Beyond Probiotics: Heat-Treated Probiotics in Companion Animal Health

Julie K. Spears, PhD, Gail Czarnecki-Maulden, PhD, Clement Ameho, PhD, Arleigh Reynolds, DVM, PhD, DACVN
Nestlé Purina Research
St. Louis, MO
Julie.Spears@rd.nestle.com

Abstract
Probiotics have demonstrated digestive health and immune-boosting efficacy in dogs and cats, though maintaining viability of the bacteria limits their application in pet foods. Certain inactivated probiotics and their soluble supernatants can modulate immune response and have been shown to have similar functionalities as their live counterparts. Studies have confirmed that the combination of heat-treated *Lactobacillus delbruekii*, heat-treated *Lactobacillus fermentum*, and their supernatant improves diarrhea symptoms in children and adults. A series of trials have confirmed that this heat-treated *Lactobacillus* blend improves immune response and gastrointestinal health in healthy dogs and cats, even during times of naturally occurring stress.

Glossary of Abbreviations
ACTH: Adrenocorticotrophic Hormone
CRP: C-Reactive Protein
EFSA: European Food Safety Authority
GALT: Gut-Associated Lymphoid Tissue
IBS: Irritable Bowel Syndrome
IBS-D: Diarrhea-Predominant IBS
OPLS-DA: Orthogonal Partial Least Squares Discriminant Analysis
QPS: Qualified Presumption of Safety
TTGE: Temporal Temperature Gel Electrophoresis

Probiotics
Gastrointestinal health is integral for the health and quality of life of companion animals. The gastrointestinal tract of mammals contains a diverse commensal population of bacteria that plays a crucial role in health and disease. These commensal bacteria generally live in equilibrium and interact with host cells. Additionally, the presence of commensal bacteria plays an important role in priming both the innate and adaptive immune responses.[1] The beneficial role of intestinal microbiota in animals can be strengthened by probiotic supplementation.

Probiotics are defined as live microorganisms that when administered in adequate amounts confer a health effect on the host.[2] These beneficial bacteria confer their benefits both directly and indirectly. Direct effects include inhibiting pathogens via phagocytosis, blocking attachment of pathogens to the intestinal wall, and priming the local immune system. The gut-associated lymphoid tissue (GALT) acts through secretory IgA to improve the gut mucosa response to pathogens. Indirect effects include producing short-chain fatty acids that serve as a source of energy for intestinal cells, increasing intestinal barrier strength, and lowering intestinal pH, thus making the environment inhospitable to pathogens.[3] Probiotics have demonstrated efficacy in the treatment of diarrhea and in stimulating the immune system.[4] Efficacy is contingent on a number of factors including the strain, conditions of preparation and storage, product matrices, the dietary, cultural and health differences of consumers, as well as genetics.[5]

The question of viability limits application of probiotics in pet foods. Although certain probiotics have demonstrated efficacy in dogs and cats, product forms for pets have largely been limited to supplements to ensure the bacteria remains viable for its entire shelf life.[6] Factors affecting viability include exposure of probiotics to oxygen, high water activity of pet foods, and storage time and temperature. Studies have shown that few commercial probiotic-containing products available for pets meet the viable bacteria guarantees stated on their primary packaging.[7,8]

Although probiotics are, by definition, live bacteria that confer a health benefit, there is growing evidence suggesting that supplementation of certain inactivated probiotics may confer health benefits as well.

Inactivated Probiotics
Inactivated probiotics or paraprobiotics have been defined as "nonviable microbial cells (intact or broken) or crude cell extracts (i.e., with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer."[9] Inactivated probiotics cannot divide, and the bacteria are not considered alive. Probiotics may be rendered inactive through heat-treatment, UV- or γ-irradiation or by chemical agents.

