

Biofilm Formation and Immunomodulatory Response of a Probiotic Blend

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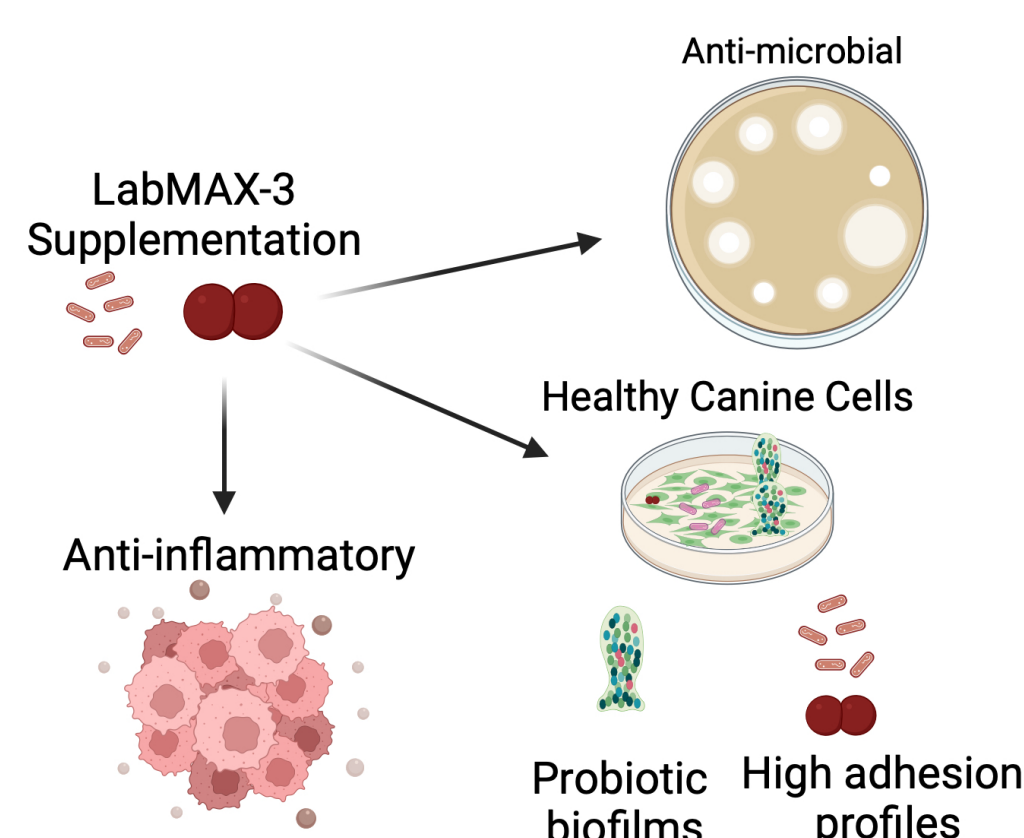
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Background

Gut dysbiosis and an inflamed bowel are growing concerns within mammals, including dogs. Probiotic supplements are safe and effective for restoring the natural microbial community and improving gastrointestinal health. The biofilm formation, antimicrobial activities, and immunological responses of probiotics are crucial to improving gut health and thus were analyzed in this study. We tested a commercial probiotic blend (LabMax-3), a canine kibble additive, comprising *Lactobacillus acidophilus*, *Lactisacibacillus casei*, and *Enterococcus faecium*, for its ability to inactivate common enteric pathogens on agar plates, its ability to form biofilms on plastics, and its epithelial cell adhesion and immunomodulatory response on the Madin-Darby Canine Kidney (MDCK) cell line. The probiotic LabMax-3 blend or individual isolates showed a strong inhibitory effect against *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, enterotoxigenic *Escherichia coli*, and *Campylobacter jejuni*. In contrast, heat-killed and probiotic cell-free supernatants did not show any inhibition. LabMax-3 formed a moderate biofilm compared to *Staphylococcus aureus* cultured for 72 h, further confirmed by microscopic imaging. LabMax-3 adhesion to the MDCK cell line (with or without lipopolysaccharide (LPS) pretreatment showed comparable adhesion ($P < 0.05$) to *Lactisacibacillus casei* ATCC 334, used as a control, which was further verified by Giemsa staining. LabMax-3 also did not show any cytotoxic effects on the MDCK cell line, as measured by lactate dehydrogenase assay. The IL-10 and TNF α ratio of LabMax-3, compared to the *L. casei* control, showed a significant increase ($P < 0.05$), indicating a more pronounced immunomodulatory effect. TGF β showed no significant differences between LabMax-3 and *L. casei* treatment. LabMax-3, a canine kibble additive, can potentially improve canine gastrointestinal health.



