the 29 days.

Figure 5. *Bifidobacteria* (0.05 uL) or 0.0025% concentration at Stage 47 on Day 17. Tadpoles from this group exhibited very fast activity (activity level ranked 3 on our scale).

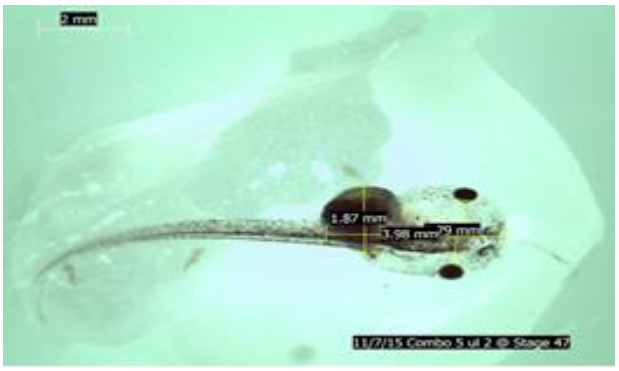

Figure 6. Combo (5uL) or 0.025% concentration at Stage 47 on Day 17. Tadpoles were relatively slow moving (activity level ranked 1 on our scale).

We detected five species of opportunistic pathologic bacteria in the experimental and control (Table 1) groups. We screened for bacteria rather than used a more comprehensive metagenomics analyses as our data are preliminary. Present in all groups, except for the experimental group 0.025% *L. acidophilus*, was one bacterium species (*Pantoa agglomerans*), a common enterobacteria. Of interest, the probiotics did not significantly out-compete the opportunist pathogenic bacteria.

Table 1: Showing species of pathogenic bacteria found, with pathogenic bacteria found in every tadpole tested. Data for each individual trial were combined in the graph for ease of understanding/overall comparison.

Probiotic/Concentration	*Pantoea agglomerans*	*Citrobacter freundii*	*Cedecea neteri*	*Cedecea lapagei*	*Cedecea sp. 3*
Control	x				
L. acidophilus 0.5 ul	x	x			
L. acidophilus 5 ul			x		
Bifidobacteria 0.5 ul	x				
Bifidobacteria 5 ul	x				
Combo 0.5 ul	x	x			
Combo 5 ul				x	x

Discussion

Probiotics supplementation has become increasingly popular as a means to treat minor ailments and promote better health. *Lactobacillus acidophilus* and *Bifidobacteria* are common probiotics used for these purposes. Preliminary data seem to indicate that *L. acidophilus* is better than *Bifidobacteria* for probiotic use to enhance amphibian development.

Xenopus laevis (African clawed frog) embryos were used as a model organism for this experiment. *X. laevis* is an excellent model organism for embryonic and bacterial studies because their embryos develop in an aqueous environment. Early on in development, *Xenopus* embryos are exposed to a variety of microbes, making them especially responsive to the presence of microbes such as probiotics [21]. Skin microbiota depends on a species' habitat, thus exposure to bacteria in an aqueous environment affects the bacteria that live on or near the surface of the frog's skin [21]. Additionally, *Xenopus* develops a gut mucosal immune system that is similar to that of a human, with symbiosis occurring between the healthy bacteria located in the gut and the frog itself. In the absence of an ability to conduct a mammalian study, these organisms served as the best model organism to examine the impacts of probiotics on embryonic development with the hope to obtain results similar to that in a human.

Bifidobacteria at 0.0025% yielded inhibited frog embryo growth, which was the only group that showed a significant statistical difference for all three measured size parameters. A smaller embryo size implies a lower birth weight; lower birth weight can be detrimental to the vitality of an organism. In humans, there is a correlation between low birth weight and incidence of diabetes and obesity later in life [22]. Low birth weight causes higher leptin levels and a higher leptin-to-fat mass ratio [22]. Additionally, low birth weight men showed a high rate of cardiovascular disease, ultimately leading to premature death [23]. In women, low birth weight is associated with higher incidences of mortality at any age [23]. In light of these studies, the small size of frog embryos treated with *Bifidobacteria* at 0.0025% proves concerning. These results could suggest that *Bifidobacteria* at 0.0025% could have an effect on birth weight in mammals, perhaps even humans. If this is the case, expectant mothers should avoid the use *Bifidobacteria* during pregnancy. However, data are preliminary and require more research to understand if and/or how this probiotic may interact with genes involved in embryo size.

Intraocular distance was a size parameter measured to analyze overall embryo size and potentially brain size. Results indicate that the intraocular distance was shorter for *Bifidobacteria* at 0.0025%, *L. acidophilus* at 0.025%, and Combo at 0.025%, but average activity levels for *L. acidophilus* at 0.025%, Combo at 0.0025%, and Combo at 0.025% were all significantly lower than that of the control. *Bifidobacteria* at 0.0025% was the only group with significantly higher activity levels than the control. Intraocular distance in concert with activity levels may indicate a neurological difference between control and experimental groups, particularly in *Bifidobacteria*, which is not easily visible in photos. Perhaps synaptic connections or other neuronal changes vary between groups thereby forbidding the use of distance data as a sole measurement of brain development. Regardless, *Bifidobacteria* at 0.0025% appears to show too much activity, potentially affecting overall brain size.

