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The Intake of Probiotics Mixture Disturbs Lipid Profile Serum Levels and Affects the Morphometric State of Adipocytes in A Piglet Model

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ABSTRACT

Probiotics are microorganisms that contribute to the health of the host. Our study was carried out in an F2 hybrid breed piglet model to analyze probiotic intake on metabolic outcome, adding to the diet piglet a mixture of the three probiotics: Limosilactobacillus fermentum LH01, Lactiplantibacillus plantarum LH03, and Limosilactobacilus reuteri LH05, called P/LH-010305. The serum levels of metabolic markers were determined, and the histopathological state of the adipose and small and large intestinal tissue segments was evaluated. The results showed that the consumption of P/LH-010305 dismissed triglyceride levels, and the adipose tissue morphometric study was improved. In pigs that consumed P/LH-010305, a tendency to regulate glucose and cholesterol serum levels was observed. We suggest that probiotic mixture intake may improve triglyceride levels, which indicates a metabolic regulation, likewise corroborated by the diameter and number of adipocytes on adipose tissue observed. We consider that the mixture of probiotics can benefit the growth and health of piglets.

Keywords
Piglet, Probiotics, Metabolic regulation, Adipose tissue.

Introduction
Piglet models, due to their organs that exhibit similarity with humans, in terms of their genetics, body composition, anatomical and histological organization, and physiology, provide important information for science and based on these findings, the two species may show similar alterations in their intestinal microbiota, in response to environmental factors, such as stress, antibiotic consumption, which is why it is suggested that pigs are an animal model with translational power to explain physiological and pathological phenomena in humans [1].

In pig nutrition, probiotics are of great interest since they are known for their ability to modulate the immune response, increase performance at all stages of pig production, improve performance during gestation, farrowing, and lactation in sows, and can enhance and maintain the intestinal health of the animal. It has been shown that probiotics have antimicrobial effects against pathogenic strains. In addition, they can influence meat quality in the finishing phase and help reduce environmental contaminants [2].

Proper microbiota development is essential for intestinal function and influences a pig's health, growth, and productivity. The diversity of the intestinal microbiota is linked to the diet that changes abruptly after weaning, affecting the microbiota and physiology of the animal. Situations such as stress contribute to the appearance of intestinal disorders, possibly modifying the microbiota and the structure of epithelial cells, facilitating the colonization of pathogenic bacteria, decreasing performance, and increasing the use of antibiotics. Antibiotics reduce the intestinal microbial population, and probiotics and prebiotics are used to recolonize to help maintain intestinal balance and inhibit the development of pathogens. Understanding the dynamics of the animal microbiome helps to improve immunity, productive performance, and welfare and reduce the use of antibiotics in pigs [3].

Rodríguez-Arreola et al. [4] evaluated the in vitro probiotic activity of *L. fermentum* LH01, *L. plantarum* LH03, and *L. reuteri* LH05, isolated from human milk samples. The results showed that these bacteria exhibit survival in the presence of digestive enzymes, at low pH conditions and bile salt concentrations, and showed a high degree of adhesion and hydrophobicity, indicating their potential for intestinal colonization. They also showed the ability to produce bacteriocin against pathogenic bacteria. On the other hand, the combined use of *L. fermentum* LH01, *L. plantarum* LH03, and *L. reuteri* LH05 was studied by Solís-Pacheco et al. [5] for the control of gastrointestinal problems. This study showed that the administration of probiotics in combination with other products in people with gastrointestinal complications can be effective against intestinal inflammation, reduce abdominal pain, and promote recovery in people with constipation problems.

It is well known that the gut microbiome plays a fundamental role in human and animal health and well-being. Establishing and maintaining a beneficial gut microbiota in the early stages of life is crucial for humans and animals, as early gut colonizers establish permanent microbial community structures in the gut and can affect the health and growth of weaned piglets later in life. Other factors such as diet, antibiotics, feed type, probiotics, and prebiotic administration have an impact on the modulation of the gut microbiome. Therefore, this study may provide a new strategy for feeding weaned piglets with probiotics to improve the gastrointestinal health, growth, and well-being of pigs in adulthood.

