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Use of a microbial endocrinology designed dopamine-producing probiotic to control gut neurochemical levels associated with the development of gut inflammation

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ABSTRACT

Neurochemicals, such as the catecholamines, serve as critical regulators controlling the immune response in the pathogenesis of inflammation. Overproduction of one of these neurochemicals, namely norepinephrine, has been previously shown to occur concomitant to the development of diet-induced gut inflammation. As a pro-inflammatory neuroimmune modulatory chemical, norepinephrine can dysregulate the immune response to inflammation. In order to ameliorate the increased production of norepinephrine, we have utilized a microbial endocrinology-based approach that utilizes an *Enterococcus faecium* probiotic to produce the anti-inflammatory neurochemical dopamine. As shown in the present study, the feed incorporated *E. faecium* probiotic converts the precursor L-dopa to dopamine with high efficiency to produce significant amounts of dopamine within the gastrointestinal tract. In replicate broiler feeding trials utilizing a high non-starch polysaccharide (NSP) inflammation-inducing diet in combination or not with L-dopa alone or in combination with the dopamine-producing *E. faecium* probiotic, the NSP diet induced a large increase in norepinephrine concomitant to the development of inflammation that was abrogated in the groups fed the L-dopa precursor in combination with the dopamine-producing *E. faecium*. Less, though still significant, amelioration of the norepinephrine increase was achieved in the group only fed the L-dopa precursor. The present report represents the first use of a dopamine-producing probiotic to mechanistically influence the production of another neurochemical that is intimately involved in the pathophysiology of gut inflammation. As such, this study demonstrates that the use of a Microbial Endocrinology-designed probiotic can serve as a means by which to prevent and/or control the development of gut inflammation in poultry.

Introduction

The widespread discontinuance of antibiotics in poultry feed as a means by which to improve growth performance has led to the emergence of low-grade, chronic inflammation within the intestinal tract. This has become the one of the most pressing issues negatively impacting poultry production (Morgan, 2017). The intestine is constantly exposed to a number of environmental triggers including reused litter, intestinal pathogens, poor quality feed ingredients, high energy diets, and changes in feed formulation that stimulate inflammation and lead to a reduction in performance. The ability of neurochemicals within the gut, especially those that are involved in the regulation of immunity during inflammation, are poorly understood.

One approach that has been used industry-wide to ameliorate gut inflammation has been through the use of probiotics added to the feed or drinking water (Shehata, et al., 2022). While there have been reports of some success, the widespread use of probiotics is plagued with inconsistent results. Much of this is due to an incomplete mechanistic understanding of how a specific probiotic may benefit gut health and improve overall growth performance. Without knowing the mechanism

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of action once the probiotic is fed, especially as no quantitative biological measures are performed when sold as a poultry feed additive, there cannot be any guarantee of batch-to-batch consistency. This situation leads to inconsitent impact of probiotics as a means to consistently and predictably improve growth performance.

Thus, the use of a probiotic that provides a mechanistic, quantifiable means by which to treat and or prevent the development of gut inflammation would represent a more viable means by which to consistently improve poultry gut health and growth performance. In order to accomplish this goal, we have employed a Microbial Endocrinology-designed approach to identify a probiotic that leverages intestinal neurochemistry as a means to control the immune elements that drive the pathogenesis of gut inflammation.

Microbial Endocrinology represents the union of microbiology and neurobiology (Lyte, 2016). We propose Microbial Endocrinology can be used for the design of probiotics that can be employed in a directed, mechanistic fashion in which the probiotics are chosen by their ability to influence key immune elements that control the pathogenesis of inflammation (Lyte, 2011). Prior *in vitro* work demonstrated that certain *Enterococcus* spp., in particular *E. faecium*, are capable of the efficient conversion of L-dopa to dopamine (Villageliu and Lyte, 2018). Using our previously described diet-induced gut inflammation model, we herein report on the use of an *E. faecium* dopamine-secreting probiotic to suppress the production of a key gut neurochemical, namely norepinephrine, that is, in part, responsible for driving the immune inflammatory process within the gut.

