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Article

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Nicholas L.F. Gallina [1,2,*,] **Nicole Irizarry Tardi** [1,2*,] **Xilin Li** [1,2], **Alvin Cai** [1,2], **Mandy J. Horn** [3], **Bruce M. Applegate** [2,4,5], **Lavanya Reddivari** [2,4,5] **Arun K. Bhunia** [1,2,4,5**]

[1] Molecular Food Microbiology Laboratory, Department of Food Science, Purdue University, West Lafayette, IN 47907, USA; ngallin@purdue.edu; irizarrn@purdue.edu; li2807@purdue.edu; cai325@purdue.edu; bhunia@purdue.edu

[2] Purdue Institute of Inflammation, Immunology, and Infectious Disease, Purdue University, West Lafayette, IN, USA

[3] CH2 Animal Solutions, 21 Bear Creek Estates Dr. Ottumwa, IA 52501, USA; Mandy.Horn@ch2animalsolutions.com

[4] Purdue University Interdisciplinary Life Sciences Program, Purdue University, West Lafayette, IN 47907, USA; applegate@purdue.edu

[5] Department of Food Science, Purdue University, West Lafayette, IN 47907, USA; lreddiva@purdue.edu

[6] Department of Comparative Pathology, Purdue University, West Lafayette, IN 47907, USA

* Equal contributors

** Correspondence**:** bhunia@purdue.edu

Abstract: Gut dysbiosis and an inflamed bowel are growing concerns in mammals, including dogs. Probiotic supplements have been used to restore the natural microbial community and improve gastrointestinal health. Biofilm formation, antimicrobial activities, and immunological responses of probiotics are crucial to improving gut health. Thus, we tested a commercial probiotic blend (LabMAX-3), a canine kibble additive comprising *Lactobacillus acidophilus*, *Lacticasibacillus casei*, and *Enterococcus faecium* for their ability to inactivate common enteric pathogens; their ability to form biofilms, epithelial cell adhesion and anti-inflammatory response on Madin-Darby Canine Kidney (MDCK) cell line. Probiotic LabMAX-3 blend, or individual isolates, showed a strong inhibitory effect against *Salmonella enterica*, *Listeria monocytogenes*, enterotoxigenic *Escherichia coli*, and *Campylobacter jejuni*. LabMAX-3 formed biofilms comparable to *Staphylococcus aureus*. LabMAX-3 adhesion to the MDCK cell line (with or without lipopolysaccharide, LPS) pretreatment showed comparable adhesion and biofilm formation ($p < 0.05$) to *L. casei* ATCC 334 used as a control. LabMAX-3 had no cytotoxic effects on the MDCK cell line during 1 h exposure. The IL-10 and TNFα ratio of LabMAX-3, compared to the *L. casei* control, showed a significant increase ($p < 0.05$), indicating a more pronounced anti-inflammatory response. The data show that LabMAX-3, a canine kibble supplement, can improve gastrointestinal health.

Keywords: probiotics; biofilm; antimicrobial activity; adhesion; pathogens; canine gut health; anti-inflammatory response

1. Introduction

Probiotic application is widespread in humans and animals for their anti-inflammatory and immunomodulatory response, as well as improved gut health [1-3]. The definition of probiotic in the root sense means "for life," and the critical expectation is that live and active cultures should be delivered in substantial amounts to observe health benefits [4]. Probiotics are Gram-positive bacilli or cocci with oxygen requirements ranging from obligate to facultative anaerobes. Some genera, such as *Bacillus*, are spore formers. Lactic acid bacteria (LAB) are non-spore formers, and some are probiotics and predominant residents of the gut [5]. Probiotics have desirable attributes that benefit the host, such as producing short-chain fatty acids (SCFAs), immunomodulation, surviving acidic environments, pathogen exclusion, and influencing the microbiome composition [6, 7]. Probiotics are now used as an additive to kibble and wet foods for companion animal gut health [2, 8]. Their

beneficial effects are either transient or ineffective [2, 9] primarily due to their inability to colonize the inflamed bowel [10-13].

In a mammalian dysbiotic gut, pockets of inflammation and dysregulated epithelial cell tight junction permeability are evident [14]. The inflamed state of the bowel propels the reconstruction of the enteric flora to that of a negative state, termed dysbiosis [15]. Canine gut dysbiosis is marked by pockets of inflammation and an imbalance in the standard phyla of bacteria *Firmicutes, Fusobacteria, Bacteroidetes, Proteobacteria,* and *Actinobacteria* [16, 17]. Their metabolic byproducts, such as SCFAs, reduce intestinal inflammation and regulate the commensal community [17].

Canines are exposed to numerous pathogenic and environmental insults that make them vulnerable to illness. Dog food has been linked to multiple outbreaks associated with *Salmonella enterica, Listeria monocytogenes, Escherichia coli,* and other pathogens [18-20]. Using probiotic bacteria with their beneficial properties as a canine food additive is one of the attractive methods of reducing pathogen exposure, as well as antibiotic use and promoting gut health [13].

Probiotic bacteria have been used in human food or animal diets to prevent pathogen colonization and gut health through competitive exclusion [21-23]. Probiotic *Bacillus* lipopeptides have been used to eliminate *S. aureus* colonization within a rural Thai population [24, 25]. *Lacticaseibacillus rhamnosus* was shown to produce acetic acid and surfactants that reduce biofilm formation by *Acinetobacter baumannii* [26]. *Lacticaseibacillus rhamnosus* GG was also shown to reduce *Salmonella enterica* Typhimurium colonization in a heat-stress cell culture model [27]. Probiotics have also been used to sequester mycotoxin from feed [28-30].

The addition of probiotics as a canine food additive has gained notoriety for promoting the overall health of the companion animal [2]. Clinical trials for probiotics in pet food have been carried out, and several products on the market contain stable probiotics that may promote pathogen clearance and improve gut health while promoting overall animal fitness [31].

Natural probiotics may have limited capabilities of colonizing the inflamed-damaged tissue; therefore, recombinant bioengineering has been used as a novel strategy to enhance probiotic epithelial adhesion, biofilm formation, immunomodulation, and pathogen colonization resistance [10, 11, 32-35]. Nevertheless, genetically engineered and native probiotics possess the same inherent properties for pathogen exclusion, such as nutrient partitioning, biofilm formation, and clusters of abundance within the gastrointestinal (GI) tract. This is an intrinsic property of the gut flora [36].

