The LinkSkin heat-killed probiotic strains stimulate an anti-allergic cytokine profile in canine peripheral blood cells

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Background: The effect of probiotics (i.e., live bacteria) and postbiotics (i.e., inactivated bacteria) on the immune system is heterogeneous among bacterial genus, species or even strains [1-3].

For live and heat-killed probiotics to have a beneficial clinical effect in allergic dogs, these bacterial strains should have the following immune-stimulatory profile:
- a stimulation of Th1 and Treg immune responses, and,
- a lack of stimulation of Th2 immune response.

Combined, the above properties would be expected to have the desired anti-allergic effect.

Objectives: In this study, we wished to determine the cytokine response after incubating canine peripheral blood mononuclear cells (PBMCs) with the two heat-killed probiotic strains included in LinkSkin (DRN, Nextmune, Italy).

Methods:

Animals

Blood in EDTA was collected from five healthy dogs without a history of allergy or digestive signs. The samples were collected at the time of vaccination or routine spay/neuter in dogs that had not received any immunomodulating drugs (e.g., cyclosporine, oclacitinib, or oral or injectable glucocorticoids) in the preceding month.

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation using Histopaque 1077 Hybri-Max (Sigma-Aldrich/Merck Life Sciences, Madrid, Spain). Firstly, 5 mL of blood in EDTA was diluted and mixed 1:1 in 0.9% saline before the addition of 3 mL of Histopaque 1077. This mixture was centrifuged for 30 mins at 2000 rpm without braking. The PBMC layer was aspirated and subjected to three successive cycles of saline wash. After the last centrifugation, the supernatant was removed, and PBMCs were resuspended in 5 mL of Gibco AIM-V medium (Fisher Scientific Spain, Madrid, Spain). The cell viability was verified by mixing 20 µL of the PBMC suspension with 20 µL of trypan blue (Fisher Scientific) and counting live cells on a TC20 automated cell counter (Bio-Rad, Barcelona, Spain); were only kept samples with over 70% of viable cells for food allergen stimulation.

Finally, the PBMC suspension was adjusted to 1 million cells/mL in the AIM-V medium.

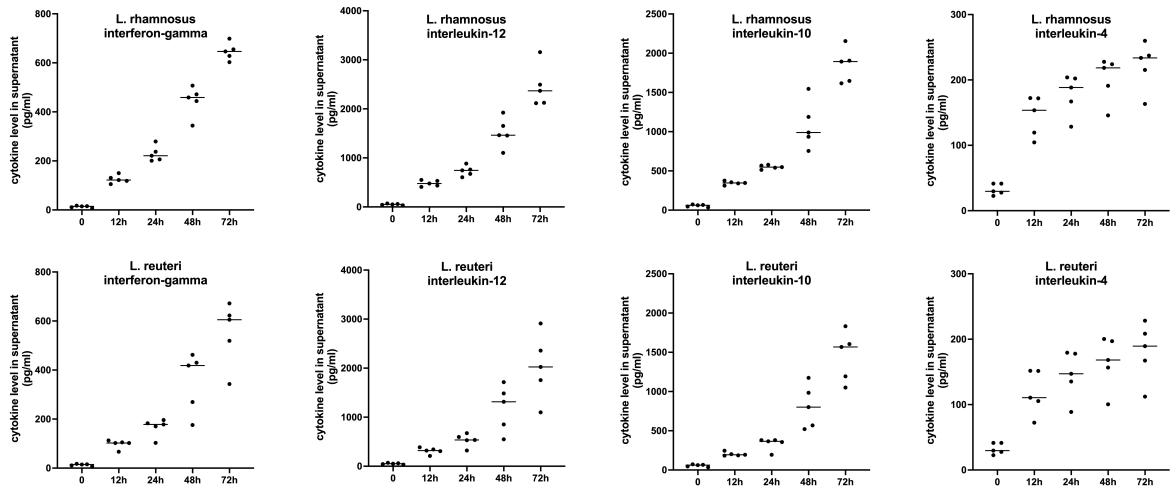
Bacterial stimulation
The PBMCs were aliquoted and put in different wells so that cells could be co-cultured with the two different heat-killed (i.e., tyndallized) probiotic strains (*Lactobacillus rhamnosus SGL01* and *Lactobacillus reuteri, SGL06*), with one well serving as the no-bacteria control.
The PBMC:bacteria stimulation ratio was 1:5 (e.g., 2 x 10^5 PBMC and 1x10^6 bacteria). The cells were co-cultured for 3 days with supernatant collected before and after 12, 24, 48 et 72h.