PCR Confirmation

Organism	Target gene	Product size (bp)
<i>L. acidophilus</i>	16S-23S	129
<i>L. casei</i>	Putative protein	115
<i>E. faecium</i>	Rhomboid protease GluP (serine protease)	427

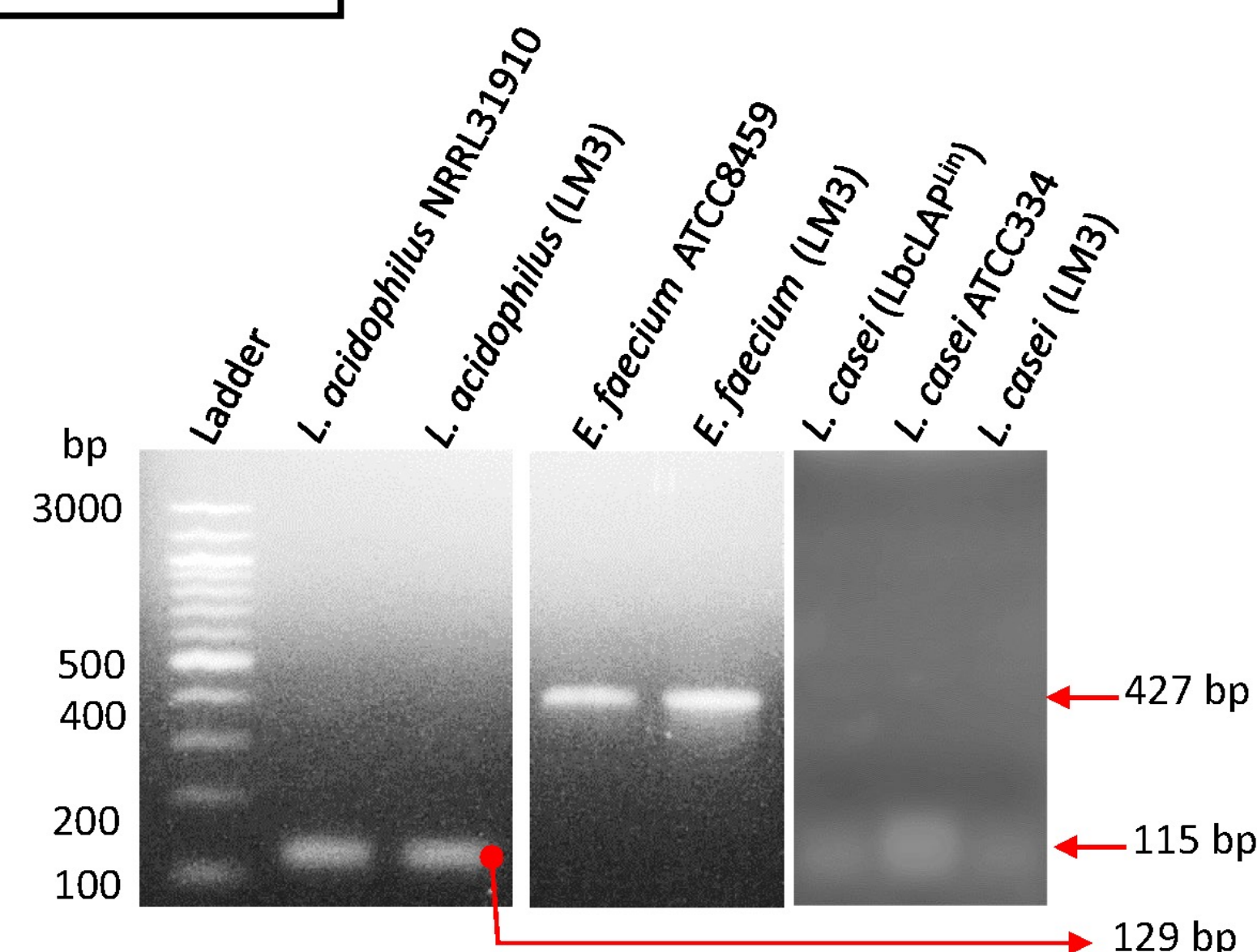
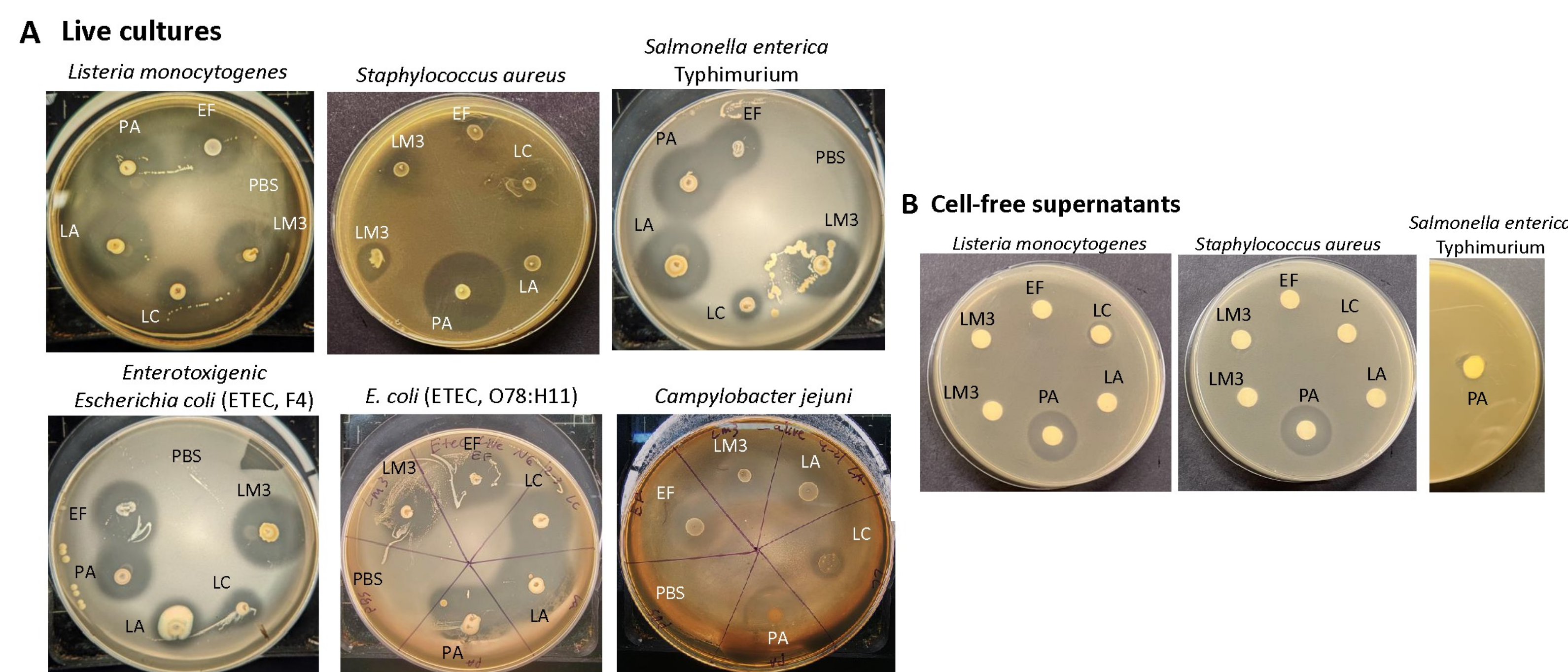