Materials and methods

Ethical statement

The experiments were approved by, conducted in accordance with guidelines set by, the United States Department of Agriculture Animal Care and Use Committee. The trials were conducted at the Agricultural Research Service Facility of the United States Department of Agriculture (ARS-USDA), College Station, Texas, US.

Experimental design

The experimental design utilized the exact same conditions as previously reported (Kogut, et al., 2024). In brief, Cobb by-product day--of-hatch chickens were randomly divided into experimental treatment groups of 25 animals per group and raised to 28-days of age. The individual chicken was considered as the experimental unit. Treatment groups were 1) Inflammation diet (non-starch polysaccharides; NSP), 2) standard corn/soybean diet, 3) NSP diet + L-dopa prebiotic, and 4) NSP diet + L-dopa prebiotic + probiotic. Two replicate trials were performed (i.e. the entire study was performed twice). Diets, fed from Day 1 until sacrifice, consisted of either a corn/soybean standard diet or a chronic low-grade inflammation inducing diet composed of high amounts of NSP. The composition of the NSP diet, and its ability to induce a progressive chronic low-grade inflammation in the intestinal tract, has been previously described (Dal Pont, et al., 2021), and the feed compositions of the corn/soybean and NSP diets used in the present study are identical to those previously reported, hence Table 1 is reproduced here. All diets were iso-energetic, iso-nitrogenous, and formulated to meet or exceed the chicken requirements (Cobb-Vantress, 2021). The composition of the corn-soy and NSP diets are presented in Table 1. Probiotic was incorporated into the basal NSP diet at a final concentration of 3×10^{11} CFU per kg of feed. The prebiotic mucuna powder was added as top-dressing to the basal NSP diet. The NSP inflammation-inducing diet was either supplemented with mucuna powder in the absence or presence of the dopamine-producing probiotic *E. faecium*. Feed and water were available *ad libitum* to all groups throughout the trials.

Table 1
Ingredients and calculated nutritional composition of experimental diets.

Ingredients (%)	Starter (1-21 d)		Grower (22-36 d)	
	Corn-soy	NSP	Corn-soy	NSP
Corn	58.87	31.08	65.97	38.185
Soybean meal[1]	34.75	29.93	27.87	22.515
Rice bran	0	30	0	30
Soybean oil	2.40	5.62	2.74	5.95
Monocalcium phosphate	1.72	1.333	1.42	1.03
Limestone	1.08	1.255	0.93	1.10
NaCl	0.37	0.35	0.37	0.35
DL-Methionine	0.35	0.35	0.27	0.30
L-Lysine HCl	0.22	0.29	0.18	0.259
L-Threonine	0.098	0.165	0.012	0.079
Choline chloride	0.05	0.05	0.05	0.05
Vitamin premix[2]	0.05	0.05	0.1	0.1
Mineral premix[3]	0.03	0.03	0.05	0.05
Calculated composition				
Metabolizable energy (kcal/kg)	2990	2990	3100	3100
Crude protein (%)	22	22	19	19
Lysine dig. (%)	1.22	1.22	1.02	1.02
Methionine (%)	0.61	0.63	0.53	0.55
Meth. + cysteine dig. (%)	0.91	0.91	0.80	0.80
Threonine dig. (%)	0.83	0.83	0.66	0.66
Av. Phosphorus (%)	0.45	0.45	0.38	0.38
Calcium (%)	0.90	0.90	0.76	0.76
Potassium (%)	0.92	1.14	0.79	1.02
Sodium (%)	0.16	0.16	0.16	0.16

[1] Soybean meal 49 % of crude protein
[2] Composition of minimum (per kg of feed): Vit A 8,818,342 IU; Vit D3 3,086,420 IU; Vit E 3,674 IU; Vit B12 130 mg; Vit K 1,177mg; Vit B2 4,775 mg; pantothenic acid 16,168 mg; Vit B1 2,350 mg; Vit B3 36,742 mg; Vit B6 5,732 mg; folic acid 1.1,398 mg; choline 104,460 mg; biotin 441mg.
[3] Minimum of Fe 12 %; Cu 1.4 %; I 800ppm; Zn 12 %; Mn 173.0 mg; Mg 12 %
Av.: available; dig.: digestible.

