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Integrative analysis of probiotic-mediated remodeling in canine gut microbiota and metabolites using a fermenter for an intestinal microbiota model

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Abstract

In contemporary society, the rising number of pet-owning households has significantly heightened interest in companion animal health, catalyzing the expansion of the probiotics market aimed at enhancing pet well-being. This burgeoning interest has propelled research into the gut microbiota of companion animals, although the breadth of research in this area is still evolving. Given the ethical and societal challenges associated with experiments on highly intelligent and pain-sensitive animals, there is an imperative need for alternative research methodologies that diminish reliance on live animal testing. Against this backdrop, the Fermenter for Intestinal Microbiota Model (FIMM) is investigated as a practical in vitro tool designed to replicate the gastrointestinal conditions of living animals in this study, offering a means to study the gut microbiota while minimizing animal experimentation. The FIMM system was employed to explore the interactions between intestinal microbiota and probiotics within a simulated gut environment. Two strains of commercial probiotic bacteria, *Enterococcus faecium* IDCC2102 and *Bifidobacterium lactis* IDCC4301, along with a newly isolated strain from domestic dogs, *Lactobacillus acidophilus* SLAM AK001, were introduced into the FIMM system in conjunction with the gut microbiota from a beagle model. Findings underscore the system's capacity to effectively mirror and modulate the gut environment, evidenced by a marked increase in beneficial bacteria like *Lactobacillus* and *Faecalibacterium* and a decrease in the opportunistic pathogen *Clostridium*. This study also verified the system's ability to facilitate accurate interactions between probiotics and commensal bacteria, demonstrated by the expected production of short-chain fatty acids and critical bacterial metabolites, including amino acids and GABA precursors. Thus, the results advocate for the application of FIMM as an in vitro cultivation system that authentically simulates the intestinal environment, presenting a viable alternative for examining the dynamics of gut microbiota and metabolites in companion animals.

Keywords: in vitro culturomics; lactic acid bacteria; canines; FIMM; microbiome

Introduction

The gut microbiome, an intricate community of microorganisms inhabiting the gastrointestinal tracts of animals, exerts a profound influence on the health and well-being of its hosts. The critical role of the gut microbiome in human health has been well-documented, leading to a parallel increase in research focusing on the microbiological aspects of both industrial and domestic animal health (Lee et al., 2023; Song et al., 2023). This burgeoning field, situated at the intersection of microbiology and veterinary science, explores how dietary components, particularly probiotics, influence the gut microbiota, contributing to enhanced health and growth in animals (Lee et al., 2022b; Quinn et al., 2015). The incorporation of probiotics into pet diets aims not only to maintain a balanced microbial ecosystem but also to enhance immune function and provide therapeutic benefits in various conditions, including gastrointestinal disorders and resistance to antibiotics. The rising awareness of these benefits has spurred a notable expansion in the probiotics market, tailored to meet the nutritional needs of companion animals, with a significant emphasis on gram-positive bacterial strains like *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, and *Streptococcus* (Harel and Tang, 2023; Lee et al., 2022a; Mugwanya et al., 2021).

Despite the valuable insights gained from animal-based microbiological research, such studies are fraught with ethical, logistical, and financial challenges (Lee et al., 2022a; Mun et al., 2021). The ethical debate surrounding animal experimentation, especially with animals that exhibit high levels of intelligence and sensitivity to pain, underscores the necessity for humane and sustainable research methodologies. Additionally, the limitations inherent in animal models, particularly in their ability to accurately replicate complex human diseases or conditions, highlight the need for innovative research approaches that can offer reliable and ethically sound alternatives.

In response to these challenges, this study introduces the Fermenter for Intestinal Microbiota Model (FIMM), an advanced in vitro tool engineered to replicate the physiological conditions of the animal gastrointestinal tract, including optimal pH, temperature, and resistance time. The FIMM system offers a distinctive platform for examining the interactions between probiotics and gut microbiota under controlled

conditions, allowing for the exploration of these intricate relationships without the ethical and logistical complexities associated with live animal testing.

In this study, a meticulous selection of probiotic strains was employed to elucidate the operational dynamics of the FIMM system. Two commercial probiotic strains, *Enterococcus faecium* IDCC2102 and *Bifidobacterium lactis* IDCC4301 (Kang et al., 2024), along with *Lactobacillus acidophilus* SLAM AK001 (Kang et al., 2022), a strain newly isolated from domestic dogs, were integrated into the FIMM system. This integration was performed alongside gut microbiota sourced from a laboratory beagle model, selected for its uniform living conditions, diet, and species consistency, which are crucial for minimizing experimental variability. The incorporation of diverse probiotic species aims to provide a comprehensive understanding of the FIMM's capability to simulate the canine gastrointestinal environment accurately. This approach is designed to not only test the system's efficacy in replicating complex gut microbial interactions but also to evaluate the potential influence of these probiotics on the gut microbiota within a controlled, in vitro setting. Through this strategic selection of probiotic strains and a well-defined animal model, the study endeavors to enhance the precision and applicability of the FIMM, contributing valuable insights into the interplay between probiotics and gut microbiota, and ultimately facilitating the development of more targeted and effective strategies for animal health and nutrition.

Materials and Methods

Bacterial cultivation and study design

In this study, fecal samples were collected from domestic canines (n=3; Maltese and Jindo) aged between 6-8 years old. These samples were subsequently pooled for analysis. The strain *Lactobacillus acidophilus* SLAM AK001 (LA), isolated from domestic canines, was identified in a prior investigation (Kang et al., 2022). Additionally commercial strains *Enterococcus faecium* IDCC 2102 (*E.faecium* IDCC2102) and *Bifidobacterium lactis* IDCC4301 (*B. lactis* IDCC 4301) were supplied by ILDONG Bioscience CO., LTD (Gyeonggi-do, Republic of Korea). To culture these probiotic strains, de Man, Rogosa & Sharpe (MRS; BD Difco, Franklin Lakes, NJ, USA) medium was utilized. The culturing process lasted 48 hours at a temperature of 37 ℃ under aerobic conditions. The collection of samples and subsequent experimentation involving domestic canines and laboratory-raised beagles were carried out with the endorsement of the Institutional Animal Care and Use Committee (IACUC) at Chungnam National University (202109A-CNU-149).

