Honorable Mention*, The Navigator's *Best Writing Award

The Effect of Dietary Probiotics on Tight Junction Gene Expression in *Danio rerio*

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Abstract

Probiotics are microbes found within the diet which partake in positive interactions with their host via the digestive tract (Roberfroid, 2000). Recent research on probiotics has shown that they induce improvement to the intestinal tight junctions by bolstering the commensal bacteria of the colon (Karczewski et al., 2010; Maldonado-Galdeano et al., 2019). Probiotic research of this nature often sees results through administration of probiotics throughout a period of prolonged stress or disease state, but with the recent popularization of probiotic supplements as a health trend, more research is needed to understand the effects of probiotics on a healthy gut. Male zebrafish were fed a diet supplemented with *Lactobacillus plantarum* for a period of eight weeks. Gene expression levels of *claudin-12* and *occludin* were then analyzed by PCR. Results indicated that consumption of probiotics in a non-disease state does not alter the expression of *occludin* and *claudin-12* with any statistical significance, suggesting that dietary probiotics only exhibit a direct influence on intestinal tight junctions during a disease state.

Introduction

Intestinal Function and Probiotics

The alimentary canal represents the barrier between the external environment and the body's vital metabolic and physiological functions. The lining of the digestive tract is structured to serve as a semipermeable veil between the external environment of the canal's lumen and the internal metabolic and physiological pathways of the body. This selective permeability prevents unwanted substances, such as disease-causing pathogens, from entering the bloodstream while allowing for the absorption of beneficial nutrients. The discrimination between which substances are absorbed makes the gut, and subsequently the diet, a targeted medium for regulating overall health through alteration of gut permeability. Commensal bacteria present on the epithelial lining of the colon significantly impact which materials are permitted to cross the intestinal walls (Buffie & Pamer, 2013). These commensal bacteria are often referred to collectively as the gut microbiome or microflora. It has been hypothesized that altering the population size or diversity of the gut microbiome can impact overall health by regulating the permeability of the intestines (Buffie & Pamer, 2013; Ohland & MacNaughton, 2010).

Probiotics are living microbes found in the diet which maintain positive interactions with their host via the digestive tract (Roberfroid, 2000). Numerous strains of probiotic bacteria have been identified as beneficial to human health, with the two most common groups being *Bifidobacterium* and *Lactobacillus* (Gibson and Roberfroid, 1995). The *Lactobacillus* family in particular is often included in dietary probiotic supplements because it has been shown to alter the intestinal environment,

promote the proliferation of existing gut bacteria, and increase the production of intestinal enzymes (Maldonado-Galdeano et al., 2019). Probiotics have become a growing field in diet and health research because of their hypothesized benefits to human health.

Epithelial Tight Junctions

Innate immune defense is a crucial function of epithelial tissue, and this defense is largely governed by the tight junction. Located near the apical face of columnar intestinal cells, tight junctions serve as impermeable points of contact between the membranes of adjacent cells. More than 40 proteins compose tight junctions in order to pinch cell membranes together to form a barrier against paracellular transport (Anderson and Van Itallie, 2009). Transmembrane proteins, such as claudins and occludins, extend from the interior of the cell through the cell membrane to connect to one another in the extracellular matrix, joining adjacent cell membranes (Anderson & Van Itallie, 2009). Intracellularly, claudins and occludins are anchored to cytoskeletal actin by scaffold proteins, such as zonula occludins (Anderson & Van Itallie, 2009). The resulting structure forces pathogens to attempt to enter the bloodstream through transcellular transport, which allows for the pathogens to be identified and destroyed within the cell (Anderson and Van Itallie, 2009).

Increased permeability of tight junctions is often referred to as "leaky gut," and this condition has been observed in many inflammatory conditions such as inflammatory bowel disease, irritable bowel syndrome, celiac disease, and early colon cancer (Karczewski et al., 2010). Heightened permeability of the intestinal wall can also be present in those with diabetes, HIV-related diarrhea, atopic eczema, and food sensitivity (Karczewski et al., 2010). Probiotics, especially *Lactobacillus plantarum*, have been shown in previous research to increase the concentration of proteins that compose the tight junction, claudins, and occludins, near the area of the cell at which the junction would be located (Karczewski et al., 2010). The increased concentrations of transmembrane and scaffold protein strengthen tight junctions, thereby bolstering the intestinal barrier to inflammatory agonists which could otherwise enter the bloodstream through the leaky junction.