Probiotic and prebiotic preparations

Lactiferm® (Chr. Hansen, Hørsholm, Denmark), an *E. faecium* commercially available product approved by EFSA and the FDA for use in a range of farm production animals including poultry, was utilized as the probiotic for *in vitro* and *in vivo* experiments. For a source of the prebiotic, L-dopa, finely ground *Mucuna pruriens* (mucuna) beans (Purest Botanical, Sodiko-Douala, LT 9667, Republic of Cameroon) which contained 6.4 g of L-dopa per 100 g of dry bean weight were utilized. Mucuna is a well-recognized source of pure L-dopa and has been utilized in the treatment of neurodegenerative diseases such as Parkinson's disease (Cassani, et al., 2016). For incorporation into feed, Lactiferm® was used at a final concentration of 3×10^{11} CFU per kg of feed. Finely ground mucuna was employed at 10 g per kg of feed which provided a L-dopa final concentration in the feed of 0.064 %. It is important to clarify that a prebiotic is not that exclusively utilizable by bacteria, but rather a compound or substrate that has known action on the host through intermediary bacterial metabolism. Hence, *Mucuna pruriens* containing L-dopa serves as a prebiotic for utilization by the probiotic bacteria for conversion into dopamine to ultimately impact the host. Numerous poultry feeding studies which have utilized mucuna as a source of protein have shown no adverse physiological effects at the concentrations used in the present study (Vadivel and Pugalenthi, 2010). In addition, mucuna has been used in livestock species including swine and cattle (Pugalenthi, et al., 2005). Since the submission of this report, we have transitioned from mucuna to the use of proprietary developed food grade coated pure L-dopa for future trials. In corn/-soybean meal feeding trials, as well as *in vitro* experiments, the coated L-dopa has performed equally to that observed with mucuna in its ability to be converted by *E. faecium* to dopamine (data not shown).

Determination of catecholamine levels

The quantitative determination of norepinephrine and dopamine was performed by ultra-high performance liquid chromatography (UHPLC) with electrochemical detection as previously described (Villageliu, et al., 2018). In brief, following euthanasia, cecal tissues were rapidly dissected free of mesentery. Full thickness ceca tissue from the blind end of a ceca pouch was immediately acidified in 0.2N perchloric acid, placed into a reinforced tube containing 6 (2.8mm) ceramic beads, and snap frozen on dry ice until analysis. Tissues were homogenized using a beadmill and then centrifuged (15min, 4°C, 3000xg). Supernatant was collected and passed through a 2-3kDa spin filter, the flow-through of which was collected and analyzed for norepinephrine using ultra-high performance liquid chromatography (UHPLC) with electrochemical detection (ECD) as previously described (Lyte, et al., 2022). Similarly, broth cultures of the probiotic *E. faecium* in the presence, or absence, of the prebiotic L-dopa were centrifuged, passed through a spin filter, and flow-through was analyzed for dopamine using UHPLC-ECD. Chromeleon (v7.2, ThermoFisher Scientific) software was used for data analysis. Norepinephrine and dopamine identification were verified using corresponding analytical standards (Millipore-Sigma, MA).