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Although probiotic bacteria confer some of their benefit through direct interaction with other bacteria (which is dependent upon the cells remaining viable), interactions with host cells still can occur regardless of probiotic viability.[9] Although inactive, components of the bacteria can still interact with the intestinal epithelium through cell signal receptors.[10] Through interaction with GALT, inactivated probiotics can also modulate the immune system.[11]

Thorough reviews of research studies of inactivated probiotics have shown efficacious responses in immune modulation, allergy and inflammatory bowel disease.[9-12] However, the health benefits of inactivated probiotics must be evaluated individually. The efficacy of inactivated bacteria is not predicted by the efficacy of its live counterpart since inactivation often leads to the loss of benefits.[13-18] In some cases, inactivated bacteria can exert the opposite effects of their live counterparts, as observed with *in vitro* cytokine stimulation from live and heat-treated *L. paracasei* NCC2461.[19]

An additional source of immune system modulators includes the supernatants that are used to grow probiotic bacteria. The complex composition of supernatant is dependent on the bacteria species and culture conditions. Components of the supernatant include short-chain fatty acids, phospholipids, bacteriocins, and other proteins.[12] These metabolites also can exert immunomodulatory effects. Reviews of research studies of probiotic supernatant and soluble factors have shown efficacious responses *in vitro* and *in vivo*.[9,12,20]

Supplementation of inactivated probiotics and their supernatant is advantageous in immunocompromised populations. Because inactivated probiotics are not viable, the bacteria cannot translocate to the bloodstream. Additionally, inactivated probiotics provide an opportunity to include a functional ingredient with probiotic-like effects in a variety of food forms. Although there are several inactive probiotics available, a blend of heat-treated *Lactobacillus* species has demonstrated efficacy in adults and children.

Efficacy of Heat-Treated *Lactobacillus* Blend in Humans

A heat-treated probiotic that has been widely studied is the combination of *Lactobacillus fermentum* and *Lactobacillus delbruekii*. Both *Lactobacillus* species are common in whey starter cultures and have a long history of use in the production of cheese and other fermented foods.[21,22] Both *L. delbruekii* and *L. fermentum* are on the European Food Safety Authority (EFSA) Qualified Presumption of Safety (QPS) positive list based on their documented history of use.[23]

A combination of heat-treated *L. delbruekii* and *L. fermentum* in a 10:1 ratio and their culture supernatant medium (referred to in this article as heat-treated *Lactobacillus* blend) has been commercially available in more than 40 markets for over 100 years as a treatment for diarrhea. Several randomized controlled studies have been published evaluating the efficacy of the heat-treated *Lactobacillus* blend in both children and adults with a focus on resolution of diarrhea symptoms.

Supplementation of the heat-treated *Lactobacillus* blend for four days in children with acute diarrhea reduced the time to obtain a normal bowel movement by 27 hours ($P<0.05$).[24] Children with noncharacterized diarrhea were given a sachet containing the heat-treated *Lactobacillus* blend every 12 hours for four days. The statistical difference was only noted when used in conjunction with oral rehydration therapy.

In a study conducted in 2000, the heat-treated *Lactobacillus* blend was supplemented in children with acute diarrhea.[25] Both control and heat-treated *Lactobacillus* blend groups received oral rehydration therapy, while a subset of children received antibiotic therapy prior to initiation of the study. Compared to the control group, children receiving the heat-treated *Lactobacillus* blend had a significantly reduced duration of diarrhea (57.0 vs 43.4 hours, respectively, $P<0.05$). A significant number of children receiving the heat-treated *Lactobacillus* blend had "formed" stools after 24 hours ($P<0.05$). By 72 hours posttreatment, 97% of the children receiving the heat-treated *Lactobacillus* blend had recovered as compared to 75% of children receiving the control ($P<0.05$). Antibiotic treatment did not impact results.

The effect of the heat-treated *Lactobacillus* blend was evaluated in babies with acute nonrotavirus diarrhea.[26] Babies received either a control sachet or a sachet containing the heat-treated *Lactobacillus* blend every 12 hours for 72 hours. Duration of diarrhea was significantly reduced in babies receiving the heat-treated *Lactobacillus* blend compared to control (39.5 vs 63.4 hours, respectively, $P<0.05$). After 72 hours, cessation of diarrhea was noted in 86% of babies receiving the heat-treated *Lactobacillus* treatment as compared to 53% of control. The authors concluded that the heat-treated *Lactobacillus* blend provided a clinically significant benefit to children with nonrotaviral diarrhea.

A study examining the impact of the heat-treated *Lactobacillus* blend in children with diarrhea was conducted in Peru.[27] The children enrolled in the study included a cohort that had diarrhea for longer than 24 hours at the beginning of supplementation. Duration of diarrhea in children that had symptoms for longer than 24 hours was significantly decreased with the heat-treated *Lactobacillus* blend group compared to control (9.1 vs 36.0 hours, $P<0.05$). The authors concluded that the heat-treated *Lactobacillus* blend was effective in children with well-established diarrhea.