Cytokine measurement
For each bacterial stimulant and each time point, the cytokine levels were determined by ELISA (Bio-Techne R&D Systems, Minneapolis, MN, USA) validated for the measurement of canine IFN-gamma, IL-4, IL-10, and IL-12. After obtaining the cytokine concentrations in the supernatant, we calculated the stimulation indices (SI) of cytokine levels after co-culture with each of the two probiotic strains over those cultured with saline at the same time point.

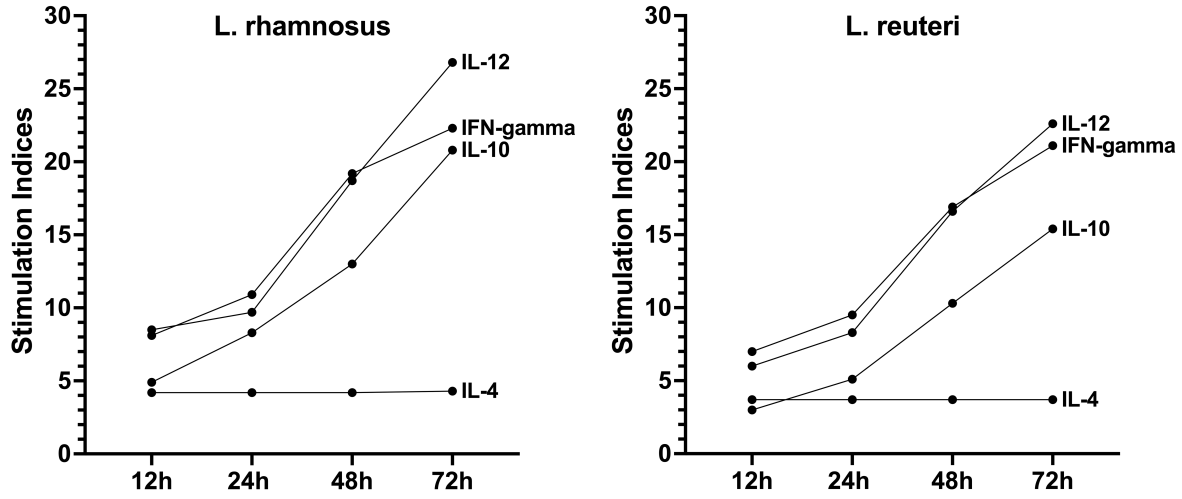
Statistical analyses
The cytokine levels were compared by repeated-measures ANOVA with post-hoc tests comparing each time point to that without stimulation.

Results:
The incubation of the canine PBMCs with both strains of heat-killed probiotics led to a significant increase (repeated-measures ANOVA, $P < 0,001$) in the levels of cytokines released in the supernatants, starting already 12 hours after the start of incubation and continuing throughout the 72 hours of co-culture (see figure below).

Both *Lactobacillus rhamnosus* and *reuteri* strains led to similar stimulations of cytokine production, being highest for IL-12 (around 2,000 – 2,500 pg/ml at 72h) and IL-10 (1500-2000) than with interferon-gamma (500-700); they were lowest with IL-4 (about 200). In fact, after 72 hours of culture, the IL-12:IL-4 ratio was higher than 8, indicating a strong anti-Th2 effect.



We then compared the stimulation indices (SI) of the cytokine release after probiotic stimulation over that without probiotic. As can be seen below, the two strains of *Lactobacillus* markedly stimulated IL-12, interferon-gamma and IL-10 compared to IL-4. While the IL-4 amount increased slightly over time after the probiotic stimulation, it also did so without stimulation, with the SI ratio remaining constant and inferior to 5.



Conclusions:

The two Lactobacillus strains included in LinkSkin, even tyndallized (or heat-killed), stimulate canine lymphocytes to have an anti-allergic immune profile, that is, a stimulation of Th1 (IL-12, interferon-gamma) and Treg (IL-10) cytokines compared to the Th2 cytokine IL-4. These strains thus possess the desired profile to serve as a relevant adjuvant in the multimodal therapy of allergic diseases of dogs.

References

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