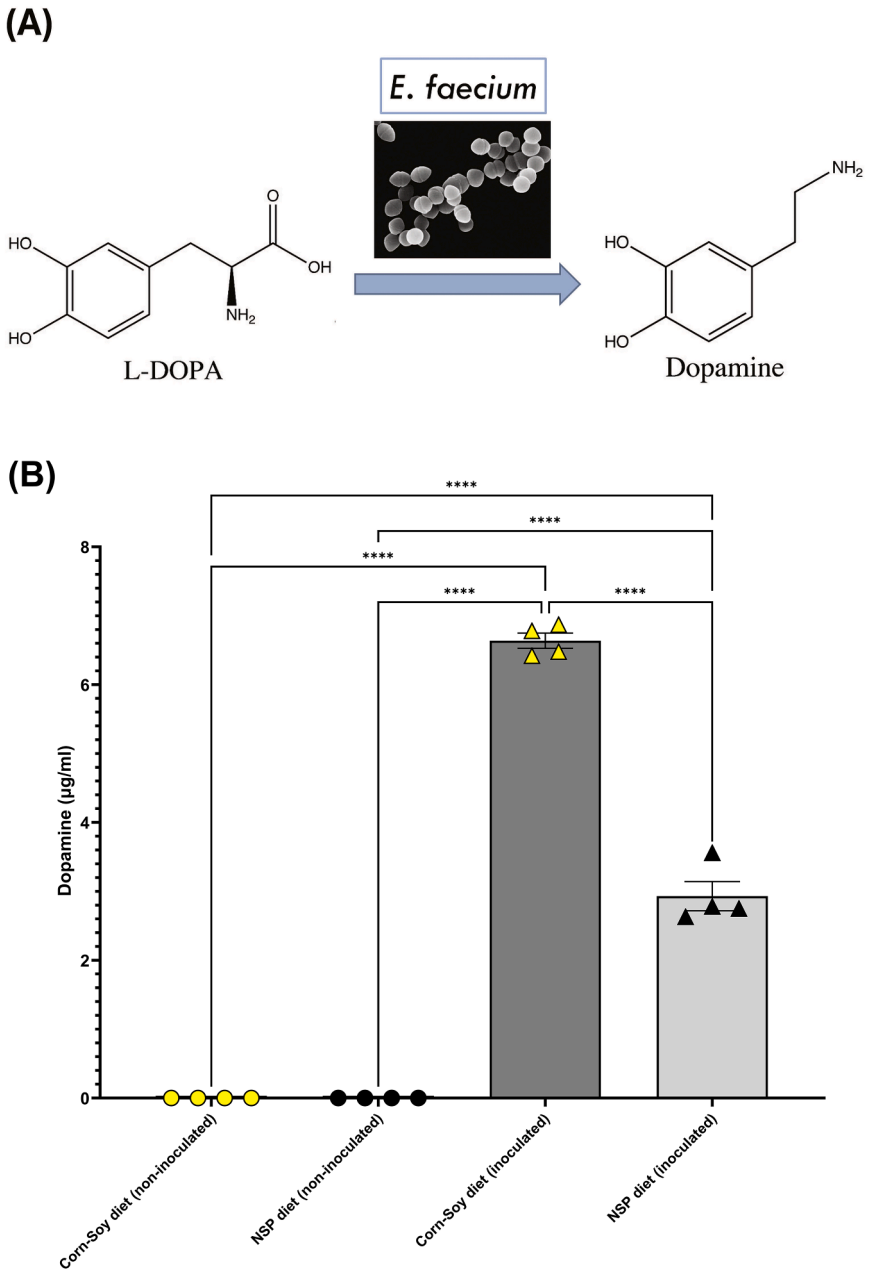

Fig. 1. *E. faecium* converts L-dopa to dopamine in a feed-based intestinal simulated media (sSIM)
(1A) Conversion of L-dopa to dopamine by *E. faecium.* The ability of *E. faecium* to convert the precursor (prebiotic) molecule L-dopa to dopamine is due to the *E. faecium* bound enzyme dopa decarboxylase (also known as aromatic-L-amino-acid decarboxylase). Within the animal kingdom, dopa decarboxylase is also utilized for the synthesis of other neurochemicals in addition to dopamine such as serotonin.
(1B) *In vitro* production of dopamine by *E. faecium* in sSIM medium in the presence of added L-dopa. Simulated small intestinal medium (sSIM) was prepared from a corn/soybean standard poultry diet and an NSP-inflammation inducing diet using methodology as previously described (Villageliu, et al., 2018). sSIM more accurately approximates the *in vivo* compositional food environment in the small intestine than does the use of standard rich microbiological medium. Data were analyzed by one way ANOVA followed by Tukey's posthoc as described in Methods. The results shown are the mean±SEM, statistical significance was set at $p<0.05$.