Probiotics in Popular Culture

In recent years, probiotic bacteria have been exploited by health and diet companies as a fitness trend marketed toward those pursuing a healthy lifestyle. Popular media sources present the consumption of probiotic supplements and food products as a passive way to boost the immune system and improve overall health. In this context, probiotics are administered through manufactured tablets and pills to be taken orally, or as live cultures in fermented foods such as kombucha. Research, on the other hand, may contradict the idea that this method of consuming probiotics is effective, as studies that observed the increased localization and expression of tight junction proteins due to probiotics delivered the live bacterial cultures grown in a lab directly to the intestine through a duodenal catheter (Karczewski et al., 2010). Furthermore, many of the probiotic studies conducted on humans as well as other model organisms have been conducted in the presence of an existing or induced disease state, usually, an inflammatory condition (Pérez-Ramos et al., 2018). Probiotics, though, are marketed as a diet trend to already healthy individuals

looking to boost their immunity or digestive health. More research is required to better understand the physiological effects of dietary probiotics in those without an inflammatory condition and how that relates to the reputation of probiotic supplements as a diet trend.

Model Organisms

Danio rerio, more commonly known as zebrafish, are small, freshwater teleosts that have become popular both as pets and research subjects (Briggs, 2002). Molecular biologist George Streisinger pioneered the use of zebrafish as a model organism, identifying the traits of the species which made them optimal candidates for research, such as ease of care and clarity of observation throughout development (Briggs, 2002). The species' genome is sequenced, with many gene sequence and gene map resources available (Briggs, 2002). Genomic sequencing has illuminated that 71% of human genes share an ancestral root with zebrafish, and 69% of zebrafish genes have a minimum of one human ortholog (Howe et al., 2017). Additionally, 82% of disease genes found in the catalog "Online Mendelian Inheritance in Man" have a zebrafish ortholog (Howe et al., 2017). These genetic similarities between humans and zebrafish, in both diseased and normal specimens, in tandem with the practical benefits of studying and caring for zebrafish, make *Danio rerio* an important vertebrate model upon which to study human physiology.

Recent studies observing the influence of probiotics on overall health have been conducted utilizing zebrafish as model organisms. Research has also confirmed that many strains of probiotic bacteria which colonize the human colon can colonize the colon of zebrafish as well (Valenzuela et al., 2018). *Lactobacillus plantarum* has been shown to induce local and systemic benefits in *Danio rerio,* such as improving the local intestinal environment as well as attenuating anxiety and preventing oxidative damage to cells (Davis et al., 2016; Zang et al., 2019). Many studies observing the impact of probiotics on the overall health of zebrafish utilized live cultures of probiotics to inoculate larval or juvenile zebrafish rather than adults (Pérez-Ramos et al., 2018). Few studies have been conducted utilizing adult zebrafish as model organisms for dietary probiotic administration; however, adult zebrafish have demonstrated physiological changes, such as reduced systemic inflammation, after dietary administration of *Lactobacillus plantarum* (Wang et al., 2019).

Specific Aims

This study aims to establish a methodology for studying changes in gene expression for the tight junction proteins *occludin* and *claudin-12* in a non-diseased, adult zebrafish model system. Adult, wild-type zebrafish are intended to represent a non-diseased adult human, and they can be considered an acceptable model organism due to their gene orthologs and previous research outcomes indicating their susceptibility to probiotic-induced health changes. Changes in the tight junctions will be determined by using PCR to quantify mRNA expression levels of *occludin* and *claudin-12* genes in the intestinal tissue of zebrafish that have been exposed to a dietary probiotic supplement. Fish who have been exposed to the dietary probiotic will be compared to a control group that was not given a probiotic. The system being observed is intended to model the effects of non-diseased humans taking dietary probiotics recreationally for a short-term period of time. Establishing a methodology

for studying the effects of probiotics in a non-diseased model organism will provide insight into whether probiotics are effective or necessary to an average, healthy human gut.