Biofilm formation by pathogenic or beneficial bacteria aids in their adhesion and colonization to biotic and abiotic surfaces [37-39]. Since probiotics are natural gut inhabitants, they can form a biofilm on the mucosal surface, often in mixed communities, which is a vital and desirable attribute [39]. During biofilm formation, bacteria communicate via the messaging system termed quorum sensing [39-41]. Probiotic biofilms promote gastrointestinal healing, community structure, and immunomodulatory response [42]. Bacteria produce exopolysaccharides, proteins, and extracellular DNA to aid in biofilm formation [43]. Probiotic-based biofilms are often localized in specific pockets within the GI tract. They can competitively inhibit pathogen colonization, such as seen with the *Listeria monocytogenes* challenge in a mouse model [10]. Furthermore, biosurfactants from *Lactobacillus jensenii* and *Lacticaseibacillus rhamnosus* have shown antimicrobial and antibiofilm formation properties against clinically relevant strains of *Acinetobacter baumannii, Escherichia coli* and *Staphylococcus aureus* (MRSA) [44]. A cocktail of *Lactobacillus fermentum* and *Lactiplantibacillus plantarum* could also reduce *S. aureus* (MRSA) biofilm production [45].

Therefore, this study evaluated the antimicrobial activities, epithelial cell adhesion, biofilm formation, and anti-inflammatory response of a canine probiotic blend (LabMAX-3). Madin-Darby Canine Kidney (MDCK) cell line was used as a gut epithelial cell model. MDCK cells are derived from the renal tubule of the canine kidney and display enterocyte-like absorbing properties [46, 47]. Our data show that the canine kibble probiotic additive with biofilm-forming capabilities, robust antimicrobial activities, and anti-inflammatory response can potentially improve canine gastrointestinal health.

2. Materials and Methods

2.1. Probiotic Preparation

A commercial proprietary probiotic blend, LabMAX-3, containing three probiotic strains of *Enterococcus faecium*, *Lactobacillus acidophilus,* and *Lacticaseibacillus casei*, was received from CH2 Animal Solutions (Ottumwa, IA, USA). The lyophilized powder was plated on DeMann Rogosa Sharpe (MRS, Thermo Fisher Scientific, Walthan, MA, USA) agar plates and incubated at 37°C for 48 h under anaerobic conditions using a Gaspak (Thermo Fisher Scientific). The colonies were picked based on phenotype, and each bacterial culture morphology was verified by microscopy (Leica, Deerfield, IL, USA). *L. acidophilus* NRRL 31910, *Pediococcus acidilactici* H and *E. faecium* ATCC 8459, as controls, were propagated in MRS under anaerobic conditions. *Lacticaseibacillus casei* ATCC 334 was cultivated in modified MRS agar (MMRS) containing 1% w/v proteose peptone, 0.5% w/v yeast extract, 0.1% v/v Tween 80, 0.2% w/v meat extract, 37 mM $C_2H_3NaO_2$, 8.8 mM $C_6H_{14}N_2O_7$ dissolved in 0.2 M potassium phosphate (dibasic, pH 7.0), 0.8 mM $MgSO_4$, 0.24 mM $MnSO_4$ and 1% w/v mannitol and supplemented with erythromycin (2 µg/mL, Thermo Fisher Scientific) and grown in anaerobic conditions [10].

Table 1. Bacterial cultures used in the study.

Bacteria	Identification	Source	Provided By
Enterotoxigenic *Escherichia coli*	F4 (K88)	Swine rectal isolate	Paul Ebner/Animal Sciences Purdue University
Enterotoxigenic *Escherichia coli*	O78:H11	Human fecal isolate	ATCC 35401
Listeria monocytogenes	F4244	Clinical isolate, Human Central Nervous System (CNS) isolate	CDC, Our lab collection
Campylobacter jejuni	ATCC 29428	Human fecal isolate	ATCC
Salmonella enterica serovar Typhimurium	ST-1		Our collection
Lactobacillus acidophilus	LabMAX-3	CH2 Animal Solutions	CH2 Animal Solutions
Lacticaseibacillus casei	LabMAX-3	CH2 Animal Solutions	CH2 Animal Solutions
Enterococcus faecium	LabMAX-3	CH2 Animal Solutions	CH2 Animal Solutions
Lactobacillus acidophilus	NRRL 31910	ATCC	Our lab collection
Lacticaseibacillus casei	ATCC 334	Cheese	Our collection
Enterococcus faecium	ATCC 8459	Cheese	Dharmendra Mishra, Purdue University
Pediococcus acidilactici	H	Fermented sausage	Our Lab collection [48]
Staphylococcus aureus	ATCC 25923 (Rosenbach)	Clinical Isolate	Our Lab collection

2.2. PCR Confirmation of Probiotic Strains

Probiotic culture isolates in LabMAX-3 were confirmed by PCR amplification of species-specific target genes listed in Table 2. Target gene amplification was performed using PCR Master Mix (Platinum™ Hot Start, Thermo Fisher Cat# 130000). Equal volume of template (2 µL of resuspended colony) was added, and DNA was amplified via a thermocycler (Applied Biosystems, Carlsbad, CA, USA). Thermocycling conditions are as follows: 94°C for 10 min, 30 cycles at 94°C at 30s per cycle, 30 s at 60°C, 72°C at 30s and 5 min at 72°C. PCR products were analyzed by agarose gel electrophoresis

(2%) and DNA bands were visualized under UV exposure using a Kodak EDAS 290 camera with ID LE 3.6.3 software.

Table 2. PCR confirmation of probiotic strains.

Organism	Primer	Target gene	Product size (bp)	Ref
L. acidophilus	F-CCT TTC TAA GGA AGC GAA GGA T R-ACG CTT GGT ATT CCA AAT CGC	16S-23S	129	[49]
L. casei	F-CCA CAA TCC TTG GCT GTT CT R-GCT TGA GGC GAT TGT AAT CC	Putative protein	115	[49]
E. faecium	F-GCGTGCATGGTTAAGACGAC R-CTGCTGGATCGCTGGGTTAT	Rhomboid protease GluP (serine protease)	427	[50]

2.3. Pathogen Propagation

Cultures of enterotoxigenic *Escherichia. coli* (ETEC) strains F4 and O78:H11, *Staphylococcus aureus* ATCC 25923 *Salmonella enterica* serovar Typhimurium ST1, and *Listeria monocytogenes* F4244 from our culture collection (Table 1) were cultivated in Tryptic Soy Broth supplemented with 0.6% yeast extract (TSBYE) at 37°C for 18-24 h. *Campylobacter jejuni* ATCC 29428 was cultivated in microaerophilic conditions (5% CO_2, 37°C, 7 days) in Bolton Broth (Neogen, Lansing, MI, USA).