Culturomic analysis

In this research, culturomic and metagenomic techniques were employed to identify prevalent lactic acid bacteria within the gut microbiota of domestic dogs, specifically Maltese and Jindo breeds (n = 3), aged between 6 to 8 years. Fecal samples were meticulously collected, with 10 grams from each sample being aseptically transferred into a sample bag (3 M, St. Paul, MN, USA). Each sample was then diluted with 90 mL of 0.1% buffered peptone water (Oxoid, Hampshire, UK) and subjected to homogenization by stomaching for two minutes at speed level 10. The resulting homogenate was serially diluted and inoculated onto various selective media, including MRS (BD Difco), phenylethyl alcohol agar (PEA; BD Difco), and Bifidobacterium selective agar (BS; BD Difco) plates, which were further enriched with 7.5% BactoTM Agar medium (BD Difco). These plates were incubated under both aerobic and anaerobic conditions at 37 ℃ for 48 hours to promote bacterial growth (Cho et al., 2022; Choi et al., 2016; Sornplang and Piyadeatsoontorn, 2016). The lactic acid bacteria isolated were then prepared for further experimental use,

underpinning the study's objective to explore the gut microbiota dynamics and probiotic interactions within the FIMM system.

Fermenter for intestine microbiota model (FIMM)

The Fermenter for Intestinal Microbiota Model (FIMM) is an advanced in vitro system designed to simulate the canine gastrointestinal environment, facilitating detailed studies of gut microbiota interactions. This system was developed based on methodologies outlined in our previous study (Kang et al., 2022), and took inspiration from the well-established Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model (Van de Wiele et al., 2015). For the incubation of canine fecal samples within the FIMM system, pooled feces of laboratory raised beagles (n=6) were aseptically homogenized in filter bags using a stomacher (JumboMix, Interscience, Saint Nom, France). Following homogenization, the supernatant was collected and introduced into the FIMM medium at a 10% inoculation rate. Concurrently, the selected probiotics—*Lactobacillus acidophilus* SLAM AK001, *Enterococcus faecium* IDCC2102, and *Bifidobacterium lactis* IDCC4301—were inoculated to achieve a final concentration of 1% within the system. The FIMM medium employed in these experiments was based on a modified Gifu Anaerobic Medium (mGAM; HIMEDIA, DB Maarn, Netherlands), recognized for its suitability in cultivating anaerobic bacteria (Javdan et al., 2020). To closely mimic the conditions of the canine gut, the medium's pH was adjusted to 7.3, and the temperature was maintained at 38℃, aligning with the physiological parameters noted in canine intestinal research (Sagawa et al., 2009; Tochio et al., 2022). Through this meticulous replication of the canine gut environment, the FIMM system provides a robust platform for investigating the complex dynamics of gut microbiota and the impact of probiotics on gastrointestinal health.

Metagenomic analysis

After the FIMM incubation, the cultivates were collected, and genomic DNA was extracted with the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). The 16S rRNA gene, including the V4 region, was amplified, and the PCR product was sequenced using iSeq 100 (Illumina, Inc. San Diego, CA, USA) following the manufacturer’s protocols. The amplicon primer sequences were as follows: 515F,

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA; 806R, GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT for the 16S rRNA gene including the V4 region. The correlations and taxonomy of the obtained pair-end sequences were analyzed using Mothur v. 4.18.0 following the standard operating procedure suggested by the Schloss laboratory (Kozich et al., 2013; Son et al., 2021) and demonstrated using GraphPad Prism v. 9.4.1 (GraphPad Software, LLC, San Diego, CA, USA). For the comparative analysis, the study utilized alpha diversity metrics, notably the Chao and Shannon indices, to reveal patterns of relative abundance across different groups. This approach provided a deeper understanding of microbial diversity. Additionally, Principal Coordinates Analysis (PCoA) diagrams, based on both weighted and unweighted UniFrac distances, were developed to illustrate the spatial distribution of the fecal microbiome samples.

Metabolomic analysis

The samples were cultivated in triplicate on FIMM medium before being separated into pellets for metagenomics analysis and supernatants for metabolite analysis. A PVDF syringe filter with a pore size of 0.2 m was used to filter the supernatants. Samples of 200 µl of the filtered supernatant were dried in a vacuum concentrator and kept at -81 °C for GC–MS analysis. Derivatization of the extract involved 30 µL of 20 mg/mL methoxyamine hydrochloride in pyridine (Sigma) at 30 ℃ for 90 min, followed by 50 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma) at 60 ℃ or 30 min. The internal standard fluoranthene was added to the extract. A Thermo Trace 1310 GC (Waltham, MA, USA) and Thermo ISQ LT single quadrupole mass spectrometer were used for GC−MS analysis. A 60-m DB-5MS column (Agilent, Santa Clara, CA, USA) with 0.2-mm i.d. A 0.25-µm film thickness was utilized for separation. The sample was injected at 300 ℃ with a 1:60 split ratio and 90 mL/min helium split flow for analysis. The metabolites were separated using 1.5 mL continuous flow helium in an oven ramp from 50 ℃ (2 min hold) to 180 ℃ (8 min hold) at 5 ℃/min, 210 ℃ at 2.5 ℃/min, and 325 ℃ (10 min hold) at 5 ℃/min. The mass spectra were obtained at 5 spectra per second from 35-650 m/z. Electron impact and 270 ℃ ion source temperature were used in ionization mode. The metabolites were identified by comparing the mass spectra

and retention indices of the NIST Mass spectral search tool (version 2.0, Gaithersburg, MD, USA) with Thermo Xcalibur software's automatic peak detection. The fluoranthene internal standard intensity standardized the metabolite data(Jung et al., 2023; Ku et al., 2023; Liu et al., 2023; Muhizi et al., 2022).