Figure 1: PCR confirmation of individual probiotic cultures from canine kibble supplement LabMAX-3 (LM3). The remaining cultures were used as positive controls

Antimicrobial Activity of Probiotic Against Pathogens



Bacteria	Strain	Avg zone of inhibition (mm) \pm SEM				
		LM3	LC	LA	EF	PA
<i>Listeria monocytogenes</i>	F4244	15.83 \pm 1.64	8.50 \pm 0.00	19.00 \pm 1.44	17.67 \pm 0.72	20.67 \pm 0.44
<i>Salmonella enterica</i> serovar Typhimurium	ST-1	22.67 \pm 1.59	10.33 \pm 0.34	15.50 \pm 0.50	18.00 \pm 1.26	19.83 \pm 0.34
Enterotoxigenic <i>Escherichia coli</i>	F4 (K88)	19.83 \pm 1.69	8.17 \pm 0.88	21.17 \pm 0.17	19.50 \pm 1.04	22.67 \pm 0.17
Enterotoxigenic <i>Escherichia coli</i> *	O78:H11	25.0	25.0	27.0	26.50	22.50
<i>Campylobacter jejuni</i> *	ATCC 29428	15.00	10.00	0.0	13.50	22.00

*Indicates one trial; EE, *Enterococcus faecium*; LA, *Lactobacillus acidophilus*; LC, *Lactisacibacillus casei*; PA, *Pediococcus acidilactici*; and LM3, LabMax-3 contains EE, LA and LC (1:1:1).