2.4. Antimicrobial Activity Testing on Agar Plates

Antimicrobial activity of individual probiotic cultures, *Enterococcus faecium*, *Lactobacillus acidophilus*, and *Lacticaseibacillus casei*, mixture (1:1:1) of three designated, LabMAX-3, *P. acidilactici* H and *L. casei* ATCC334 was tested against pathogens (Table 1) as before [48]. Briefly, overnight-grown (37°C for 18 h) cultures of probiotic strains were inoculated (1 µL, stab-method) on a base layer of MRS agar and incubated for 18-20 h at 37°C, anaerobically. Pathogens were grown at 37°C for 24 h in TSBYE aerobically. Tryptic Soy Agar supplemented with 0.6% yeast extract (TSAYE) soft top agar (0.8% agar) was prepared, and 10 µL of the respective pathogens were inoculated and vortexed. The inoculated soft agar (5 mL) was then poured onto the base layer, swirled, air-dried, and incubated at 37°C for 24 h. The zones of inhibition around the stab culture were measured.

For the preparation of cell-free supernatants, probiotic bacterial culture (18-20 h grown as above) supernatants were collected after centrifugation (14,000x, 5 min), and passed through 0.45 µm membrane filters. A 20 µl aliquot was tested on a sterile blotting paper disc against the test pathogens that were overlaid on TSA agar plates [48].

In separate experiments, each 18-20-h grown probiotic cultures were heat-treated (80°C for 10 min) and 10 µl of each culture was placed on the BHI agar surface and overlaid with the test pathogens as above [48].

2.5. Biofilm Formation by Probiotics

Biofilm formation by probiotic blend LabMAX-3 was analyzed in multi-well polystyrene plates as before [51]. Briefly, anaerobically grown probiotic bacteria were suspended in 1:1 ratio of MRS and MMRS and dispensed into 96-well tissue culture plates at 1.0×10^8/well (TPP, Switzerland) and incubated at 37°C for 24, 48, and 72 h anaerobically. *Lacticaseibacillus casei* ATCC 334 and *Staphylococcus aureus* were used as positive controls and grown in MRS and TSBYE, respectively, and inoculated at 1×10^8/well in 1:1 ratio of MRS: MMRS or MRS:TSB, respectively and incubated at 37°C for 24, 48, 72 h aerobically. Every 24 h interval, the old media was aseptically replaced with fresh

media. Biofilms were washed three times in 0.2 mM phosphate buffered saline, pH 7.2 (PBS) to remove planktonic bacteria. Bacterial counts in the biofilm were enumerated after scraping the biofilms from the well, collecting the cells in an Eppendorf tube, sonication for 20 min using iSonic (Chicago, IL, USA), and serially diluted for plating on MRS or TSAYE agar plates [38].

The formation of biofilms was also assayed by crystal violet staining. Briefly, washed biofilms were stained with 1% crystal violet (Sigma), washed three times in PBS, air-dried for 15 min at room temperature (~25°C, RT), treated with 95% ethanol, and the absorbance ($OD590_{nm}$) of supernatant was measured [51].

In a separate experiment, bacterial biofilms were allowed to form in multiwall chambered glass slides (LabTek II, Cat# 154534, Thermo Fisher) that were UV-pretreated for 45 min. Wells were washed 3x in PBS, air-dried for 15 min at RT, heat fixed, and subjected to Gram staining using crystal violet without the counter staining and visualized under Leica Microscope (Deerfield, IL, USA) with 1000x magnification.

2.6. MDCK Cell Line Preparation

The Madin-Darby Canine Kidney (MDCK, NBL-2, CCL-34, ATCC, Manasas, VA, USA) cell line was cultivated in Dulbecco's Modified Eagles Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco), termed D10F. The canine cell line was grown at 37°C at 5% CO_2, passaged from 4-15, and propagated in T-75 flasks (TPP, Switzerland), till 75% confluence. The cells were treated with Trypsin-EDTA (0.25%) (Gibco), centrifuged (800xg for 2.5 min), and resuspended in D10F. The MDCK cells were counted by 0.4% Trypan blue (Gibco) staining and seeded at densities of 10^4/well.

2.7. Treatment of MDCK with Lipopolysaccharide

Lipopolysaccharide (LPS, 1 mg/mL, Sigma, St Louis, MO, USA) was reconstituted in sterile deionized (d) H_2O and aliquoted for one-time usage. MDCK cells were treated with LPS at 1 µg/mL in D10F for 24 h at 37°C and 5% CO_2 to induce modest inflammation [47]. The monolayers were washed three times in DMEM to remove the LPS present and treated with probiotics. Control wells (no LPS), received equivalent amounts of dH_2O to serve as a vehicle control.

2.8. Probiotic Adhesion to MDCK Cell Line by Plate Counting

MDCK cells were seeded at 1×10^4 cells/ml/well and cultured for 9 days to allow monolayer formation. Cell monolayers were washed three times in DMEM and treated without (no LPS groups) or with freshly prepared D10F containing 1 µg/mL LPS and incubated for 24 h (37°C, 5% CO_2). Monolayers were then washed three times in DMEM to remove residual LPS, and cells were examined for monolayer integrity by microscope.

Probiotics were grown anaerobically for 24 h in MRS or MMRS as before and added to MDCK cell monolayers with a multiplicity of exposure (MOE) of 1000 and incubated at 37°C with 5% CO_2 under humidified conditions for 24 h. Supernatants were collected for lactate dehydrogenase (LDH) and cytokine profiles (see below). MDCK cell monolayers were washed three times in DMEM to remove unattached bacteria. Cell monolayers were detached using Triton-X 100 (Sigma) treatment (0.1% for 5 min), vortexed, serially diluted, plated, and incubated at 37°C for 48 h, anaerobically. Colony counts were plotted to determine bacterial adhesion.

2.9. Adhesion and Biofilm Analysis by Giemsa Staining

MDCK cells were seeded (1×10^4 cells/ml/chambered well) in UV-pretreated (45 min) cassettes (LabTek II, Cat# 154534, Thermo Fisher) and incubated at 37°C at 5% CO_2 for 9 days and inoculated with bacteria at MOI, 1000 as above. The cell monolayers were fixed with 100% methanol for 10 min, air-dried, and stained with Giemsa stain (1:20 dilution in dH_2O and methanol and stained for 45 min, air-dried, and visualized under the Leica microscope.