Activity levels seem to support the newfound phenomenon known as the gut-brain axis in amphibians. Bienenstock et al. (2016) noted that while gut microbiota does indeed affect nervous system development, especially the ENS, much is left to be discovered [6]. We may conceptualize gut-brain axis development via activity levels displayed by *Bifidobacteria*, where greater activity may indicate greater impact to neurological development. Altering the gut microbiota via antibiotic treatment can affect the amount of glial cells present in the intestinal mucosa [24]. Perhaps, this connection between the gut microbiota and glial cells may help illuminate how *Bifidobacteria* accelerates the development of the amphibious nervous system. Higher activity levels in organisms exposed to *Bifidobacteria* may indicate that *Bifidobacteria* is essential for maintaining or even increasing this number of glial cells, which contribute to neural upkeep. Since gut bacteria may use serotonin, GABA, histamine, noradrenaline, and adrenaline to interact with the gut-brain axis [25, 26]**,** *Bifidobacteria* may increase use of adrenaline for communication and thereby increase activity levels. However, *Bifidobacteria's* apparent ability to quicken the development of the nervous system and gut-brain axis may be the reason for its negative impact on amphibian embryo size. In these embryos, the development of the nervous system uses energy via the gut-brain axis rather than using it for growth. Additionally, developing calves fed *Bacillus subtilis* exhibited a fraction of the amount of bacteria from the family *Bifidobacteriaceae* as well as an increased amount of microbes from *Lactobacillaceae* compared to the control. As well, the average daily increase in body mass of the experimental groups of calves, with low amount of ruminal *Bifidobacteriaceae*, was significantly higher ($p \leq 0.05$) than that of the control [27]. When correlated with our results, this may be due to the negative effect of *Bifidobacteria* on size. Due to the negative effects low birth weight [22, 23], this is not a beneficial trade-off for the embryo's future vitality. If these results correlate with future studies done on mammalian species and ultimately humans, *Bifidobacteria* would not be a good choice as a probiotic supplement during pregnancy.

L. acidophilus consumed at a 0.0025% concentration (recommended dosage) seems to provide an excellent option for positively enhancing amphibian development. Although *L. acidophilus* at a 0.025% concentration (10 times the recommended dosage) had a statistically significantly shorter intraocular distance and lower activity levels, *L. acidophilus* at 0.0025% was statistically quite similar to the control in both morphological and activity levels. The *Lactobacillus* genus provides numerous undetectable benefits to the several host species. For example, *L. rhamnosus* treatment improved lipid metabolism in zebrafish by via inducing the development of longer microvilli and enterocytes [28]. Further, administration of probiotic created healthy GI tract via developing intricate architecture and increasing surface area [29]. Such internal effects may be present in the embryos in the present study; however, we did not examine the lumen of the GI tract. Future studies in this realm could prove promising in understanding how *Lactobacilli* affect development and whether *L. acidophilus* produces the same increase in intestinal absorptive surface area as *L. rhamnosus. L. rhamnosus* treatment may also be key in modulating glucose in the blood using

transcriptional control via up-regulation of genes involved in reduced glucose levels during zebrafish development [29]. *Leptin* gene expression, which is involved in feelings of satiety, appetite control, and energy homeostasis, was significantly increased by probiotics supplementation in all examined zebrafish developmental stages [29]. *L. acidophilus* specifically helps fortify the intestinal barrier [30]. Interestingly, *Lactobacilli* can convert glutamic acid to GABA [31, 32, 33], an inhibitory neurotransmitter in the CNS that could reduce pain [34]. Our results may suggest that an expectant mother could safely use *L. acidophilus* at normal daily dosages during pregnancy, minimizing impacts to embryonic development. Examining our data in concert with the above studies, *L. acidophilus* could even imbue positive effects on developing embryos in manners not observable in this study.

The gut bacteria data showed a variety of different species of opportunistic pathogenic bacteria, demonstrating that opportunistically pathogenic bacteria remain in the gut of amphibians regardless of the probiotic. Only *Pantoea agglomerans* was present in the control and *Bifidobacteria* at 0.0025% and 0.025% concentrations. Every other group and/or concentration had one or more other opportunistic pathogenic bacteria present. Gut microflora are a complex mixture of microorganisms that thrive in animal digestive tracts via a mutualistic relationship. All bacteria found from this screening are common to the GI tract, in small numbers, which was expected. *Bifidobacteria*, however, showed levels of species richness similar to the control. *Bifidobacteria* is a representative of one of the most common genera comprising the gut microflora [3]. As such, *Bifidobacteria*'s role likely has developed in response to the complexity of microorganisms and interactions within the gut thereby developing the ability to outcompete many opportunistic pathogens. Few pathogenic species likely persist in its presence.

As mentioned earlier, our results must be tested on a mammalian model before being applied to humans. Although the similarity between *Xenopus* and mammals in gut microbiota makes *Xenopus* an excellent option for a model organism in the absence of an ability to use mammals [20], several glaring characteristics differ between *Xenopus* development and mammalian development—and, even more so, human development. First, amphibians undergo external fertilization while mammals undergo internal fertilization, which changes how the developing embryo is exposed to bacteria. In amphibians, this exposure is more direct, making amphibians an easier model organism to utilize for bacterial studies [20]; however, this direct exposure could amplify the effects of the bacteria on the organism. In placental mammals, such as humans, bacterial exposure must occur via the placenta, providing a barrier that could absorb some of the effects of the bacteria. Second, amphibians are an r species, producing a large amount of offspring with little parental care, while humans are a k species, producing usually only one offspring at a time paired with immense parental investment. This difference could also alter the effects of probiotics on embryo development. Due to these two differences in addition to others, further studies on mammals and eventually humans are essential before making concrete recommendations for human maternal probiotic supplementation.

Ultimately, this study provides an excellent baseline on the effects of probiotics on developing frog embryos that might correlate to human fetal development during the prenatal period. However, the limitations of this study make further studies crucial. Data presented herein provide a preliminary lens into the effects of these specific probiotics species on development, but more research needs to be conducted to confirm these results and understand how probiotics interact with genes during development.

Acknowledgements

A sincere thank you to the NSU Math and Science Department for the resource assistance, including necessary funding and supplies. And, a special thanks to the NSU Honors Program for the guidance and mentorship throughout this research.

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