Isolation of primary intestinal epithelial cells and adhesion assay

The experiment began with the retrieval of intestines, which were then placed in ice-cold HBSS devoid of Mg and Cl ions (Gibco, NY, USA). These intestines underwent meticulous cleaning to eliminate mesenteric fat and external mucus. Subsequently, the duodenal tract was harvested, longitudinally opened, cut into 1-2 mm pieces, and rinsed in ice-cold HBSS. The prepared tissue pieces underwent a 30-minute digestion at 37 ℃ using digestion medium. After the digestion process, the tissue was subjected to centrifugation at 100 × g for 3 min, and the resulting pellet was resuspended in a 37 ℃ washing medium. The resuspended pellet was subsequently filtered through a 100 μm cell strainer, followed by a second filtration using a 40 μm cell strainer in reverse. The aggregates recovered from the filtration were resuspended in basal medium. These aggregates were then diluted to a concentration of 0.8 mg/ml, with a density of 1000 aggregates per well, and plated in 24-well plates coated with a Matrigel matrix (Corning, NY, USA). The cells were cultured at 37 ℃ with 5% CO_2 for 24 h. During this time, the cell clusters were identified, and the degree of endothelial cell contamination was assessed. The cultures were meticulously washed with HBSS to eliminate unattached and dead cells, and any foci of proliferating enterocytes were replenished with fresh medium. Different growth factors were introduced at specific time points following seeding. For passaging, trypsin-EDTA was employed, and the cells were seeded into newly coated wells at a density of $3.5x10^5$ cells per cm^2 (Ghiselli et al., 2021; Marks et al., 2022).

Before the adhesion assay, primary cell monolayers were washed 3 times with PBS to remove culture medium and nonattached cells. Bacterial strains were treated with medium without FBS and incubated at 37 ℃ for 2 h in an atmosphere of 5% CO2. After 2 h, the monolayers were washed 5 times with PBS to remove the nonattached bacteria. The attached cells were lysed using trypsin-EDTA. Serial dilutions of the mixture were plated on MRS agar and incubated at 37 ℃ for 48 h. The adhesion ability was determined by

206 counting CFU/mL. *Lacticaseibacillus rhamnosus* GG was used as a positive control.

207

208 **Statistics**

209 This study used triplicate data points, expressed as the mean ± standard deviation, and determined
210 significant differences using Student's t test, one-way ANOVA, and SigmaPlot 13 (GraphPad Software,
211 CA, USA), followed by Tukey's post hoc test. The abundance of metabolites of each sample was analyzed
212 using the M^2IA server (http://m2ia.met-bioinformatics.cn/) and MetaboAnalyst 5.0
213 (https://www.metaboanalyst.ca).

214

Results and discussion

Metagenomic and culturomic analysis of domestic canines

To identify prospective probiotic candidates that may be beneficial to canines, we compared the culture-dependent and culture-independent gut microbiota of domestic canines. In pursuit of practical insights in culturomic analysis, an assortment of 138 distinct lactic acid bacteria was collected from three distinct media types. These isolates belonged to twenty different species. Fig. 1A illustrates that the four predominant bacterial species were as follows: *Enterococcus hirae* (5.1%), *Lactobacillus acidophilus* (21.7%), *Lactobacillus agilis* (13.8%), and *Ligilactobacillus animalis* (6.5%). We determined that the potential spectrum of probiotics should be restricted to *Lactobacillus* species, as they comprised the majority of the bacteria that were isolated (Fig. 1B, C). To achieve this, we monitored the number of *Lactobacillus* species that overlapped between the culturomic and metagenomics methodologies. As shown in a Venn diagram (Fig. 1D), four species of *Lactobacillus* (*L. acidophilus*, *L. amylolyticus*, *L. fermentum*, and *L. murinus*) were identified through both culturomic and metagenomics analyses. In light of this result, we sought to investigate what probiotic changes *L. acidophilus* SLAM AK001, which has the highest proportion, could make through FIMM incubation.

FIMM incubation increased the microbial diversity

The study meticulously analyzed the effects of FIMM incubation on microbial diversity by integrating specific canine-derived probiotics, *L. acidophilus* SLAM AK001, and marketed probiotics, *E. faecium* IDCC 2102, and *B. lactis* IDCC 4301 were integrated into the FIMM system with fecal samples from laboratory-raised beagles to simulate the gut environment and assess the ensuing microbial alterations. Utilizing next-generation sequencing, the research identified a comprehensive array of 46,016 operational taxonomic units (OTUs) and 872 distinct taxonomic bacterial entities. Through the application of the alpha-diversity index, specifically the Chao and Shannon indices, a significant elevation in species diversity was observed (Chao and Shen, 2003). The Chao index revealed a 46.9 ± 7.4% enhancement in mean species diversity attributable to the FIMM incubation, with an additional increase of 103.6 ± 31.6% following

probiotic supplementation. Concurrently, the Shannon index recorded a 23.83 ± 5.1% rise in diversity post-FIMM incubation, and a further augmentation of 66.1 ± 1.8% with the introduction of probiotics (Fig. 2A). Moreover, the diversified microbiota was found to be unique to each other according to the beta diversity analysis. Unweighted and weighted UniFrac used in beta diversity represent qualitative and quantitative variants, respectively. Each plot represents a relative abundance of species of a group, and the distance between the plots represents distinctiveness (Koleff et al., 2003). From our study, the beta diversity analysis, employing both unweighted and weighted UniFrac methods, illustrated distinctive microbial assemblages resulting from FIMM incubation relative to the control, and a unique microbial configuration associated with the probiotic intervention (Fig. 2B). These results highlight the FIMM system's capability to not only enhance microbial diversity but also to cultivate specific microbial community contingent on the introduced probiotic strains.