2.10. Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) release was from MDCK cells and percent cytotoxicity was calculated as per the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI, USA) [10, 52].

2.11. Cytokine ELISA

Canine cytokines IL-10, TGFβ and TNFα was purchased from R&D Systems (Minneapolis, MN, USA), and cytokine levels in MDCK culture supernatants were quantified following the manufacturer's instruction.

2.12. Data Analysis

Data were analyzed with GraphPad Prism (La Jolla, CA, USA) with unpaired Mann-Whitney tests. All data sets are represented of at least three experimental/biological replicates. Data are presented with standard error of the mean (±) or box-whisker plots with an interquartile range.

3. Results and Discussion

3.1. Antimicrobial Activity of Probiotics Against Pathogens

Probiotic microbes have long been acknowledged to possess antimicrobial properties against human and animal pathogens [1, 53, 54]. LabMAX-3, used in this study, contained three probiotic cultures of *Lactobacillus acidophilus*, *Lacticaseibacillus casei*, and *Enterococcus faecium*. The commercial LabMAX-3 lyophilized powder blend could not be directly used in our proposed experiments due to the presence of some insoluble materials (part of formulations), which significantly interfered with our experiments (data not shown). Thus, before the experiment, we isolated each culture from the blend and mixed them at a 1:1:1 ratio, designated LabMAX-3. The identity of each culture was verified by PCR targeting 16S rRNA gene (Figure 1), and by light microscopy (Figure S1) to be *Lactobacillus acidophilus*, *Lacticaseibacillus casei*, and *Enterococcus faecium*.
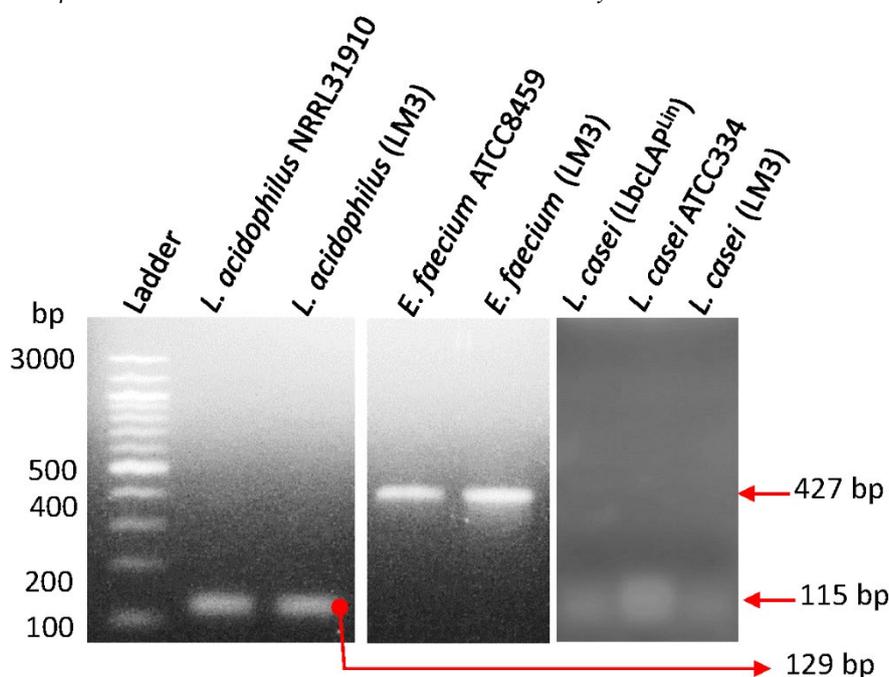
Figure 1. PCR confirmation of individual probiotic cultures from canine kibble supplement LabMAX-3 (LM3) (see Table 2). The remaining cultures were used as positive controls.

Antimicrobial activity of each live probiotic culture and the LabMAX-3 showed strong inhibitory zones against both Gram-negative *Salmonella enterica* serovar Typhimurium, enterotoxigenic

Escherichia coli (ETEC) strains F4 and O78:H11, and *Campylobacter jejuni* ATCC 29428 and Gram-positive, *L. monocytogenes* (**Figure 2, Table 3**). The zone of inhibition produced by LabMAX-3 was comparable to zones produced by *L. acidophilus*, *E. faecium*, and *P. acidilactici. L. casei* showed the weakest antimicrobial response among the lactic acid bacterial cultures tested (**Table 3**). Surprisingly, neither heat-killed probiotic bacteria nor cell-free culture supernatants of the probiotic bacteria showed any growth inhibition of test pathogens except for heat-killed *L. acidophilus* cells, which showed a faint zone of inhibition against *Listeria*, *Salmonella,* and ETEC (**Figure S1**). On the other hand, cell supernatant from *P. acidilactici*, a known bacteriocin producer [48] inhibited *L. monocytogenes* and *S. aureus* but not *Salmonella* (**Figure 2**) since bacteriocin (Pediocin AcH) produced by *P. acidilactici* is effective only against Gram-positive bacteria. These results indicate that live probiotic bacteria produce inhibitory compounds that are effective against the Gram-positive and Gram-negative pathogens tested. Since heat treatment or cell-free culture supernatants of LabMAX-3 did not show an effect suggesting, interaction with live cultures is essential for suppressing pathogens' growth (**Figure S1**).

Table 3. LabMAX-3-mediated inhibition of pathogens.

Bacteria	Strain	Avg zone of inhibition ± SEM (mm)					
		LabMAX-3*	LC*	LA*	EF*	PA	LC (ATCC 334)
Listeria monocytogenes	F4244	15.83±1.64	8.50±0.00	19.00±1.44	17.67±0.72	20.67±0.44	11.00±0.57
Staphylococcus aureus	ATCC 25923	7.33±0.67	10.67±0.67	9.33±0.67	7.33±0.67	20.67±0.67	18.33±0.88
Salmonella enterica serovar Typhimurium	ST-1	22.67±1.59	10.33±0.33	15.50±0.50	18.00±1.26	19.83±0.33	9.33±2.19
Enterotoxigenic *Escherichia coli*	F4 (K88)	19.83±1.69	8.17±0.88	21.17±0.17	19.50±1.041	22.67±0.17	13.67±2.73
Enterotoxigenic *Escherichia coli*	O78:H11	6.00±0.57	9.33±0.67	10.00±1.00	9.00±0.00	11.67±0.33	11.67±3.28
Campylobacter jejuni‖	ATCC 29428	15.00	10.00	0.0	13.50	22.00	NA