Materials and Methods

Ethical and Biosecurity Considerations

The porcine included in this study were handled in compliance with the protocols of the animal management guidelines established by the International Organization for Animal Health (OIE), reviewed in the national guide for the integration and operation of ethics committees in research (National Bioethics Commission National Guide for the Integration and Operation of Ethics Committees in Research, 2013), the Regulations of the General Health Law and NOM-062-ZOO-1999 on Research in animal species. The chemical and biological-infectious waste generated was destined for final disposal by current regulations (OFFICIAL MEXICAN STANDARD NOM-052-SEMARNAT-2005, which establishes the characteristics, identification procedure, classification, and lists of hazardous waste, by the Official Mexican Standard NOM-087-SEMARNAT-SSA1-2002, Environmental Protection-Environmental Health-Hazardous biological-infectious waste Classification and management specifications).

Probiotic Production (P/LH-010305)

Limosilactobacillus fermentum LH01, *Lactiplantibacillus plantarum* LH03, and *Limosilactobacilus reuteri* LH05 of human milk origin were sourced from the strain collection of the Human Milk Research Laboratory at CUCEI, Universidad de Guadalajara. The strains were cultured in de Man Rogosa Sharpe (MRS) medium (Difco), incubated at 37°C for 24 h, and then harvested by centrifugation at 4°C, 500 rpm for 10 min. After centrifugation, the bacteria were washed with sterile physiological saline to remove the residual medium. The bacterial pellets were suspended in sterile saline, and the mixture was made with the 3 bacteria (P/LH-010305) at a concentration of $\pm 1.4\times10^{10}$ CFU/mL for oral administration to the piglet model. The probiotics P/LH-010305 were stored at freezing temperatures (-20°C) until use.

Study Design to a Porcine model

A hybrid breed piglet model was used, resulting from crossing the Landrace-Yorkshire terminal Pietrain breeds, filial hybrid generation 2. This breed represents an exemplary case of directed hybridization to optimize desirable characteristics in pig production and stands out for its significant resistance to diseases. This study used 16 animals, 8 females (H) and 8 males (M), with weights ranging from 5.4 to 10.8 kg. The animals were obtained from the Rancho Cofradía Experimental Field of the Centro Universitario de Ciencias Biológicas y Agropecuarias (CUCBA) of the University of Guadalajara. It is divided into 2 groups: Control group (C): 4 females and four male individuals; were fed a commercial diet (Piglet Preiniciador Fase 2, 7-14 flour, Nutrimentos Ramírez). Treatment group (T): 4 females and 4 males. Fed with the commercial diet more probiotic supply (P/LH-010305). They were housed under controlled conditions with a temperature of 25 ± 2°C, a humidity of 60 ± 5%, and a light/dark cycle of 12 hours. All piglets had access to food and water. Animal care and experimental procedures were carried out according to the Government of Mexico guidelines on animal welfare (NOM-062-ZOO-1999). This protocol was evaluated by the Bioethics Committee of the University Center for Biological and Agricultural Sciences of the University of Guadalajara with registration number CINV/014/2024.

Biological Sample Collection

Blood samples were collected by venipuncture into vacutainer® tubes (BD Diagnostic Systems, Montenegro 1402, C1427AND, Buenos Aires, Argentina). After collection, the samples were allowed to coagulate at room temperature. Once coagulation was complete, the samples were centrifuged at 2000 g (Rotanta 460R, Andreas Hettich GmbH & Co.K.) for 10 min. The resulting serum was separated into aliquots and stored at −80°C for future analysis. The piglet was kindly euthanized by electrosensitization and exsanguination. Necropsy was then performed, and tissue samples were collected from various sections of the digestive system, including the duodenum, jejunum, ileum (small intestine), and cecum and colon (large intestine). In addition, samples of adipose tissue were collected. These samples were preserved for subsequent histomorphometry analysis.

Metabolic Evaluation

Serum chemistry analyses: glucose and lipid profile (total cholesterol, triglycerides) were performed using immunoturbidimetry based on the GOD/POD method (RANDOX Laboratories Ltd., Crumlin, UK).