The supplementation with probiotics plays a pivotal role in enhancing the diversity of gut microbiota, a factor that is intrinsically linked to the overall health of the host. The gut microbiota's diversity is crucial, starting with its fundamental role in the digestion and absorption of nutrients. The myriad of microorganisms residing in the gastrointestinal tract play a critical role in breaking down a broad spectrum of dietary fibers and nutrients, leading to enhanced nutrient uptake and improved digestive efficiency (Yu et al., 2022; Zhong et al., 2023). This microbial diversity extends its benefits beyond digestion to bolster the immune system. It orchestrates a range of immune responses, strengthening the host's defense mechanisms against opportunistic and pathogenic microbes. The balanced interplay among various microbial strains is also vital for regulating inflammatory processes, potentially reducing the incidence of inflammation-related disorders and supporting metabolic health and weight management (Kim et al., 2020; Liu et al., 2018; Ritchie and Romanuk, 2012; Sánchez et al., 2017). The FIMM experiments provided insightful data, demonstrating that the in vitro fermentation process could enrich the diversity of bacterial strains within the canine gut microbiota. This enhancement closely mirrors the beneficial effects observed with probiotic consumption in vivo. The distinctive clustering patterns observed in the FIMM system, which varied with each bacterial strain, offer evidence of the system's ability to foster specific interactions and associations within the microbial community. These findings underscore the potential of FIMM as a

valuable model for exploring the intricate dynamics of gut microbiota and the impact of probiotics, offering a deeper understanding of how probiotic supplementation can modulate microbial ecosystems to support host health.

FIMM incubation altered the microbial composition

The investigation into the impact of the FIMM incubation on microbial composition revealed significant alterations in the fecal microbiota, which might have been affected during sample collection. An in-depth examination of the 15 most abundant genera demonstrated that FIMM incubation induced notable changes in microbial composition. Specifically, when the FIMM system was supplemented with probiotics *L. acidophilus* SLAM AK001, *E. faecium* IDCC 2102, and *B. lactis* IDCC 4301, there was a substantial shift in microbial communities compared to the control group. Probiotics significantly increased the populations of *Ruminococcus, Blautia, Dorea,* and lactic acid bacteria, such as *Lactobacillus* and *Faecalibacterium* (Grześkowiak et al., 2015; Lee et al., 2022a). These genera are recognized as beneficial commensal probiotics in canines. Concurrently, there was a reduction in the abundance of potential opportunistic pathogens, including *Clostridium* (Ghose, 2013), *Streptococcus* (Xu et al., 2007), and *Prevotella* (Larsen, 2017) (Fig. 2C), showcasing the probiotics' ability to modulate the gut microbiota favorably. Noteworthy is the observation that the microbial changes induced by *L. acidophilus* SLAM AK001 were in alignment with those noted in an in vivo canine model previously studied by our group (Kang et al., 2024; Kang et al., 2022), suggesting that this strain's effects are consistent across different experimental settings. This consistency enhances the validation of the FIMM system as a reliable model for studying probiotic effects.

Lactobacillus, Bifidobacterium, and *Enterococcus* are well-established probiotics. In a prior study, supplementation with *Lactobacillus* and *Bifidobacterium* was found to reduce *Clostridium* and increase commensal bacteria such as *Faecalibacterium* and *Lactobacillus* in individuals with conditions such as diarrhea, inflammatory bowel diseases, and colorectal cancer (Alcon-Giner et al., 2020; Gerasimov et al., 2016; Lopez-Siles et al., 2017). While research in canines is relatively limited compared to human studies, the administration of *Enterococcus faecium* and *Bifidobacterium* in canines also resulted in an increase in *Lactobacillus, Enterococcus,* and *Enterobacteriaceae* while reducing the presence of *Salmonella,*

296 *Campylobacter*, and *Clostridium* (Sabbioni et al., 2016; Vahjen and Männer, 2003). The FIMM cultivation
297 method employed in this study mimics the probiotic effects of *L. acidophilus* SLAM AK001, *E. faecium*
298 IDCC 2102, and *B. lactis* IDCC4301, as observed in real-life scenarios where they are administered to
299 mammals. This underscores the reliability of the FIMM in vitro cultivation system.
300 Overall, the FIMM system's ability to mimic real-life probiotic effects in an in vitro setting underscores
301 its potential as a valuable tool for exploring the intricate dynamics of gut microbiota and assessing the
302 impacts of various probiotic strains on microbial communities. This system offers a promising avenue for
303 advancing our understanding of probiotic interactions within the gut ecosystem, providing insights that
304 could inform the development of targeted probiotic therapies for canines.
305
306 **FIMM incubation can imply changes in intestinal robustness**
307 Additionally, the FIMM incubation process not only influenced the microbial composition but also
308 significantly impacted the metabolic profile within the system, suggesting changes in intestinal robustness.
309 Detailed metabolic analysis categorized nine distinct types of metabolites: alcohols, alkylamines, amino
310 acids, carbohydrates, fatty acids, indoles, lipids, nucleotides, organic acids, and others. Remarkably,
311 compared to the control group, the introduction of probiotics led to an overall increase in these metabolites,
312 with notable surges in amino acids and organic acids, including 4-hydroxybutyric acid, L-norleucine, and
313 isovaleric acid. This metabolic enhancement, particularly in essential amino acids such as isoleucine,
314 leucine, lysine, methionine, phenylalanine, and valine, underscores the broad-reaching impact of probiotics
315 on metabolic processes. These amino acids are vital for protein synthesis across all living organisms,
316 indicating a systemic effect of probiotic treatment on fundamental biological functions (Amorim Franco
317 and Blanchard, 2017; Neis et al., 2015; Oh et al., 2021; Yoo et al., 2022). Organic acids, integral to primary
318 metabolism, play pivotal roles in various biochemical pathways (Ramachandran et al., 2006; Sauer et al.,
319 2008; Vasquez et al., 2022). The FIMM incubation results showed that probiotic administration could
320 influence the production of key organic acids like propionic acid, acetic acid, and lactic acid (Fig. 3). These
321 acids are crucial for numerous metabolic processes, including energy production and regulatory functions
322 within the gut environment.

Bifidobacterium plays a significant role in the fermentation of dietary fibers and carbohydrates, resulting in the production of short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate. These SCFAs offer various health benefits, including serving as an energy source for colonocytes, promoting gastrointestinal health, and exhibiting anti-inflammatory properties(Kim et al., 2022). *Bifidobacterium*, as a type of lactic acid bacteria, generates lactic acid as a metabolic byproduct, which contributes to the maintenance of an acidic gut environment, thereby restraining the proliferation of pathogenic microorganisms (de Souza Oliveira et al., 2012; Pokusaeva et al., 2011). Moreover, select strains of *Bifidobacterium* have the capacity to synthesize gamma-aminobutyric acid (GABA), a neurotransmitter with potential anxiolytic and calming effects on the central nervous system (Duranti et al., 2020).