*LabMAX-3 contains EF, LA and LC (1:1:1), *denotes origin from LabMAX-3,EF, *Enterococcus faecium*; LA, *Lactobacillus acidophilus*; LC, *Lacticaseibacillus casei*; PA, *Pediococcus acidilactici*; and LC(ATCC 334), *Lacticaseibacillus casei* ATCC 334. ‖Indicates one trial.
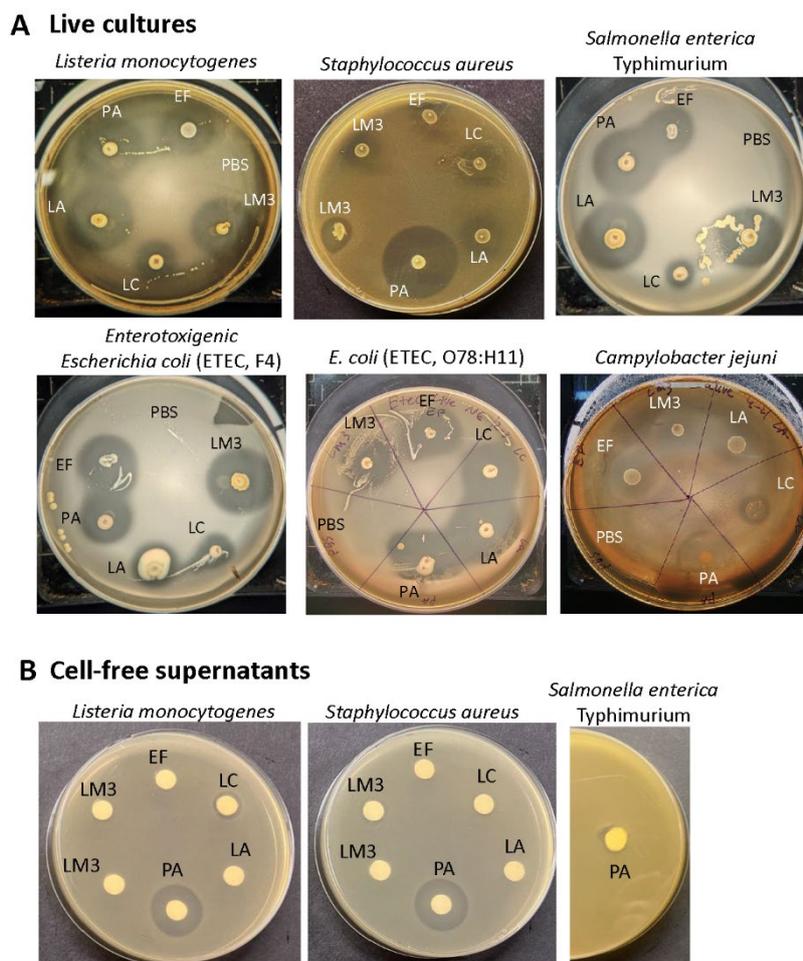
Figure 2. Antimicrobial activity of live LabMAX-3 and other lactic acid bacterial strains against pathogens. (**A**) Antimicrobial activity of 18-h MRS-plate grown cultures against pathogens. (**B**) Antimicrobial activity of cell-free culture supernatants of LabMAX-3 and other lactic acid bacterial strains against *L. monocytogenes*, *S. aureus*, and *Salmonella*. Antimicrobial activity was observed after 24 h. LM3, LabMAX-3; LA, *Lactobacillus acidophilus*; LC, *Lacticasiebacillus casei*; EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*.

Probiotic bacteria inhibit pathogens by competing with colonization sites, modulating microbial communities, and inhibiting pathogen growth by producing acids, hydrogen peroxide, bacteriocins, and other inhibitors [1, 54]. Since cell-free culture supernatants from LabMAX-3 showed no inhibition, compared to *P. acidilactici* supernatant, bacteriocin may not significantly contribute to the inhibitory effects. Previous studies have demonstrated that probiotic supplementation can reduce hemorrhagic diarrhea and inflammatory bowel disease-like symptoms in dogs [2, 55, 56]. Panja et al. [57] reported that feeding a probiotic-supplemented diet to dogs increased creatinine levels in the serum. Likewise, a probiotic blend of *Lacticaseibacillus casei* Zhang, *Lactiplantibacillus plantarum* P-8 and *Bifidobacterium animalis* improved cytokine profiles and serum immunoglobulins and altered microbial community. Microbiota profile showed increased *Lactobacillus* spp. and *Faecalibacterium prausnitzii* presence and decreased *Sutterella stercoricanisn* and *Escherichia coli* in the feces [58]. *Lacticaseibacillus rhamnosus* GG (LGG) was also reduced *Escherichia coli* (EHEC) O157:H7-mediated attachment-effacement lesion and improved tight junction integrity by redistributing claudin-1 and Zo-1 on MDCK cell line [59].

3.2. LabMAX-3 Forms Biofilm on Abiotic Surface

Analysis of biofilm formation by probiotic blend LabMAX-3 on polystyrene 96-well microtiter plate as measured by crystal violet staining showed a gradual increase in cell mass over 72 h (**Figure. 3**). LabMAX-3-mediated biofilm formation was compared with *S. aureus* and *L. casei* ATCC334. At 48 h, biofilm produced by *S. aureus* was significantly higher than LabMAX-3 ($p < 0.05$), while at 72 h, there were no statistical differences among the three cultures. Further analysis shows LabMAX-3 and *L. casei* ATCC334 biofilm-mass grew over 72 h and followed similar growth kinetics (**Figure 3AB**).