Figure 2: (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, *Lactisacibacillus casei*; EF, *Enterococcus faecium*; PA, *Pediococcus acidilactici*.

LabMAX-3 Forms Biofilm on Abiotic Surface

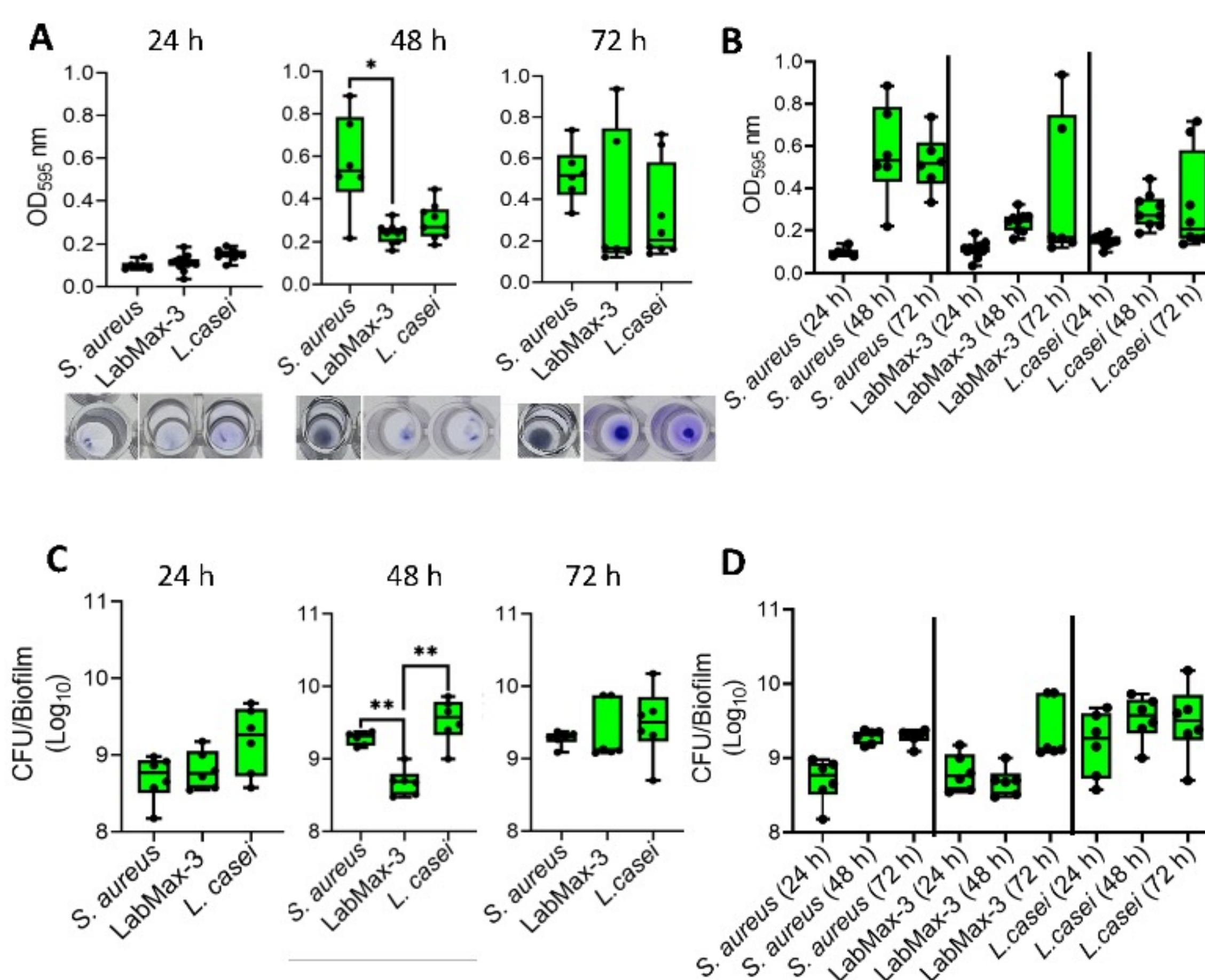


Figure 3: Biofilm formation by probiotics blends LabMAX-3 quantified by (A, B) crystal violet staining and (C, D) counting colony-forming units (CFU) after 24, 48, and 72 h incubation at 37°C. Biofilm formation by LabMAX-3 was compared with *Staphylococcus aureus* and *Lactisacibacillus casei* ATCC334.