Likewise, *E. faecium*, another beneficial gut bacterium, also generates lactic acid as a predominant metabolic byproduct, reinforcing the acidic conditions of the gut, which can inhibit the proliferation of pathogenic bacteria. In addition, *E. faecium* can participate in the production of various SCFAs, including acetate, propionate, and butyrate, each of which has multiple health advantages, particularly in the context of gut health. *E. faecium* is also involved in the digestion and metabolic breakdown of dietary proteins, giving rise to the production of diverse amino acids (Allameh et al., 2017; Wang et al., 2020).

Finally, *L. acidophilus* primarily produces lactic acid as part of its metabolic processes, supporting the creation of an acidic gut environment that impedes the growth of detrimental bacteria and pathogens. While *L. acidophilus* SLAM AK001 may not be as widely recognized for its SCFA production as certain other bacterial strains, it does contribute to the production of SCFAs, particularly acetate and propionate (Chamberlain et al., 2022; Hossain et al., 2021). These findings underscore the significance of the metabolites generated within the FIMM cultivation system, demonstrating that the in vitro cultivation system provides the conditions necessary for proper metabolite production by different bacterial species.

The observed metabolic changes within the FIMM system, prompted by probiotic supplementation, mirror the potential enhancements in intestinal robustness and metabolic activity, which could have significant implications for gut health and overall organismal well-being. This insight into the metabolic alterations provides a deeper understanding of the multifaceted impacts of probiotics, extending beyond

microbial diversity to include metabolic function, thereby offering a comprehensive view of the probiotic influence on the gut ecosystem.

Probiotics for canines were very specific for canine primary intestinal epithelial cells.

In the conclusive segment of the study, an in-depth evaluation was conducted to ascertain the host specificity of the lactic acid bacteria used, a factor that is paramount in determining their potential effectiveness as probiotics in canine hosts. Host specificity is a critical attribute that influences a bacterium's ability to colonize and thrive within a specific host, impacting its probiotic efficacy and interaction with the host's gut microbiome (Chaib De Mares et al., 2017; Dogi and Perdigón, 2006). To assess this, a series of host specificity tests were carried out using primary intestinal epithelial cells derived from a diverse array of species, including but not limited to dogs, chickens, laying hens, humans, and pigs. The aim was to investigate the cell adhesion capabilities of the probiotic strains, which is indicative of their potential to colonize and establish within the host's gastrointestinal tract effectively. The study utilized the control strain, *L. rhamnosus* GG, known for its broad host specificity, as a comparative baseline, exhibiting an 88.3 ± 0.7% specificity rate across various cell types. A significant affinity for primary intestinal epithelial cells sourced from canines was observed among the probiotic strains, an insight depicted in Figure 4. This pronounced host specificity suggests these probiotics are well-suited for adherence and potential colonization within the canine gut. Specifically, *L. acidophilus* SLAM showcased the most substantial host specificity, with a rate of 81.3 ± 2.7% when interacting with canine cells. Similarly, *E. faecium* IDCC 2102 and *B. lactis* IDCC 4301 exhibited host specificity rates of 86.2 ± 1.9% and 88.3 ± 0.6%, respectively, with canine cells (Figure 4A). Notably, these strains maintained cell counts comparable to the original CFU (colony-forming units) before inoculation, underscoring their strong adherence capabilities to canine primary intestinal cell lines. Further analysis revealed that beyond canine cells, *L. acidophilus* SLAM AK001, *E. faecium* IDCC 2102, and *B. lactis* IDCC 4301 displayed host specificity rates of 74.5 ± 6.2%, 64.4 ± 13.4%, and 75.0 ± 9.5%, respectively, towards other primary intestinal epithelial cells. A marked decrease in cell adhesion capacity was noted in avian primary enterocytes compared to the initial CFU counts, highlighting a more constrained host specificity in these cell types (Figure 4B-D). This detailed examination underlines the significant host

specificity of canine-derived probiotics, positioning them as potent candidates for in-depth in vivo studies. Their targeted adherence to canine intestinal cells intimates that these probiotics may confer specific health benefits tailored to canines, underscoring their potential value in veterinary care and probiotic formulation development.

Conclusion

To conclude, this study was initiated with the objective of mitigating the limitations associated with microbial research in live animals while identifying potential probiotics beneficial for canines. Although animal studies are pivotal in scientific discovery and pharmaceutical advancements, they are fraught with ethical dilemmas and practical challenges. There's a pronounced emphasis on animal welfare, emphasizing the reduction of animal distress and the pursuit of alternatives to circumvent the need for animal sacrifice, a subject of considerable ethical discourse. Yet, the development of in vitro methodologies capable of fully emulating the living conditions of organisms remains nascent, with a clear demand for further exploration and standardization in this domain.

Thus, the core ambition of this research was to introduce and validate a standardized in vitro cultivation approach, termed the FIMM system. This research effectively showcased the FIMM system's capability to replicate the complex interactions between gut bacteria and their host, reflecting the dynamics observed when probiotics, specifically *L. acidophilus* SLAM AK001, *E. faecium* IDCC2102, and *B. lactis* IDCC4301, derived from canine fecal samples, were introduced into the system. To claim that the FIMM system perfectly emulates the canine gut microbiota system, it would have been ideal to administer these strains to actual canines and observe the resultant effects, a step that represents a limitation in this study. Nonetheless, the findings highlight the FIMM system's efficacy as a potent tool for in-depth gut microbiota research, enhancing our comprehension of probiotics' impacts on animal health. This advancement not only facilitates a more nuanced understanding of the gut microbiome but also opens avenues for developing targeted and efficacious probiotic interventions in veterinary practice.

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Figure legends

Fig. 1 The comparison of culturomic and metagenomic characterization of domestic canine fecal microbiota. (A) This list presents the bacteria isolated from canine feces, utilizing the aforementioned medium. Subsequent to the isolation, the compositions specific to *Lactobacillus* were subjected to further examination employing (B) culturomic and (C) metagenomic analyses. (D) A Venn diagram elucidates the distribution of *Lactobacillus* species, categorized by those identified through culturomics (purple), metagenomics (yellow), and the species identified by both methods (orange).