Biofilm formation was also quantified by counting cells in the biofilm. LabMAX-3 bacterial counts gradually increased from 24 h to 72 h, showing about 1 log increase (**Figure 3CD**).
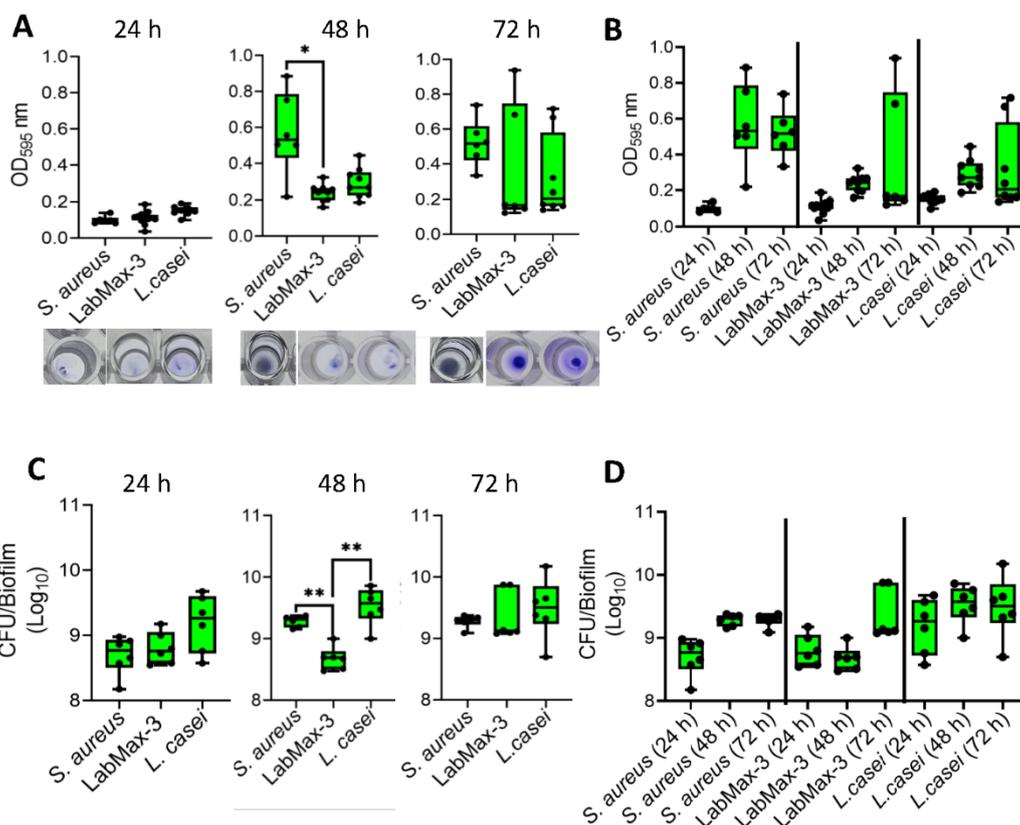
Figure 3. Biofilm formation by probiotics blends LabMAX-3 quantified by (A, B) crystal violet staining and (C, D) counting colony-forming units (CFU) after 24, 48, and 72 h incubation at 37ºC. Biofilm formation by LabMAX-3 was compared with *Staphylococcus aureus* and *Lacticaseibacillus casei* ATCC334. .

Light microscopic analysis of crystal violet-stained biofilms on glass slides shows spatial bacterial arrangement within the biofilms (**Figure 4**). LabMAX-3 forms multilayered biofilms on glass surfaces with dense and sparse areas. Close examination of biofilm architecture in the sparsed area (**Figure 4, right panel**) revealed the dominance of rod-shaped cells (lactobacilli) with a few embedded coccoid cells (*E. faceum*). Furthermore, the LabMAX-3-mediated biofilm architecture appeared very similar to that of *L. casei* ATCC334. In contrast, *S. aureus* formed multilayered dense biofilms, and only a few dispersed individual cells (cocci) are visible near the dense cell mass of the biofilm (right panel). These data indicate that a probiotic LabMAX-3 blend produces multilayered mixed culture biofilms with dense and light areas comparable to biofilms produced by *L. casei* ATCC334 and *S. aureus*.

72 h old biofilm

Dense area	Sparse area
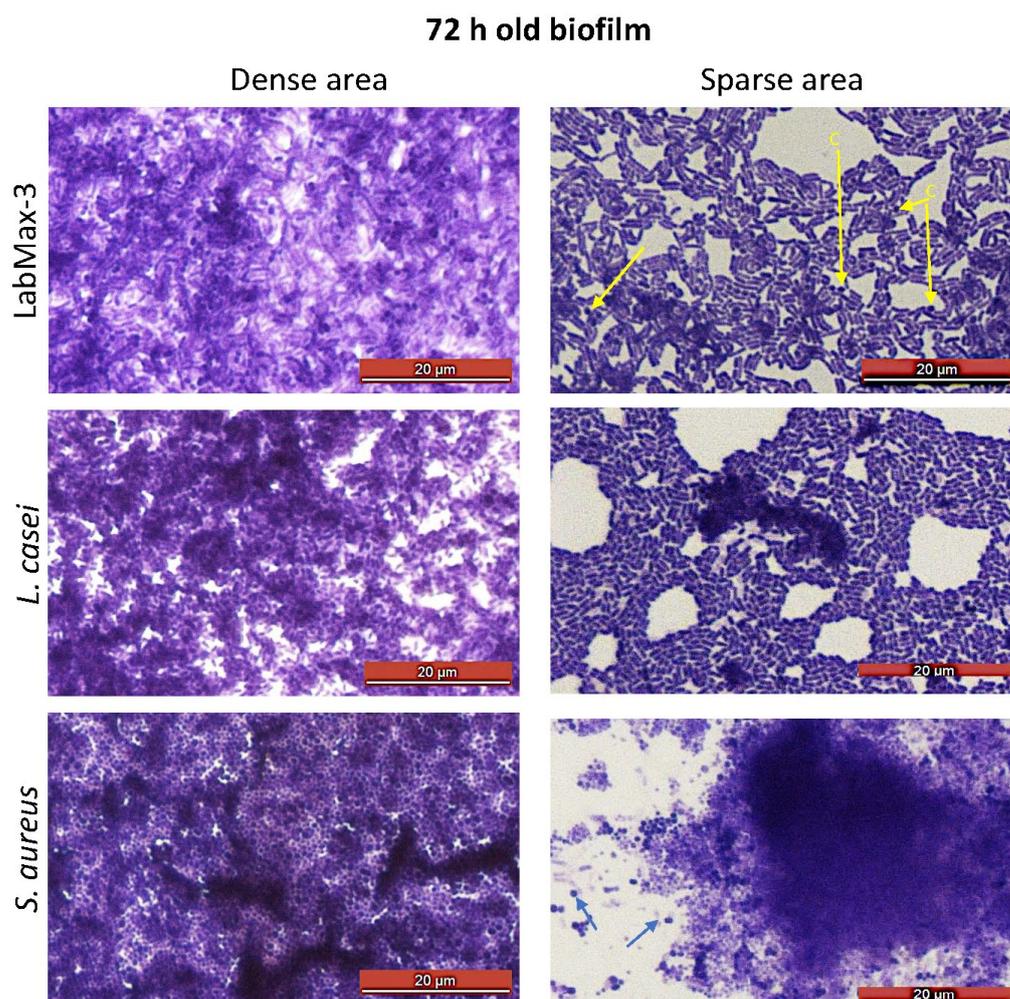
Figure 4. Microscopic examination of 72-h-old biofilms from dense and sparse areas of biofilms after staining with crystal violet. Arrows pointing to coccoid cells (c).