In vitro testing of dopamine producing capacity

The capacity of the *E. faecium* probiotic to produce dopamine in the presence of the prebiotic L-dopa was tested *in vitro* utilizing simulated small intestinal mediums (sSIM) prepared from the corn/soy and the NSP diets. The preparation of sSIM was performed as has been previously described (Villageliu, et al., 2018). Quadruplicate cultures of corn/soy and NSP feeds were prepared as sSIM were then inoculated, or not (control), with 0.1ml of a 0.2 O.D. broth culture of *E. faecium*. All cultures were additionally supplemented with 10μg of L-dopa and then incubated aerobically at 41°C for 24 hours. The quantitative determination of dopamine was then performed by UHPLC as described above in *Determination of Catecholamine levels* (Villageliu, et al., 2018).

Statistical analysis

Graphing and statistical analyses utilized the GraphPad Prism software (version 10, GraphPad, La Jolla, CA). All cecal neurochemical data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test. As we hypothesized that the chickens provided a L-dopa, L-dopa + probiotic, or corn/soy diets would have lower levels of inflammation than that caused in chickens given the NSP diet, each treatment group was compared to the NSP diet group. One-way ANOVA followed by Tukey's post-hoc test was used for analysis of *in vitro* dopamine concentrations. A P-value < 0.05 was considered statistically significant. Data are presented as mean ± SEM.

Results

E. faecium production of dopamine in sSIM medium is impacted by type of feed

Dopamine was not detected in corn-soy diet sSIM or NSP diet sSIM medias without addition of the probiotic *E. faecium* (Fig. 1A/B). The addition of *E. faecium* resulted in the detection of dopamine (ug of dopamine per mL of sSIM media) in both corn-soy and NSP diet sSIM medias ($p<0.05$). Dopamine concentrations by *E. faecium* in corn-soy sSIM were significantly ($p<0.05$) greater than that measured in *E. faecium* inoculated NSP media.

Time-course of E. faecium production of dopamine in sSIM medium

Dopamine was not detected in sSIM media that lacked the *E. faecium* probiotic (Fig. 2). The addition of the *E. faecium* probiotic to the sSIM media demonstrated rapid production of dopamine (ug of dopamine per mL of sSIM media) within 4h of inoculation (Fig. 2) reaching maximal dopamine concentrations under 24h.

Chicken cecal norepinephrine concentrations are dependent on diet and modulated by in-feed pre- and probiotics

Norepinephrine was detected in the ceca tissue of chickens from each treatment group (Fig. 3). Corn-soy diet group, NSP+prebiotic group, and NSP + prebiotic + probiotic group each had significantly lower ($p<0.05$) cecal tissue norepinephrine concentrations when compared to the NSP diet group. This statistically significant reduction in cecal tissue norepinephrine due to in-feed inclusion of pre- and probiotics was found to repeatable between both study replicates (Fig.s 3A/B).

Discussion

The present study represents the first demonstration of the utility of a microbial endocrinology-designed probiotic (Lyte, 2011) in poultry, specifically an *E. faecium* dopamine-converting probiotic to produce a neurochemical that is recognized to be responsible for the mediation of pro-inflammatory events in the gut (Channer, et al., 2023). Prior work demonstrated the ability of *Enterococcus* spp. isolates to efficiently convert the dopamine precursor, L-dopa, to dopamine (Villageliu and Lyte, 2018). As we have noted previously (Villageliu and Lyte, 2018) there is a marked difference in ability of different strains of *Enterococcus*