Histomorphometry Evaluation

The histological samples used the STP 20 Microm (Thermo Scientific), following a careful sequence of dehydration, xylol clearing, and paraffin infiltration to preserve cellular structures. Thin sections of 3-4 µm were then cut with a Leica Biosystems microtome and placed in a 45°C water bath, maintaining their integrity and readiness for examination. To capture and analyze microscopic features, we employed the LEITZ WETZLAR camera paired with Motic Images Plus 2 ML software. This combination enabled precise measurements of villi length and width in the small intestine, along with detailed crypt measurements. For quantification, we calculated villi density over a defined area of 10,000 μm^2, with comparable measurements for the large intestine, including crypt length, thickness, and density within an equivalent surface area. In addition, we measured and counted adipocytes, carefully excluding those not fully visible or overlapping to ensure accuracy in diameter and distribution assessments. This method, enhanced by the precision of the LEITZ WETZLAR camera and Motic Images Plus 2 ML software, provided a thorough and nuanced understanding of the spatial arrangement, dimensions, and morphology of microscopic structures, especially within adipose tissue.

Statistics Analysis

Two-way ANOVA followed by Tukey's post hoc test, repeated measures two-way ANOVA followed by Tukey's post hoc test, and paired t-tests were employed to identify significant differences between treatments. Pearson's correlation was utilized to evaluate relationships between the studied variables. Data are presented as mean±SD. A significance level of $P<0.05$ was considered for determining statistically significant differences.

Results

Effect of P/LH-010305 Probiotic Administration in a Piglet Model

The results obtained from the analysis of the biochemical parameters before and after probiotic supplementation are shown in Figure 1. At the end of treatment, a tendency to increase blood glucose is observed, both in the control group and in the group supplemented with probiotics. However, triglyceride and cholesterol content decreased in the group supplemented with P/LH-010305 at the end of the treatment. It has been reported that supplementation with *Lactobacillus plantarum* can alter the intestinal microbiota and bile acid profile, improving glucose homeostasis and tending to increase body weight in weaned piglets [6]. Likewise, the triglyceride-reducing effect was observed in a study carried out over 6 weeks in growing-fattening pigs, with supplementation *Lactobacillus plantarum* CJLP243, *L. fermentum* LF21, *L. salivarius* E4101, *Leuconostoc paramesenteroides* KJP421, *Bacillus subtilis* CJMPB957, and *B. licheniformis* CJMPB283. The results showed that the growth performance of the probiotic-supplemented group was improved compared with the control group by reducing hepatic triglyceride concentration, concluding that dietary probiotic supplementation may be promising for promoting growth performance and overall health of growing pigs through modulating gut microbiota [7].

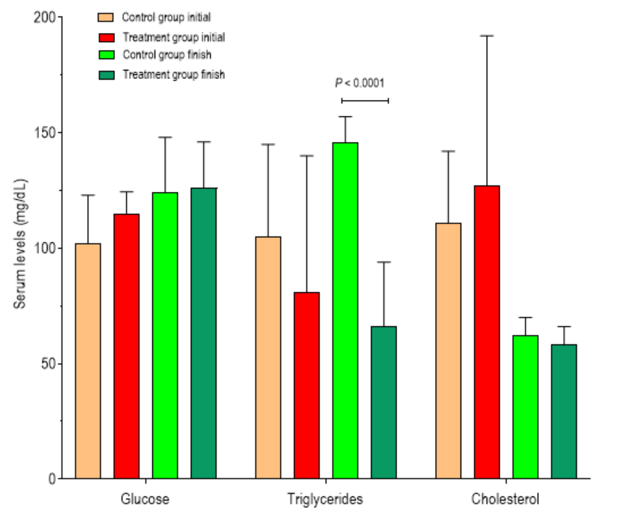

Figure 1: Biochemical parameters before and after probiotic supplementation.