Probiotic biofilm formation is critical for ensuring colonization in the gut [60]. Probiotics in the LabMAX-3 formed robust biofilms in 72 h on the abiotic surfaces, comparable to *L. casei* and *S. aureus*. These data indicate that *L. casei* in the LabMAX-3 mix would likely contribute to biofilm cell mass by producing glycocalyx [61]. Similarly, the contribution of *L. acidophilus* and *E. faecium* cannot be ruled out, akin to previous reports [62, 63].

3.3. LabMAX-3 Probiotic Blend Adhesion and Biofilm Formation on MDCK Cells

We also examined the adhesion and biofilm-forming characteristics of LabMAX-3 to MDCK cell monolayers pretreated with or without LPS. LPS was used to simulate inflamed conditions [47]. LabMAX-3 adhesion to control MDCK cells (without LPS pre-treatment) was calculated to be about 6.7 log CFU/ml, and similar counts (~6.8 log CFU/ml) to MDCK was observed when MDCK cells were pre-treated with LPS (**Figure 5**). In contrast, *L casei* showed slightly higher adhesion (7.1 log CFU/ml) to MDCK cells with or without LPS treatment.
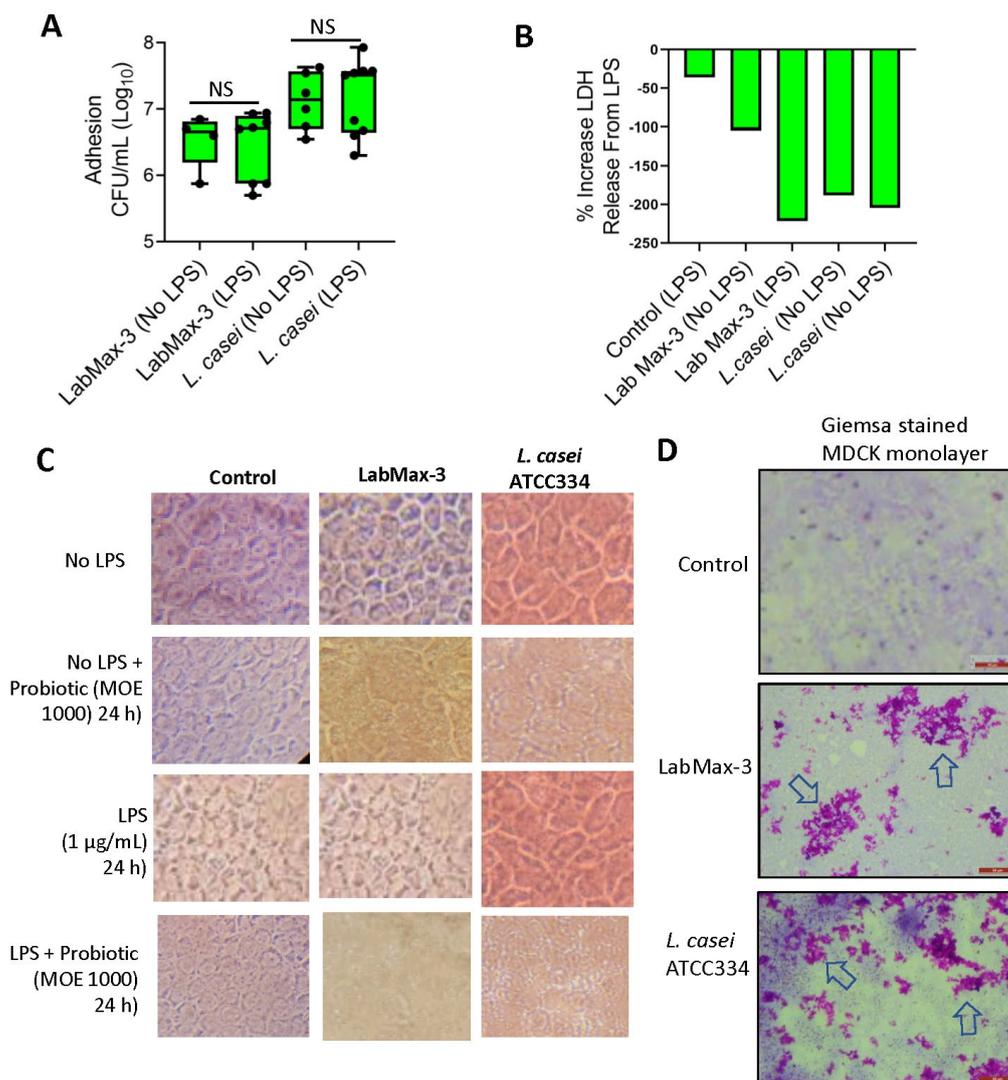
Figure 5. Adhesion characteristics of LabMAX-3 to MDCK cell line. (A) Adhesion (CFU/ml) of probiotic cultures to MDCK cells. (B) Lactate dehydrogenase (LDH) release assay from MDCK cells during the LabMAX-3 adhesion experiment. (C) Light microscopic analysis of cell monolayer integrity during probiotic adhesion experiment. (D) Giemsa staining of MDCK cells after probiotic exposure. LabMAX-3 forms biofilm-like structures in patches (arrows) on MDCK cell monolayers. .

LDH release from MDCK cells during LabMAX-3 adhesion was analyzed, and values were below zero, indicating that LabMAX-3 treatment did not cause cytotoxicity (**Figure 5B**). Further microscopic analysis of cell monolayers did not reveal any visible cell damage, and the monolayer integrity remained intact during LabMAX-3 exposure (**Figure 5C**). These data indicate that the probiotic blend LabMAX-3 efficiently adheres to MDCK cells without causing any cell damage, and the adhesion is comparable to *L. casei* ATCC334 [10]. These data further suggest that LabMAX-3 can interact with healthy and LPS-treated inflamed tissue to promote canine gut health.

Next, we analyzed the adhesion patterns of LabMAX-3 to MDCK cells by Giemsa staining (**Figure 5D**). Data show that LabMAX-3 attaches to MDCK cells, forming patchy biofilm-like structures throughout the monolayer. *L. casei* ATCC334 also forms similar patchy biofilm-like structures on MDCK cells, slightly at a higher frequency. The biofilms produced by LabMAX-3 and *L. casei* on MDCK follow a similar trend as the adhesion counts reported in **Figure 5A**. These data indicate that adhesion of LabMAX-3 is facilitated by forming biofilms, ensuring prolonged persistence and potential health benefits to the host.