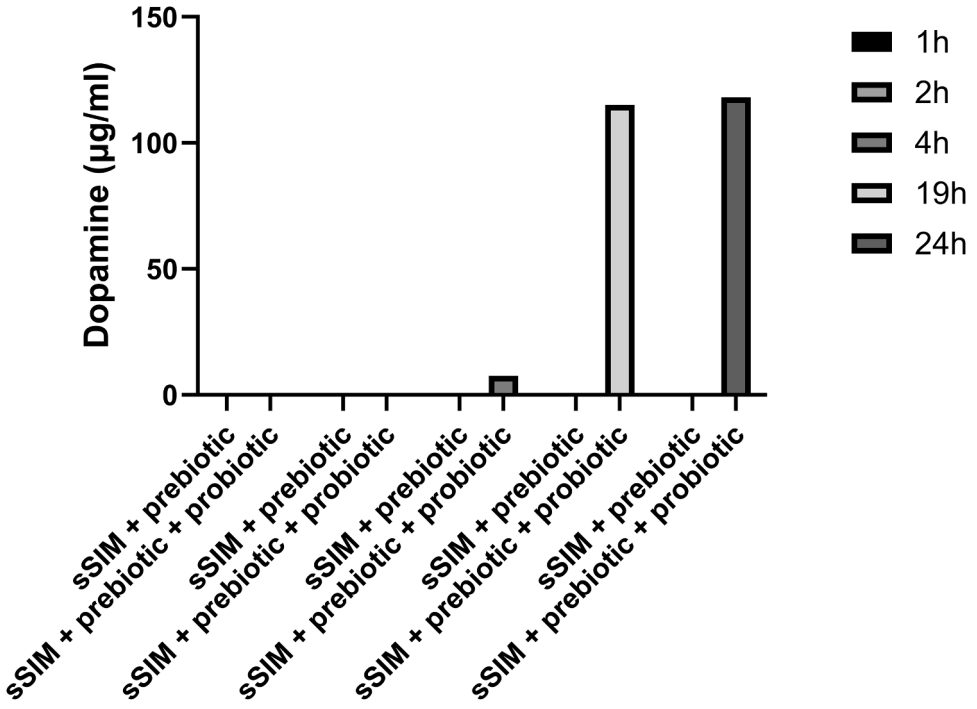

Fig. 2. Time-course of dopamine production by *E. faecium* in sSIM *in vitro*.
E. faecium was incubated for 24 hours in sSIM in the presence of the prebiotic L-dopa. Additionally, cultures without *E. faecium*, but with the prebiotic, were also performed. Dopamine production was detected as early as 4 hours following inoculation, reaching maximal concentration within 24 hours. No dopamine production was detected in sSIM cultures incubated with only the prebiotic.