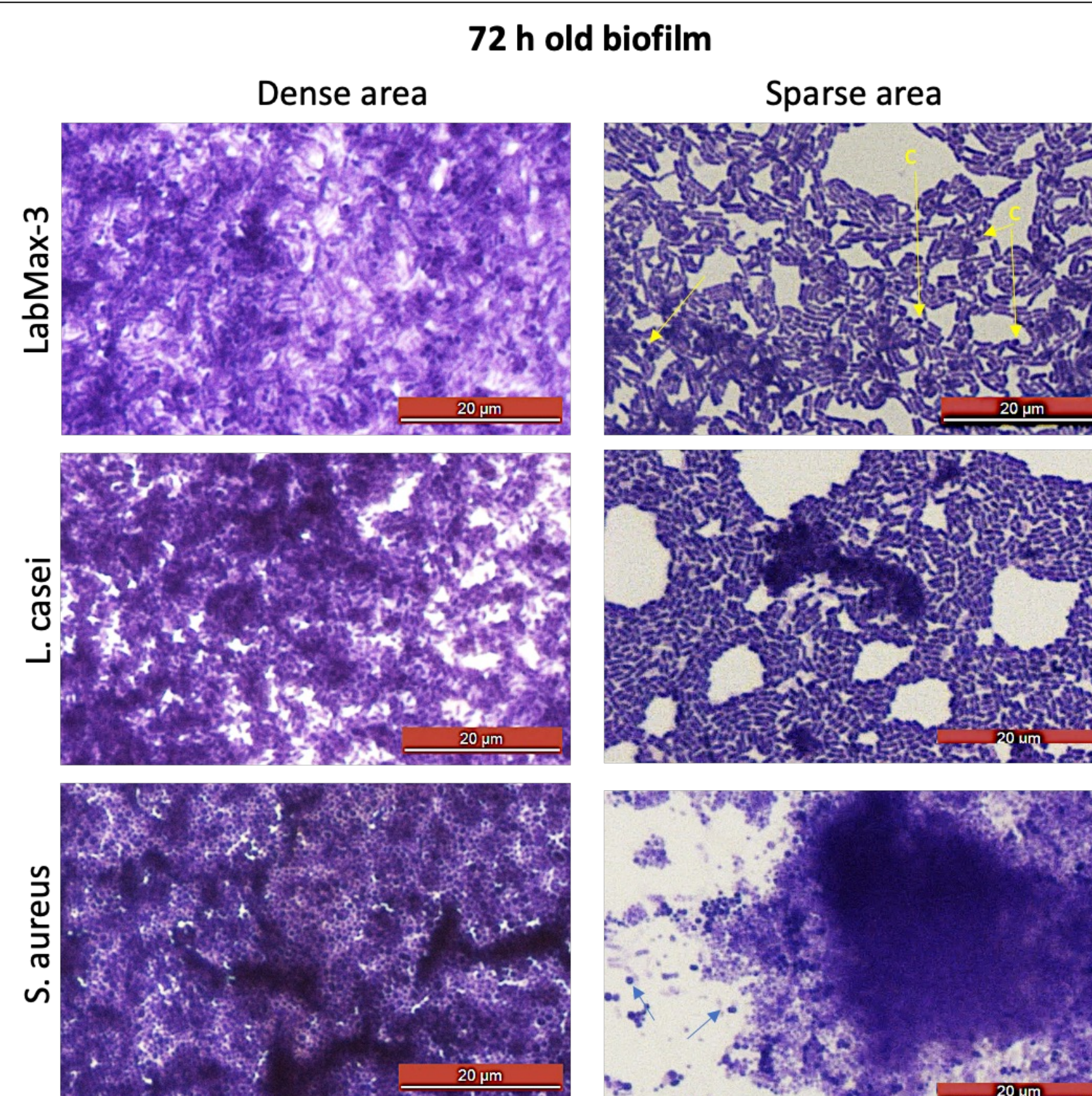


Figure 4: Microscopic examination of 72-h-old biofilms from dense and sparse areas after staining with crystal violet. Arrows pointing to coccoid cells (c).