On the other hand, this study demonstrated that probiotic supplementation P/LH-010305 can improve piglet growth, evidenced by an increase in body weight of probiotic-supplemented piglets of 19.2 kg compared to 14.5 kg in the control group after 4 weeks. Our result is similar to a study carried out by Tian et al. [8] they compared for 175 days the effect of the administration of an antibiotic (olaquindox and aureomycin) versus the supplementation with *L. reuteri* 1, at a concentration of $5x10^{10}$ CFU/kg in pigs in the longissimus thoracic. Showed that antibiotics reduced the quality of the meat of the pigs; they had a lower muscle content of free glutamic acid, inosinic acid and higher glutamine compared to control pigs and those supplemented with *L. reuteri* 1. It observed that antibiotics reduced free isoleucine, leucine, and methionine in longissimus thoraci, while pigs supplemented with *L. reuteri* 1 improved meat quality, showed greater muscle content by decreasing drip loss and shear force, increasing inosinic acid and glutamic acid that can improve flavor and muscle fiber characteristics, probably *L. reuteri* 1 improved protein synthesis by modulating the expression of amino acid transport genes and ribosomal protein kinase S6 1 (S6K1), and altered the fatty acid profile by regulating metabolic pathways

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Probiotic supplementation with two probiotic strains of *Bacillus* (BP) for 28 days on reproductive performance, nutrient digestibility, blood profile, fecal noxious gas emission, and fecal microflora in lactating sows and growth in their piglets were evaluated. Sows fed BP-supplemented diets had a reduction of NH_3 and total mercaptan concentration in fecal, improved dry matter digestibility, and reduced *E. coli* concentration. Piglet body weight was evaluated to calculate average daily gain, and it was observed that BP supplementation linearly increased piglet body weight at 21 and 25 days. The growth performance of suckling piglets increased with BP supplementation of the diet of lactating sows [9].

Morphogenic Analysis of Adipose Tissue

The analysis of adipocytes (Figure 2) in piglets supplemented with the probiotics P/LH-010305 shows an increase in both the diameter and the number of adipocytes compared to the baseline and treatment groups. These findings suggest that probiotic supplementation may favor the reduction of overall adiposity by stimulating the formation of normal-sized adipose cells without signs of atrophy. This adaptation seems to support the high energy demands of the piglets at this stage of development, potentially preventing immunometabolic stress. This effect appears to be an adaptation mechanism to the high energy demand in piglets during this stage of development, avoiding possible immunometabolic damage. The increase in the number of adipocytes could reflect a balance between the generation of new cells and the apoptosis of those not needed in response to excess energy [10].

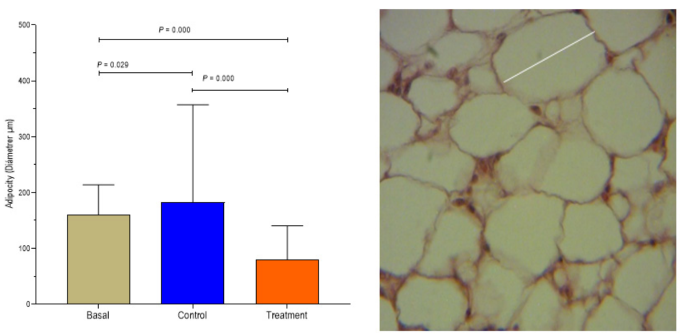

Figure 2: Morphogenic analysis of Adipose tissue.

The image was taken using an OPTIKA microscope/LEITZ-WETZLAR photo camera. The white line shows the measurement criteria.

Porchia et al. [11] reported a decrease in adipocyte diameter in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) in humans after the ingestion of *Lactobacillus* and *Bifidobacterium*. In our study, we found parallels with the work of Porchia regarding the reduction of adiposity induced by probiotics. In both studies (porcine and human), a decrease in the size and increase in the number of adipocytes were observed in response to treatment. A similar anti-adipogenic effect has been demonstrated by the strains *L. fermentum* MG4231 and MG4244, which, through the activation of the AMPK pathway in 3T3-L1 preadipocytes, showed the potential to prevent obesity by reducing the accumulation of triglycerides and the number of these cells. These findings suggest that probiotics could be effective in reducing adiposity in different models, supporting their therapeutic potential in the management of obesity and other metabolic diseases [12]. However, it is important to consider the differences between the models used in these studies. While our research focused on pigs, the other researchers evaluated the effects in humans, which could influence the interpretation and extrapolation of the results between species.