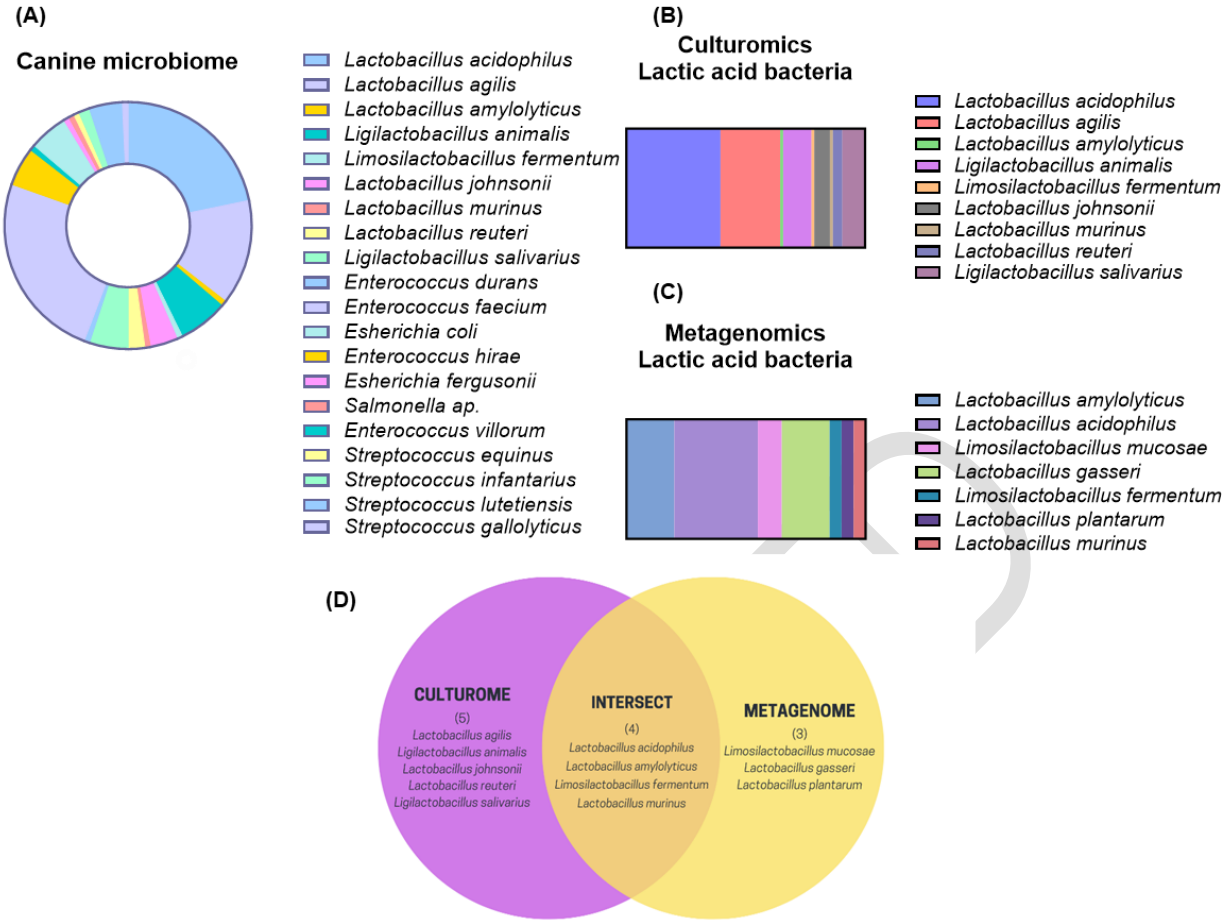
Fig. 2 The diversity and richness of fecal microbiota was altered through FIMM incubation with probiotics. The metagenomic analysis was utilized to elucidate the alterations in bacterial relative abundance subsequent to FIMM cultivation. Comparative analysis was conducted between FIMM cultivations subjected to probiotic interventions (LA, 2102, and 4301) and a control cohort devoid of any treatment (cont). (A) Indices of alpha diversity and (B) Principal Coordinates Analysis (PCoA) diagrams were constructed to elucidate the spatial distribution of fecal microbiome samples. These diagrams plot individual samples, with axes representing the principal dimensions capturing the maximal variance in microbial community structure across the groups. (C) The comparative representation of bacterial relative abundance at phylum, family, and genus levels across all groups was meticulously quantified. All values are expressed as the mean±SD; significant differences were determined using Student's t test and ANOVA compared to the cont at * $P < 0.05$ and *** $P < 0.001$. LA, *Lactobacillus acidophilus* SLAM AK001; 2102, *Enterococcus faecium* IDCC 2102; 4301, *Bifidobacterium lactis* IDCC 4301.

Fig. 3 Comparative analysis of unique metabolite production by probiotics in FIMM. Following the FIMM cultivation, variations in metabolite profiles across different groups were examined. (A) In the PCA score plots, the analysis revealed that fecal samples from groups subjected to probiotic interventions (LA, 2102, and 4301) clustered together, indicating a shared metabolic response. In contrast, the control group was distinctly clustered, highlighting significant metabolic differentiation from the treated groups. (B) The

Partial least squares discriminant analysis (PLS-DA) further analyzed these differences, identifying metabolites that drove the separation between treated and untreated groups. Additionally, the colored boxes in (B) and (C) categorized the top 50 abundant metabolites, with varying colors denoting concentration levels, offering an understanding of metabolite fluctuations resulting from FIMM cultivation and probiotic treatments. LA, *Lactobacillus acidophilus* SLAM AK001; 2102, *Enterococcus faecium* IDCC 2102; 4301, *Bifidobacterium lactis* IDCC 4301.

Fig. 4 Canine probiotics were highly selective to canine primary intestinal epithelial cells.