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

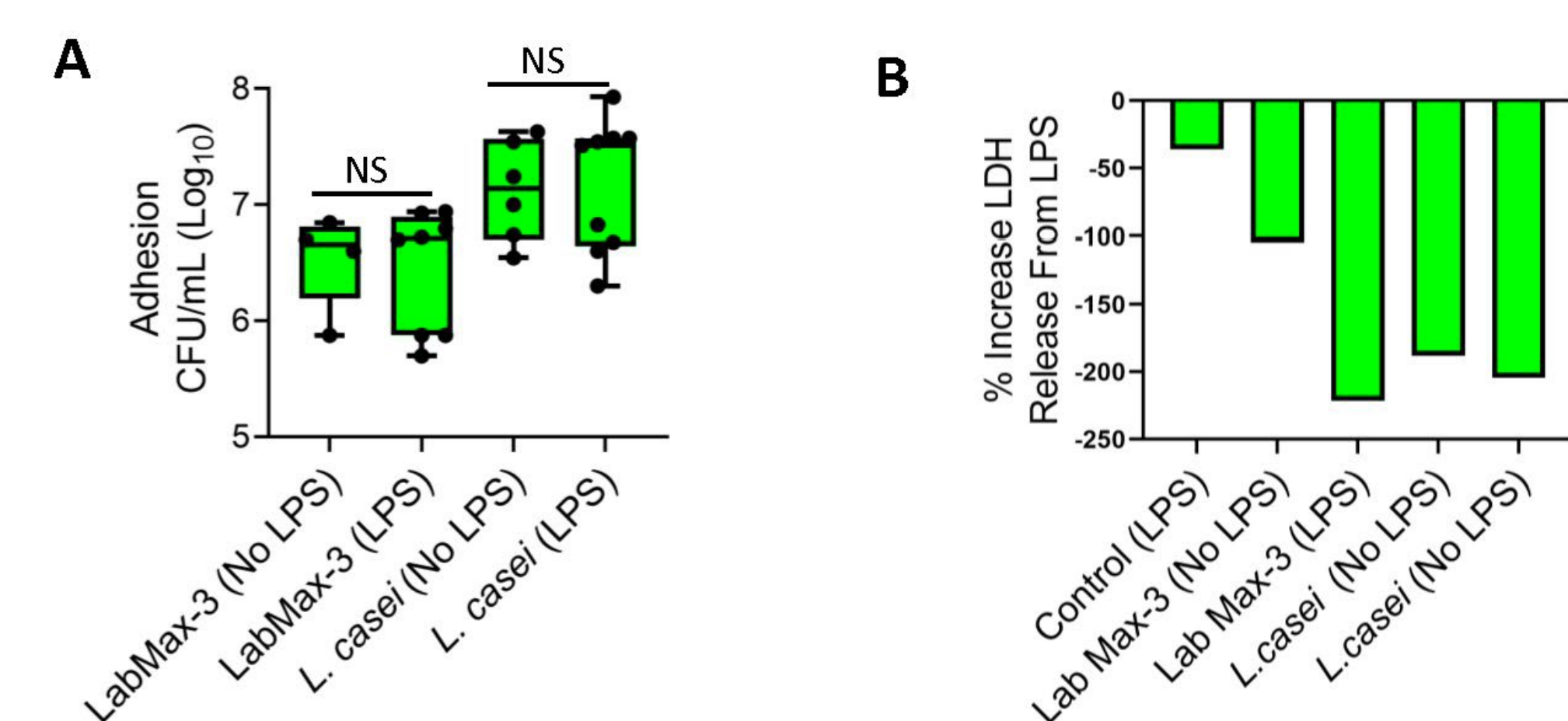


Figure 5: (A) Adhesion (CFU/ml) of probiotic cultures to MDCK cells. (B) Lactate dehydrogenase (LDH) release assay from MDCK cells during the LabMAX-3 adhesion experiment.