Materials and Methods

Animal Care and Housing

Male zebrafish were purchased from Carolina Biological Supply ®. The fish were randomly distributed into three separate tanks for a control period of one week. After this week, all fish were removed, three fish were randomly selected and sacrificed as the zero-control, and the remaining fish were randomly assigned to two tanks: a control tank and an experimental tank, respectively. The purpose of the zero-control is to serve as a point of comparison to identify differences or lack thereof in the control group at the end of the study. A total of seven fish composed the control group, and seven fish composed the experimental group. Care guidelines were determined according to the RSPCA's guide for the care and housing of zebrafish (Reed & Jennings, 2011). Each 20L tank was kept full to two centimeters from the top of the tank. The water temperature was maintained to 28.5 °C and AquaSafe ® Plus water conditioner was used anytime water was added or changed to remove chlorine and heavy metals. The full volume of water was changed weekly, with partial water changes occurring weekly or as needed if the water became clouded. In the case of a partial water change, approximately 50% of the water was removed and replaced. Fish were removed and placed in an interim container during total water replacements but remained in the tank during partial water replacements. The aquarium lights were controlled in a 12-hour light/dark cycle, and water filters were changed every two weeks.

The bottom of each tank was lined with gravel to approximately 2.5 cm. Tanks were empty aside from the gravel during the control period, but two small plants were added to each tank during week two of the eight-week experimental runtime to curtail aggressive behavior. Fish in both tanks were observed biting each other's fins, so two plants were added to each tank adjacent to one another on the side of the tank opposite the filter.

Monday through Friday, the fish were fed 0.01g of powdered food twice a day. Feedings on weekdays occurred at 7:00 am and 5:00 pm. On Saturdays and Sundays, fish were fed once a day at 5:00 pm. Fish food was handmade in order to incorporate and control probiotic culture counts into the food. Detailed methodology is contained in the *Food Synthesis Methodology* section which follows.

Food Synthesis Methodology

1g of powdered cichlid fish food was mixed with the contents of 1 Swanson ® *L. plantarum* capsule containing 10^9 CFU of *L. plantarum*. The casing was removed, and the powdered probiotic was stirred into the dry powdered food pellets. This mixture was converted to a paste by the addition of 30 mL of deionized water and extruded through the 3 mm diameter insert of a stainless-steel potato ricer and left to dry while covered for 24 hours on paper towels. Control food received the same treatment without the addition of the probiotic for consistency. Once dry, the food was ground again using a mortar and pestle and given to the fish as powder. At each

feeding, the fish received 0.01g of food, with each dose of probiotic food containing an approximate concentration of 10^6 CFU of *L. plantarum.*

Euthanasia and Sample Collection

Upon the completion of the 8-week experimental runtime, fish were euthanized by rapid transfer into ice water between 2 ℃ and 4 ℃ (Leary et al., 2020). Death was determined to be complete 10 minutes after the cessation of operculum movement. Euthanized fish were patted dry and weighed prior to dissection. The intestine of each fish was removed by dissection under a stereoscope, and the weight of each intestine was calculated. Sample tissue was flash-frozen in RNase-free microcentrifuge tubes by placing the tube containing the sample in a slurry of dry ice and 100% EtOH. After flash freezing, frozen samples were stored at a temperature of -70 °C until mRNA isolation was conducted.

mRNA Isolation

To isolate mRNA from intestine samples, the SurePrep™ Nuclear or Cytoplasmic RNA Purification Kit was utilized with the following modifications: 50μL additional lysis buffer and binding solution were added to each solution out of extra caution, and the amount of **β**-mercaptoethanol added to each was adjusted accordingly. The protocol was modified to better lyse the large tissue samples: the lysis buffer was directly applied to smaller flash-frozen samples in the microcentrifuge tube. Samples whose mass was greater than the suggested 15mg limit were lysed in multiple fractions as follows: the 200μL of lysis buffer was added to the sample as per the kit instructions. After initial homogenization with an RNase-free micropestle, the sample was divided in half and an additional 200μL of lysis buffer was added to ensure complete homogenization.

Reverse Transcription PCR

Reverse transcription (RT) was conducted utilizing Promega GoScript ™ Reverse Transcription Mix, Random Primer Protocol. RT reactions were tailored individually for each sample based on the mRNA concentration. The volume of RNA in ng/mL for each reaction was calculated for each sample from the spectrophotometer concentration reading. A subsequent volume of deionized water was added based on mRNA concentration in order to yield a total reaction volume of 20μL. The primers used can be seen below in Figure 1. The protocol used for reverse transcription was as follows: primers annealed at 25 °C for one cycle of five minutes, extension occurred at 42 °C for one cycle of 60 minutes, inactivation occurred at 70 °C for one cycle of 15 minutes, and the hold temperature was 4 °C for one cycle until removal from the thermocycler.