To investigate the host specificity of the probiotics, a cell adhesion assay was performed utilizing (A) canine primary intestinal epithelial cells, (B) porcine primary intestinal epithelial cells, and (C, D) avian primary intestinal epithelial cells. The assay determined host specificity by comparing the percentage of bacterial colony-forming units (CFUs) before and after a two-hour exposure to the seeded cells in 24-well plates. The blue bars in the graphical representation denote the CFU count of bacteria prior to exposure to each type of cell, while the purple bars indicate the CFU count of bacteria retrieved after the exposure. Each cell adhesion assay was conducted in triplicate wells. All values are expressed as the mean±SD; significant differences were determined using Student's t test and ANOVA, with each treatment's data at 2 hours compared to the baseline at 0 hour by **** $P < 0.0001$. LA, *Lactobacillus acidophilus* SLAM AK001; 2102, *Enterococcus faecium* IDCC 2102; 4301; *Bifidobacterium lactis* IDCC 4301; LGG, *lacticaseibacillus rhamnosus* GG.



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637 **Fig. 1.**

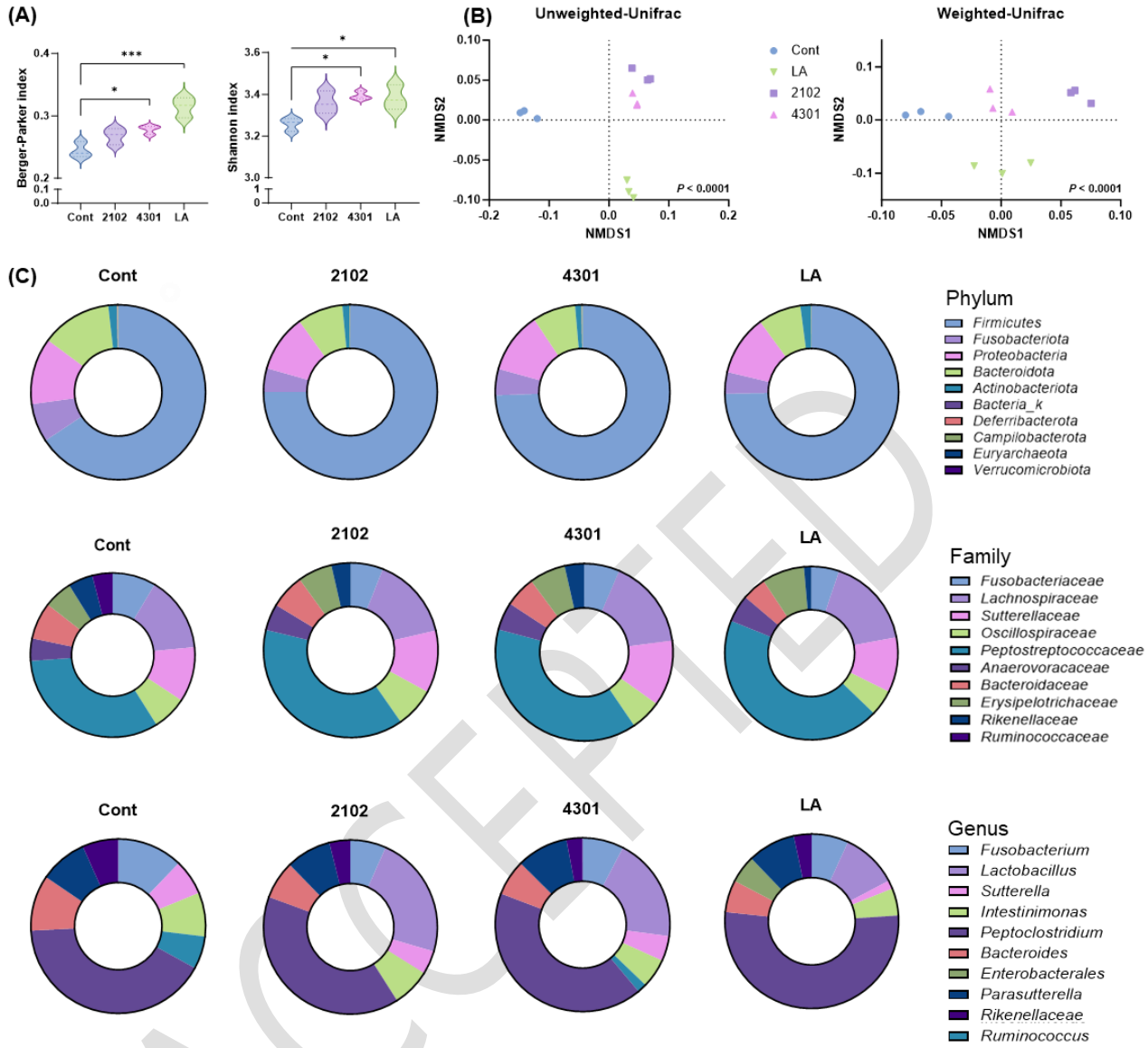
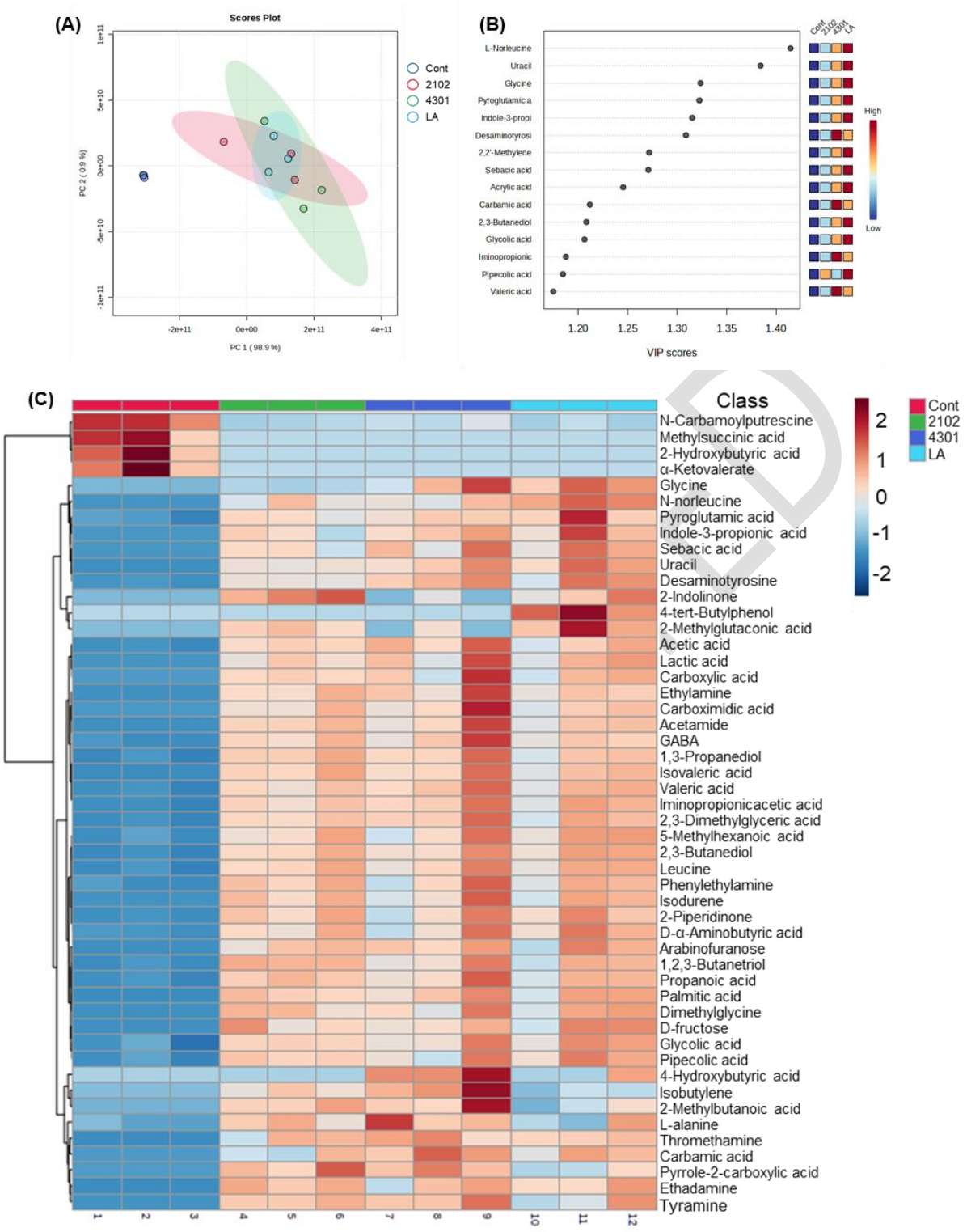