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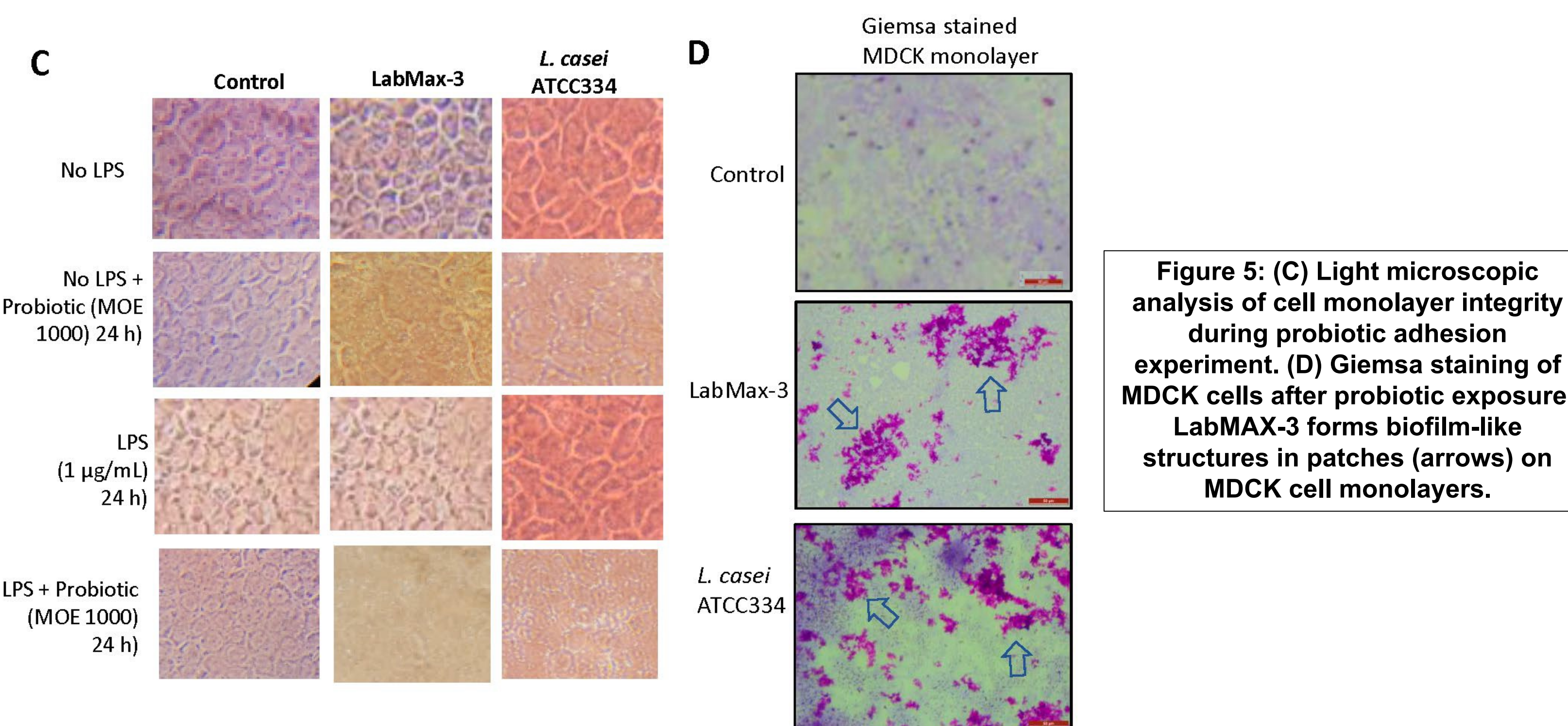


Figure 5: (C) Light microscopic analysis of cell monolayer integrity during probiotic adhesion experiment. (D) Giemsa staining of MDCK cells after probiotic exposure. LabMAX-3 forms biofilm-like structures in patches (arrows) on MDCK cell monolayers.