mRNA concentrations varied throughout the isolation process, so only samples with significant yield were utilized for PCR. Two zero control samples, four control samples, and seven experimental samples were utilized for the PCR process. Primers for *β-actin*, *occludin,* and *claudin*-12 were amplified, and the information on the three primers can be seen in Figure 1 below. The primers for PCR were diluted 10x from a 100μM stock solution to yield a 10μM working solution. PCR was conducted using a 0.3μM final primer concentration. Three protocols were utilized for PCR. Protocol A consisted of the following: 95 °C denaturation for 30 seconds, 56 °C annealing for 30 seconds, and 72 °C extension for 30 seconds. These steps were

repeated for 40 cycles, followed by a final seven-minute extension at 72 °C. Protocol B consisted of the following: one 30-second extension at 95 °C, annealing at 57 °C for 30 seconds, and a 72 °C extension for 30 seconds. These steps were repeated for 40 cycles, followed by one 7-minute cycle at 72 °C. After PCR was completed, the samples were stored at -70 °C until further use.

Gene	**Accession Number**	**Forward Sequence**	**Reverse Sequence**	**Protocol**
β-Actin	M25013	5'-GGCTGTGCTGTCCCTGTA-3'	5'-GGGCATAACCCTCGTAGAT-3'	B
Occludin	KF193855	5'-TATCTGTATCACTACTGCGTC-3'	5'-CATTCACCCAATCCTCCA-3'	A
Claudin-12	KF998571	5'-CCCTGAAGTGCCCACAA-3'	5'-GCGTATGTCACGGGAGAA-3'	B

Figure 1. Forward and reverse PCR primer sequences. Primers were adapted from a study by Wang et al., 2019.

Gel Electrophoresis

Gel electrophoresis was conducted utilizing 1.5% TAE agarose gels which were run for 20 minutes at 80V. Gels were placed on a UV lightbox and documented for later quantitation with photographs.

Quantitation of Gene Expression

Gene expression was quantified using ImageJ software, which quantitated pixel concentration of gel bands. Images were converted to black and white, and blue content was altered to minimize background interference on band intensity. Bands representing *occludin* and *claudin-12* were normalized according to *β-actin* once pixel concentrations had been calculated. Average pixel concentrations were calculated for the control and experimental gene expression of *occludin* and *claudin-12* data sets.

Statistical Analysis

Average pixel concentrations of zero control samples, control samples, and experimental samples were compared using a two-sample t-test assuming unequal variances. Two sets of t-tests were conducted: one to compare the zero control samples to the control samples, and one to compare the control samples to the experimental samples for each gene.

Results

Gel

Electrophoresis

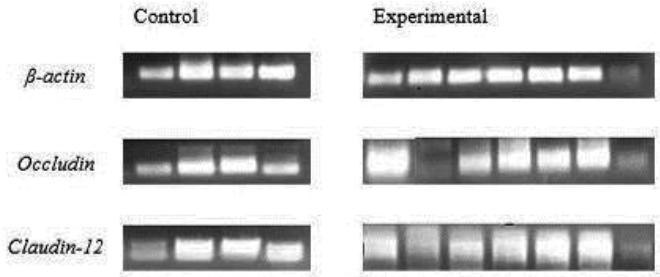
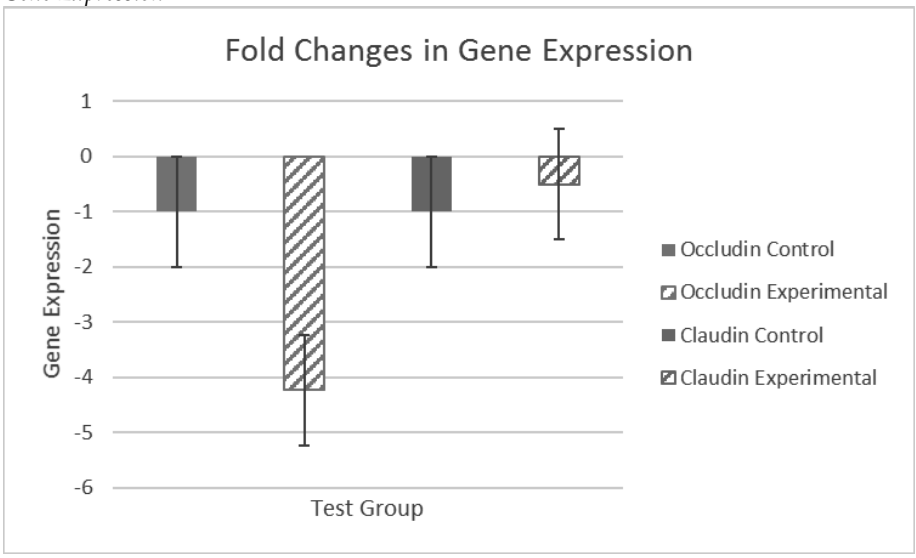


Figure 1 Gel electrophoresis. Bands were quantitated with ImageJ software.