Fig. 2.



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642 **Fig. 3.**

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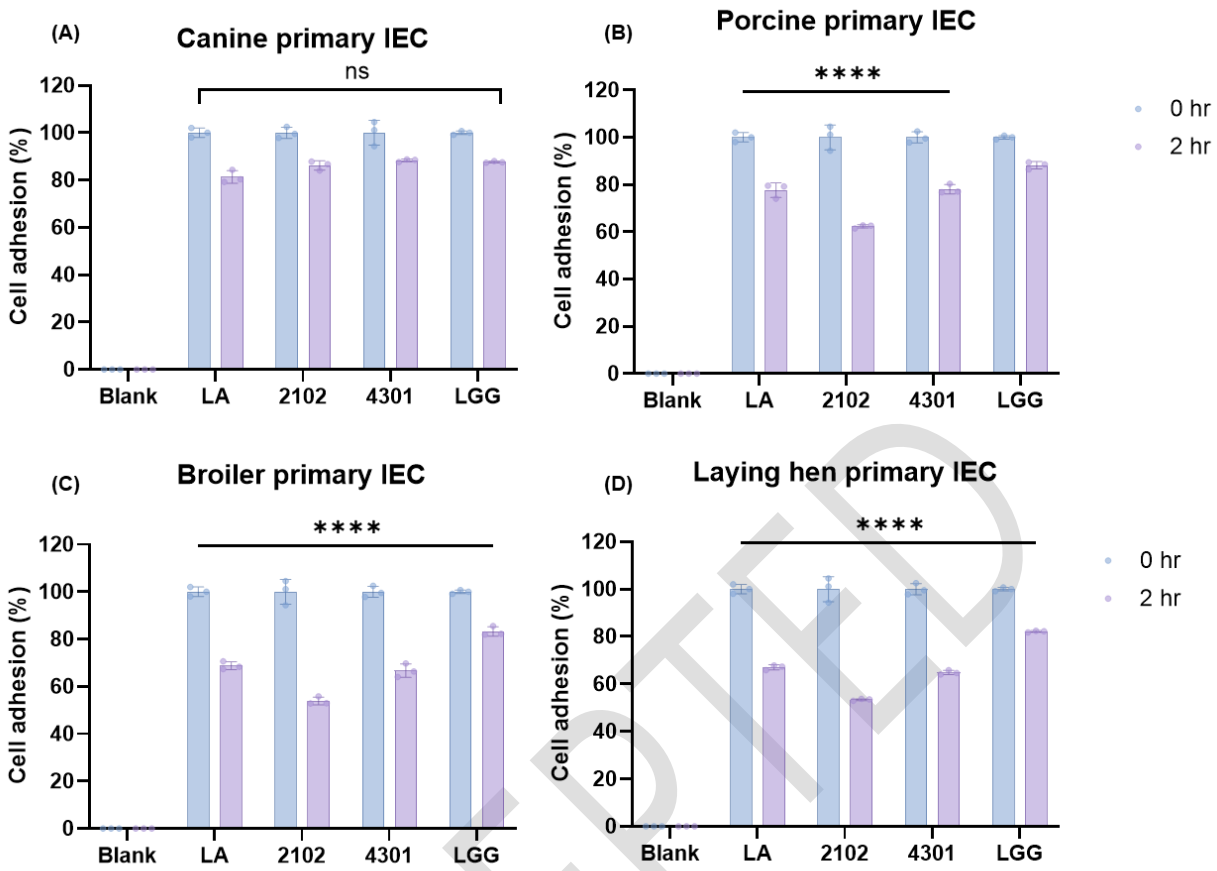

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646 **Fig. 4.**

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