Anti-inflammatory Response of LabMAX-3 to MDCK Cells

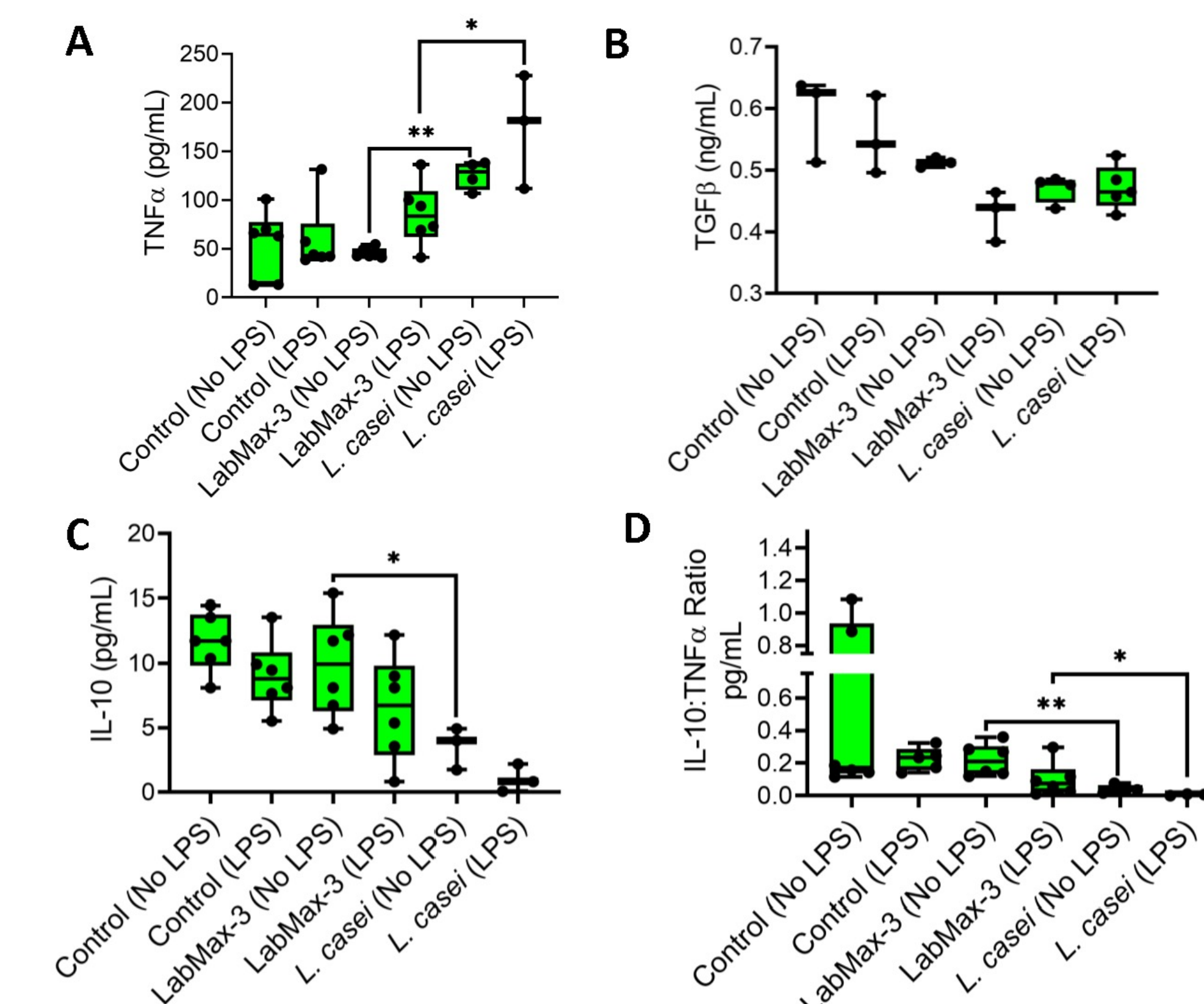


Figure 6: Cytokine secretion from the MDCK cell line by probiotics in the presence or absence of LPS pre-treatment. Data are an average of 2 experiments in triplicate. *, $p < 0.05$; **, $p < 0.005$

Conclusions

- LabMAX-3 demonstrated strong inhibition against *S. enterica*, *L. monocytogenes*, and *ETEC*.
- LabMAX-3 produced robust biofilms on both abiotic (non-living) and biotic (living) surfaces, indicating good survivability and attachment potential.
- LabMAX-3 showed strong adhesion to epithelial MDCK cells, enhancing its likelihood of colonizing the gut and providing health benefits.
- The probiotic mixture promoted favorable IL-10 to TNF α ratios, suggesting it can modulate inflammation better than single strains like *L. casei* alone.
- LabMAX-3 was non-cytotoxic to MDCK cells, even under LPS-stimulated conditions, supporting its safety for use in canine health supplements.
- Using multiple strains ensures that even if some strains do not thrive in a particular host environment, others may still provide beneficial effects.

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