Gene Expression



Images in Figure 2 show bands representing raw data from PCR results after gel electrophoresis. Following quantitation with ImageJ software, gene expression results were normalized to **β**-actin. The data illustrate no significant difference between zero control samples and control samples for either the *occludin* or the *claudin-12* controls (*occludin* expression, P = 0.39, and for *claudin-12* expression, P = 0.66). Control and experimental samples were normalized to one, and fold changes in gene expression for *occludin* and *claudin-12* were plotted in Figure 3. Figure 3 appears to show a significant fold increase in *occludin* expression and no significant change in *claudin-12* expression. Statistical analysis revealed no significant differences in fold

gene expression between control samples and experimental samples for both o*ccludin* and c*laudin-12* (*occludin* expression, P = 0.62, and for c*laudin-12,* P = 0.71).

Discussion

While research on probiotics has extensively explored the use of probiotic cultures to alleviate inflammatory damage to intestinal tissue, studies to explore the effects of probiotics on non-stressed or non-diseased subjects are lacking. Due to the presence of probiotics in trend dieting, this study attempted to use a model which mimics an average, health-focused individual taking probiotics as a diet supplement for a short-term period of time and with no inflammatory conditions. Studies that induce a disease-state to observe intestinal effects have noticed upregulation of tight junction protein genes, indicating that the mechanism by which probiotics influence intestinal permeability during an inflammatory state is through regulating gene expression. This study sought to explore whether these results occurred only in the presence of inflammation, or whether individuals without inflammatory bowel conditions would see similar changes in gene expression for tight-junction proteins. Results indicated that subjects without inflammatory conditions consuming a single-species probiotic supplement for a short-term period of time did not experience upregulation of tight junction protein genes. Further research should explore this result in conjunction with intestinal permeability to confirm not only the lack of increased gene expression, but also to understand whether this mechanism is the means through which probiotics influence intestinal permeability.

This methodology could be repeated for further study on probiotics in healthy individuals. Repetition of this study should include modifications to overcome the restrictions this study faced due to the COVID-19 pandemic, such as increasing both the sample size and the length of the study period. Despite limitations, the results achieved indicate that further study is warranted to understand the relationship between dietary probiotics and a healthy gut, an area in which current literature is lacking due to a focus on disease models. The lack of increased gene expression found in this study suggests that inflammation may serve as positive feedback to elicit upregulation of tight junction proteins mediated by probiotics, while lack of active inflammation may serve as negative feedback to prevent unnecessary translation of intestinal tight junction proteins. This suggests that probiotics may be essential to the diet rather than assistive, maintaining the commensal bacteria of the colon in times of stress without inciting negative long-term effects due to their activity being directly proportional to inflammation levels. This relationship is significant, especially when considering the spectrum at which stress-induced inflammation can be experienced.

Studies on probiotic supplementation prioritize models with inflammatory bowel conditions, suggesting that probiotics are of negligible benefit to those without diagnosable conditions. These conclusions imply a binary system in which an individual taking probiotics is either diseased or not. This conclusion neglects the commonality of inflammation-inducing stressors, which include such common experiences as exposure to environmental toxins, lack of sleep, antibiotics, among others which are of day-to-day relevance (Camilleri 2019). The consideration of daily environmental stressors generates a spectrum at which inflammation can be

experienced, but which is not being fully exploited in research on probiotics. Inflammatory bowel conditions are worsened in the face of these daily stressors, but individuals without diagnosed bowel diseases experience them as well, suggesting the "average" gut is still often subjected to increased intestinal permeability due to inflammatory damage to the gut microbiome. Furthermore, health-conscious individuals tend to habitually minimize daily stressors through diet and exercise habits and therefore may minimally benefit from probiotics, yet they are exposed to significant amounts of non-medical marketing in diet media which insinuates that probiotics, similar to collagen or other fad diet supplements, may enhance their current healthy lifestyle. These three demographics form a spectrum at which the effects of intestinal inflammation, and therefore probiotic supplements, may be experienced and should encourage the diversification of studies on probiotics to include a wider range of inflammatory and non-inflammatory models.