In a porcine model of early prepubertal obesity induced by a Western-type diet and a high-calorie diet supplemented with *Bifidobacterium breve*, rice hydrolysate, and omega-3 fatty acids, determined changes in liver and adipose tissue transcriptomes in 43 nine-week-old animals. Animals fed the Western-type diet increased body weight and total fat content and exhibited elevated serum cholesterol concentrations, while animals supplemented with *Bifidobacterium breve* showed lower body weight gain and accumulated less fat. Differential expression analyses revealed increased lipogenesis, cholesterogenesis, and inflammatory processes in animals on the Western-type diet, whilst *Bifidobacterium breve* supplementation induced fatty acid oxidation and cholesterol catabolism, decreasing adipogenesis and inflammation. These results reveal the molecular mechanisms underlying the beneficial effects of supplementation with probiotics and bioactive ingredients in an obese pig model [13].

Morphogenic Analysis of Large Intestinal Segments

Figure 3 (A and B) presents a detailed comparison of the average crypt length and thickness in the colon and cecum, and Figure 4 (A, B, and C), a villus length and thickness, and crypt length in the duodenum, jejunum, and ileum in the three study groups: basal, control, and treatment (P/LH-010305 supplementation).

A decrease in villi length was observed from the duodenum and the ileum, with differences between the control and treatment groups. The basal group presented longer villi in all sections of the small intestine compared to the control and treatment groups. The control group showed longer villi in the duodenum and jejunum, while the treatment group had longer villi in the ileum. Regarding the thickness of the villi, all three groups showed similar sizes in the jejunum and ileum. However, in the duodenum, the basal group showed thinner villi compared to the treatment group, indicating a possible difference in tissue morphology between these groups in this particular section. The number of villi per unit area showed significant differences between the groups, with a significantly higher density of villi in all three intestinal sections. A similar number of villi were seen in the control and treatment groups, which were lower than those observed in the reference group.

Concerning crypt length, the control and treatment groups showed longer crypts in all sections, suggesting that probiotics may affect crypt development. These results indicate that the reference, control, and treatment groups exhibited distinct morphological characteristics in the small intestine, with specific differences in villus and crypt morphology that may reflect underlying physiological changes. The data obtained from the measurements of dimension, thickness, and number of intestinal crypts in the cecum and colon showed an increase in dimension and thickness in all groups analyzed, however, a difference was observed in the group of piglets supplemented with P/LH-010305, observing greater dimension and thickness, while in the number of intestinal crypts, the control group showed a higher crypt count than the group supplemented with probiotics.

Some research has highlighted the positive effect of probiotic bacteria supplementation on the intestinal health of piglets [14]. Reported significant improvements in intestinal morphology, such as increased villus length and improved growth of piglets after 11 days of probiotic supplementation. On the other hand [15], demonstrated that *Lactobacillus rhamnosus* GG supplementation in piglets infected with the porcine epidemic diarrhea virus reduced crypt depth and increased villus height, which contributed to improved intestinal barrier integrity.

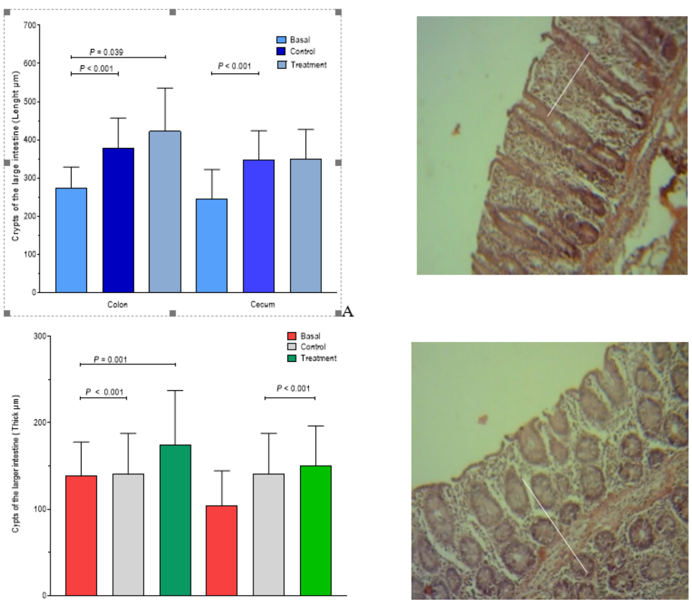

Figure 3: Morphogenic analysis of large intestinal segments.