The ability of probiotic strains to produce biofilms on host epithelial cells is critical for ensuring colonization, pathogen exclusion, and promoting health [10, 13, 60, 64]. In general, probiotic bacteria in the gut are transient, and their poor colonization in an inflamed gut environment may explain their inconsistent health benefits [65]. Therefore, LPS pretreatment on the MDCK cell line was used to create an inflamed condition in the gut [47]. LabMAX-3 could adhere and form biofilms on MDCK cell monolayers irrespective of LPS treatment.

3.4. Anti-inflammatory Response of LabMAX-3 to MDCK Cells

We quantified the proinflammatory (TNFα) and anti-inflammatory (IL-10 and TGFβ) cytokine profiles of LabMAX-3 on MDCK monolayers (**Figure 6**). TNFα expression in LPS-pretreated MDCK cells exposed to LabMAX-3 was significantly higher (p = 0.005) than in cells without LPS treatment. A similar result was also observed for *L. casei* ATCC334. In contrast, a reduced IL-10 level (numerical difference but not a statistically significant difference) was noticed in MDCK cells pretreated with LPS compared to those without LPS treatment following LabMAX-3 exposure. A similar trend was observed with *L. casei* ATCC334 treatment. IL-10 and TNFα ratio analysis demonstrated a significant (p < 0.05) reduction in values in LPS pretreated MDCK cells than those with LPS treatment following LabMAX-3 exposure. TGFβ secretion showed no significant differences between LabMAX-3 and *L. casei* ATCC334 treatment. Cytokine profile data indicate LabMAX-3 has an anti-inflammatory response towards MDCK cells consistent with probiotic functionality reported by others [3, 66].
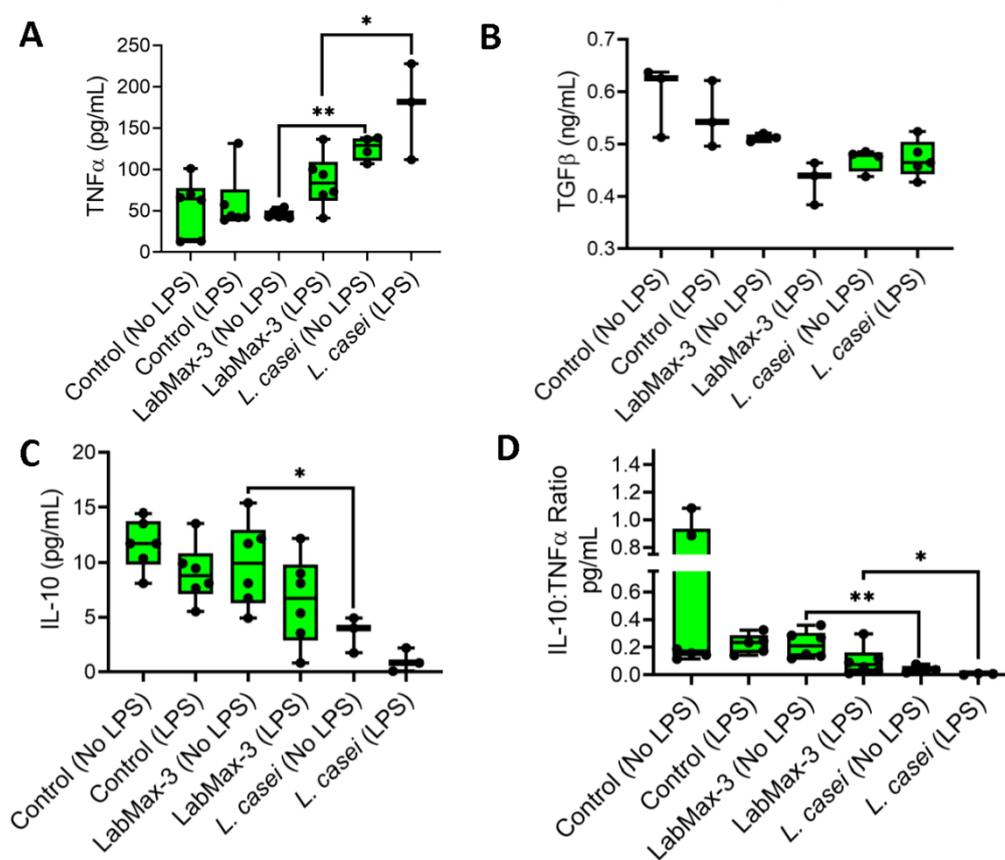
Figure 6. Cytokine secretion from the MDCK cell line by probiotics in the presence or absence of LPS pre-treatment. Data are an average of 2 experiments in triplicate. *, p < 0.05; **, p < 0.005.

4. Conclusions

Probiotic supplementation for systemic health in canines is a growing market. Here, we explored LabMAX-3 as a novel kibble additive comprising three renowned probiotics: *Lactobacillus acidophilus*, *Enterococcus faecium*, and *Lacticaseibacillus casei*. LabMAX-3 successfully inhibited the growth of notable canine-associated pathogens (*Salmonella enterica*, *Listeria monocytogenes*, enterotoxigenic

Escherichia coli). LabMAX-3 was shown to be a strong biofilm producer on both abiotic and biotic surfaces. The ability of LabMAX-3 to form biofilm-like structures and strong adhesion to epithelial MDCK cell monolayers suggests its potential to improve canine gut health. LabMAX-3 showed no cytotoxic effects on the MDCK cell line, with and without LPS stimulation. Anti-inflammatory properties of LabMAX-3 promote higher IL-10 and TNFα ratios when compared to *L. casei* strain ATCC334 alone. Together, these data indicate that LabMAX-3 is viable as a supplement to companion animal kibble for promoting systemic health.

The advantages of using a combination of three probiotic cultures in the LabMAX-3 are that they showed robust antimicrobial activity against pathogens, formed biofilms, and efficiently attached to MDCK cells, promoting an anti-inflammatory response. Another benefit of a multi-probiotic mixture is the chance of a positive effect on health in case one or more members fail to grow in an unfavorable host environment. Commercial probiotic supplements for human use often consist of multiple probiotic cultures, and a similar probiotic blend comprising multiple species and strains should be used to ensure pets' health.

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