Understanding the effects of probiotics in a non-diseased gut may reveal the need for a change in marketing: health-conscious subjects may require less supplemental probiotics, while individuals subjected to day-to-day stressors may benefit from probiotics just as much as those with diagnosable inflammatory bowel conditions. Overall, expanding probiotic research to cover the full spectrum of intestinal environments, from diseased, to average, to health-conscious would play a crucial role in expanding the field of nutrition and improving public health knowledge as a whole.

Literature Cited:

Anderson, J.M., & Van Itallie, C.M. (2009). Physiology and function of the tight junction. *Cold Spring Harbor Perspectives in Biology*. 1(2): a002584. Doi: 10.1101/cshperspect.a002584

Briggs, J.P. (2002) The zebrafish: A new model organism for integrative physiology. *American Journal of Physiology*. 282(1): R3-R9 https://doi.org/10.1152/ajpregu.00589.2001

Buffie, C., & Pamer, E. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. *Nature Reviews Immunology*. Vol. 13, 790-801. Accessed 9 February 2019.

https://www.nature.com/articles/nri3535

Gibson, G.R., & Roberfroid, M.B. (1995). Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *The Journal of Nutrition* 6: 1401-1412.

https://academic.oup.com/jn/article-abstract/125/6/1401/4730723

Howe, D.G., Bradford, Y.M., Eagle, A., Fashena, D., Frazer, K., Kalita, K., Mani, P., Martin, R., Moxon, S.T., Paddock, H., Pich, C., Ramachandran, S., Ruzicka, L., Schaper, K., Shao, X., Singer, A., Toro, S., Slyke, C.V., & Westerfield, M. (2017). The zebrafish model organism database: New support for human disease models, mutation details, gene expression phenotypes and

searching, *Nucleic Acids Research*, Volume 45, Issue D1, January 2017, Pages D758–D768, https://doi.org/10.1093/nar/gkw1116

Karczewski, J., Troost, F.J., Konings, I., Dekker, J., Kleerebezem, M., Brummer, F.J.M., & Wells, J.M. (2010). Regulation of human epithelial tight junction proteins by Lactobacillus plantarum in vivo and protective effects on the epithelial barrier. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 298: G851–G859; doi:10.1152/ajpgi.00327.2009.

Maldonado-Galdeano, C., Cazorla, S.I., Lemme-Dumit, J.M., Vélez, E., & Perdigón, G. (2019). Beneficial effects of probiotic consumption on the immune system. *Ann Nutr Metab*. 74(2):115-124. doi: 10.1159/000496426. Jan 23. PMID: 30673668.

Ohland, C.L., & MacNaughton, W.K. (2010). Probiotic bacteria and intestinal epithelial barrier function. *American Journal of Physiology*. Vol 298.6: G807-G819

Pérez-Ramos, A., Mohedano, M.L., Pardo, M.Á., & López, P. (2018). β-glucan-producing Pediococcus parvulus 2.6: Test of probiotic and immunomodulatory properties in zebrafish models. *Front. Microbiol.* 9:1684. doi: 10.3389/fmicb.2018.01684

Roberfroid, M.B. (2000). Prebiotics and probiotics: Are they functional foods? *The American Journal of Clinical Nutrition* 6: 1682S-1687S. Accessed 24 February 2019

https://academic.oup.com/ajcn/article/71/6/1682S/4729644

Valenzuela, M.J., Caruffo, M., Herrera, Y., Medina, D.A., Coronado, M., Feijóo, C.G., Muñoz, S., Garrido, D., Troncoso, M., Figueroa, G., Toro, M., Reyes-Jara, A., Magne, F. & Navarrete, P. (2018). Evaluating the capacity of human gut microorganisms to colonize the zebrafish larvae (Danio rerio). *Front. Microbiol*. 9:1032. doi: 10.3389/fmicb.2018.01032

Wang, Y. Zhou, X.Q., Jiang, W.D., Wu, P., Liu, Y., Jiang, J., Wang, S.W., Kuang, S.Y., Tang, L., & Feng, L. (2019). Effects of dietary zearalenone on oxidative stress, cell apoptosis, and tight junction in the intestine of juvenile grass carp (Ctenoparyngodon idella). *Toxins*. 11(6):E333. doi: 10.3390/toxins11060333.