Images were taken using an OPTIKA microscope/LEITZ-WETZLAR photo camera. The white line shows the measurement criteria.

The intestinal epithelial layer is made up of circular folds, villi, crypts, and enterocytes in which microvilli are observed that cover the mucosal surface of the small intestine, irrigated by a network of blood vessels. These increase in size or length of the surface area to achieve the absorption of nutrients, so they play an important role in health [16]. Intestinal crypts are critical for nutrient absorption and immune defense, so these results highlight the role of probiotics in supporting intestinal health and function in piglets. Although the number of crypts in the PL/H-010305 supplemented treatment group was lower, the crypts were larger and more robust, which could reflect tissue remodeling rather than simple proliferation. This suggests that probiotics could stimulate the maturation of existing crypts, improving their function and resilience. According to various works, probiotic supplementation in piglets is effective in preventing, controlling, and treating infections, in addition to positively influencing the modulation of the immune response, intestinal function, and growth rate. In the initial phase of growth, there is a beneficial effect on the intestinal mucosa, on immunohematological parameters, as well as effects against pathogens. However, the benefits obtained with the use of probiotics may vary at each stage of the animal's life [2].

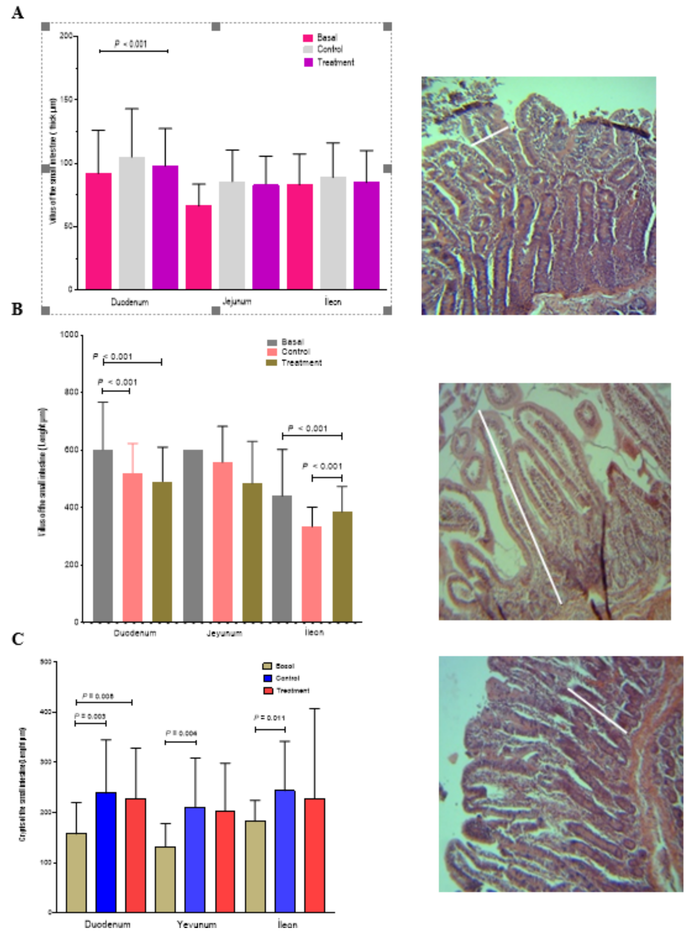

Figure 4: Morphogenic analysis of small intestinal segments. Images were taken using an OPTIKA microscope/LEITZ-WETZLAR photo camera. The white line shows the measurement criteria.

Conclusion

Probiotic supplementation in animal feed can be a favorable alternative for piglet development and weight gain. Undoubtedly, the consumption of probiotics can bring important changes in pig nutrition due to the benefits that they provide to animal welfare. However, although probiotics have been used for many years in pig farming, the mechanism of probiotics in the prevention and treatment of pig diseases still requires further research to pinpoint the effects of probiotics for better use in the pig farming industry.

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