The effect of the heat-treated *Lactobacillus* blend was evaluated in adults with acute diarrhea.[28] After one day of supplementation, 33% of adults receiving the heat-treated *Lactobacillus* blend had returned to normal stool frequency as compared to 3% of those receiving control ($P<0.05$). A similar percentage of subjects from both treatment groups had returned to normal stool frequency by day three of

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supplementation. These results indicate that the heat-treated *Lactobacillus* blend can help in resolving diarrhea symptoms quickly in otherwise healthy adults.

A controlled randomized study investigated the effect of the heat-treated *Lactobacillus* blend when given to adults with chronic diarrhea.[29] Adults received their supplement twice daily for four weeks. Daily bowel frequency was significantly reduced at weeks two and four in the heat-treated *Lactobacillus* blend group ($P<0.05$). Stool consistency, abdominal pain and distention were significantly improved in adults receiving the heat-treated *Lactobacillus* blend ($P<0.05$). The authors concluded that the heat-treated *Lactobacillus* blend can relieve symptoms of chronic diarrhea.

Although research with the heat-treated *Lactobacillus* blend has predominantly focused on populations with acute or chronic diarrhea, a small amount of work has focused on the effect of supplementation on irritable bowel syndrome (IBS). When the heat-treated *Lactobacillus* blend was supplemented twice daily for one month in adults with symptoms of diarrhea-predominant IBS (IBS-D), a significant reduction in pain score and bloating was noted ($P<0.05$).[30] The mean number of stools per week significantly decreased with heat-treated *Lactobacillus* treatment ($P<0.05$).

A randomized, double-blind, crossover trial was conducted to compare the efficacy the heat-treated *Lactobacillus* blend in adults with IBS.[31] Subjects received their treatment for six weeks followed by a two-week washout. Criteria (abdominal pain, bloating, daily number of stools, consistency, mucus content, and general physical state) were evaluated daily by the individual subjects in the study. When receiving the heat-treated *Lactobacillus* blend, 50% of subjects obtained better results compared to control. Although these data are promising, larger scale studies are needed to confirm the effects of the heat-treated *Lactobacillus* treatment on IBS.

Published research has confirmed that the heat-treated *Lactobacillus*s blend can reduce duration of diarrhea and improve gut defenses in adults and children. A small amount of work indicates that the heat-treated *Lactobacillus* blend could alleviate symptoms of IBS. Additional benefits to immune health and gastrointestinal health have not been assessed. No published work has evaluated the efficacy of daily consumption of the heat-treated *Lactobacillus* blend in a healthy population of humans or animals. Given the volume of positive results reported in compromised populations, the efficacy of the heat-treated *Lactobacillus* blend was evaluated in healthy companion animals.

Efficacy of Heat-Treated *Lactobacillus* Blend in Pets

To determine the effect of the heat-treated *Lactobacillus* blend in companion animals, several feeding studies were conducted. The overall aim of these series of nutritional studies was to evaluate the effect of the heat-treated *Lactobacillus* blend on gastrointestinal and immune health in healthy pets. Feeding studies were conducted with puppies, adult dogs, kittens, and adult cats at pet care centers in Missouri, Alaska and France. Length of feeding ranged from three to 10 months, and all dietary treatments were blinded to the staff at the pet centers until data were validated. The results of these feedings studies are summarized below:

General Health. All pets remained in good health throughout the studies. No animals were removed from the studies due to an adverse reaction to the heat-treated *Lactobacillus* blend. Complete blood count and blood chemistry data were evaluated throughout supplementation, and the values remained in normal ranges.

During a feeding study in adult dogs in Alaska, an unexpected outbreak of infectious diarrhea occurred in the kennel. The dogs had been receiving their allotted treatment for two months when the diarrhea outbreak occurred. The staff veterinarian determined that clinical signs were consistent with a rotaviral or corona virus infection There was no elevation in white blood cell count, and the duration of signs ranged from two to six days. Signs included vomiting, diarrhea and anorexia. Over 50% of dogs in the control group had diarrhea during this time. In addition, the diarrhea affected an equal percentage of a separate group of dogs that were housed in close proximity to the dogs on the feeding study. Those dogs receiving the heat-treated *Lactobacillus* blend did not show any signs of illness, and their fecal quality remained at the ideal score throughout the entire period of the infection. Fecal *Clostridia perfringens* concentrations (measured by qPCR) were significantly lower in dogs receiving the heat-treated *Lactobacillus* blend at the time of the diarrhea outbreak ($P<0.05$). These findings indicate that the heat-treated *Lactobacillus* blend provided a protective benefit when consumed daily.

Fecal Quality. Fecal quality was monitored daily in all studies using the Nestlé Purina fecal scoring system. Scores range from 1 (very hard and dry) to 7 (watery with no texture). Overall fecal quality and number of unacceptable fecal scores were analyzed. Significant differences in fecal quality were noted in puppies. Overall fecal scores were closer to an ideal score in puppies receiving the heat-treated *Lactobacillus* blend, and the number of unacceptable fecal scores were significantly higher in puppies receiving control supplements ($P<0.05$). Negative fecal quality was not observed from consumption of the heat-treated *Lactobacillus* blend in any of the feeding studies.

GI Health. Impact of the heat-treated *Lactobacillus* blend on bacteria was measured using pyrosequencing in studies with healthy adult dogs, growing kittens and healthy adult cats. Effect on microbiome populations and diversity were evaluated after 10 months of supplementation. Multivariate

analysis of the 16S rRNA gene data set was performed using orthogonal partial least squares discriminant analysis (OPLS-DA) to determine effect of dietary treatment.

Pyrosequencing was completed on feces collected from healthy adult dogs receiving can food to determine the effect of the heat-treated *Lactobacillus* treatment on the gut microbiome. Differences in microbiome populations and diversity were noted between the control group and heat-treated *Lactobacillus* blend group after 10 months of supplementation ($Q2=0.87$). Overall microbiome was affected, with a generally higher presence of the *Phylum Firmicutes* present in the heat-treated *Lactobacillus* group, and indices of diversity were significantly higher compared to control ($P<0.05$). Similar results were seen in kittens, with the heat-treated *Lactobacillus* blend significantly increasing *Firmicutes* and diversity indices compared to control ($P<0.05$). Adult cats had a similar increase in *Firmicutes, Proteobacteria* and *Bacteroidetes* with heat-treated *Lactobacillus* supplementation, and differences in the overall microbiome compared to control were noted with PCA analysis ($Q2=0.44$).

These results indicate that the fecal microbiome of healthy animals is positively affected by consumption of the heat-treated *Lactobacillus* blend. A greater level of species diversity reduces the opportunity for potential pathogens to colonize the gut and protects from gastrointestinal bacterial pathogens.[32] Daily consumption of the heat-treated *Lactobacillus* blend improves the fecal microbiome community and may be protective.

Immune Health. The impact of the heat-treated *Lactobacillus* blend on immune health was measured throughout the course of these studies. Immune function was evaluated using several methods.

Fecal IgA was used as a marker for the status of the local gut immune system. A higher level of fecal IgA is a marker for an active gut immune system, as it translates to the ability of the secreted IgA to eliminate harmful bacteria and neutralize toxins. Overall fecal IgA was significantly increased in populations of healthy dogs, healthy cats and growing puppies receiving the heat-treated *Lactobacillus* blend ($P<0.05$). An increase in fecal IgA indicates a significant positive effect on the local gut immune system.

Using a Luminex Immunoassay platform, plasma cytokine concentrations in healthy dogs consuming the heat-treated *Lactobacillus* blend were evaluated monthly. Cytokines are small soluble proteins that are used in intercellular communication to impact cellular immune responses. The Luminex technology allows simultaneous quantification of several biologically relevant cytokines using very small quantities of sample. PCA analysis demonstrated that separation of circulating cytokines between control and the heat-treated *Lactobacillus* group increased with increasing time of supplementation ($Q2=0.12$, 0.44 and 0.63 at months 2, 3 and 4, respectively). The strong separation between control and the heat-treated *Lactobacillus* blend was driven by a lower concentration of IL-18 and MCP-1 in dogs receiving the heat-treated *Lactobacillus* blend compared to control. These results indicate that supplementation of the heat-treated *Lactobacillus* blend helps prime the immune system for a potentially different response to a mild stress compared to animals receiving control.

While fecal IgA and cytokine concentrations were affected by the heat-treated *Lactobacillus* blend, vaccine response was not affected when titers were measured in adult dogs, adult cats and growing kittens. Numerical differences were noted in titer response in cats and kittens, though these changes did not reach statistical significance.

To confirm that changes noted in immune status brought about by the heat-treated *Lactobacillus* blend did not overstimulate the immune system, plasma C-reactive protein (CRP) was measured. CRP is an acute phase protein that is produced by the liver in response to inflammation. Animals receiving the heat-treated *Lactobacillus* blend had CRP levels well within the normal ranges, indicating that changes in immune status are indicative of an enhanced immune system and not due to an overactive immune system.

Overall, results achieved in these studies indicate that immune function in healthy populations of cats, dogs, kittens, and puppies were positively impacted by supplementation of the heat-treated *Lactobacillus* blend.

Response to a Naturally-Occurring Stress. The impact of the heat-treated *Lactobacillus* blend on physiological responses to a mild, naturally occurring stress was evaluated. The naturally-occurring stresses evaluated were mild restraint in small dogs, cats and kittens, while larger dogs underwent physically demanding exercise. Measurements were taken before and several times after the naturally occurring stress.

General measures of stress were impacted by the heat-treated *Lactobacillus* blend in kittens and adult cats. After 10 months of feeding, fecal adrenocorticotrophic hormone (ACTH) was significantly lower in kittens receiving the heat-treated *Lactobacillus* blend compared to control ($P<0.05$). Adult cats receiving the heat-treated *Lactobacillus* blend maintained their ACTH concentrations during 20 weeks of supplementation, while the control group had a significant increase ($P<0.05$).

The effect of mild restraint was evaluated in small dogs. Following restraint in a pet carrier for 20 minutes, fecal quality was significantly lower in control dogs when compared to dogs fed the heat-treated *Lactobacillus* blend ($P<0.05$). Dogs receiving the heat-treated *Lactobacillus* blend maintained their fecal quality immediately following the restraint and for several days after, indicating that dogs fed the blend were less affected by the natural stress of being placed in a carrier.

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The impact of the heat-treated *Lactobacillus* blend on immune and gastrointestinal health parameters following exercise was evaluated in healthy adult dogs. Microbial diversity of fecal samples was measured using temporal temperature gel electrophoresis (TTGE). Following exercise, dogs receiving the heat-treated *Lactobacillus* blend had significantly higher Shannon-Weiner and Simpson's indexes of diversity compared to control dogs ($P<0.05$). This increase in diversity indicates that the heat-treated *Lactobacillus* blend improved gut microbiota stability during times of naturally occurring stress. Exercise also impacted circulating cytokines in healthy dogs. Following exercise, there was an immediate upregulation of inflammatory cytokines regardless of treatment. By 24 hours post-exercise, separation between the treatment groups had returned ($Q2=0.74$), indicating that the control and heat-treated *Lactobacillus* blend groups were again expressing different ratios of cytokines. The difference between the treatment groups at 24 hours post-exercise was driven by a decrease in inflammatory cytokines (IL-8 and IL-18) in the heat-treated *Lactobacillus* blend group, while dogs in the control group had an increase in chemokine recruiters, indicating elevated inflammation.

Supplementation of the heat-treated *Lactobacillus* blend appears to positively impact dogs and cats during times of mild, naturally occurring stresses. The response to stress was more readily managed in healthy animals by maintaining microbial diversity, decreasing length of inflammatory response, maintaining ideal fecal quality, and decreasing markers of stress.

Consumption of the heat-treated *Lactobacillus* blend improved signs of gastrointestinal and immune health in populations of healthy companion animals. Daily intake of the heat-treated *Lactobacillus* blend may confer a protective benefit to pets during times of mild, naturally occurring stress.

Conclusion

Although probiotics have been shown to be beneficial in pets, maintaining viability of the organisms has limited their application in pet foods. Supplementation of inactivated probiotics and their supernatant growth media would allow for application in a variety of products and could be an alternative for healthy or immunocompromised populations. Inactivated probiotic bacteria may not have the same benefits as their live counterparts, so the potential benefits of each bacteria should be confirmed. A blend of heat-treated *Lactobacillus delbruekii, Lactobacillus fermentum* and their supernatant has been shown to improve duration and symptoms of diarrhea in humans. Diets supplemented with this heat-treated *Lactobacillus* blend significantly improved several indicators of gastrointestinal and immune health when included in the diets of healthy dogs and cats, even in times of naturally occurring stress.

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