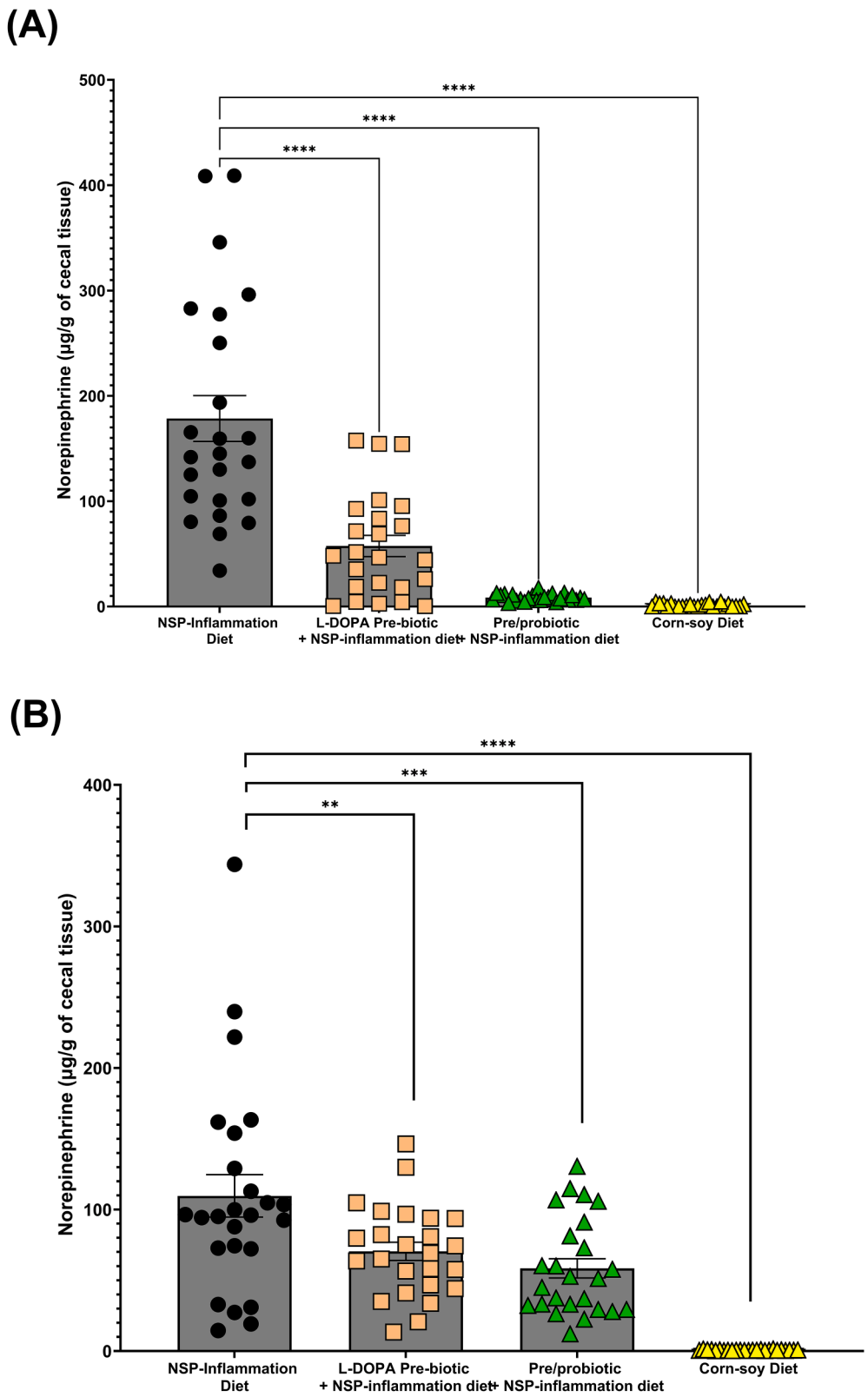

Fig. 3. Norepinephrine content in cecal tissue in birds fed corn or NSP inflammation diet in the absence or presence of L-dopa prebiotic alone or in combination with prebiotic L-dopa and dopamine-producing *E. faecium* probiotic. Concentration of norepinephrine in the cecal tissue of broilers (N=25 birds per group) fed for 28 days either a normal corn-soy diet or NSP inflammation-inducing diet during the whole experiment for Trial 1 (Fig. 3A) and Trial 2 (Fig. 3B). Cecal tissue was collected and processed from individual animals and each graph symbol is for a unique individual animal. Data were analyzed using one-way ANOVA followed by Dunnett's post-hoc test, as described in Methods. Each individual data point represents an individual unique animal. The results shown are the mean±SEM with asterisks indicating significance level. Statistical significance was set at $p<0.05$.

spp. to produce dopamine. The molecular basis for these differences has not yet been elucidated. The present study represents the first use of this dopamine-producing probiotic (Fig. 1A) in chickens. As shown in Fig. 1B, the *in vitro* production of dopamine by the dopamine-producing *E. faecium* probiotic in the presence of the prebiotic L-dopa occurred in sSIM preparations from a standard corn/soy and NSP-inflammation diets. Interestingly, dopamine production was higher in the corn/soy diet sSIM than in the sSIM prepared from the NSP diet. Although the reason for this is not clear, it is interesting to speculate that the development of inflammation may be due, in part, to the suppression of the gut microbiome that are capable of dopamine production thereby further providing an environment in which inflammation could proceed into an acute or low-grade chronic state. It should be noted that the time-course experiments examining the time needed for *E. faecium* to produce dopamine demonstrated that dopamine production occurred as early as 4 hours and reached maximal production by 24 hours (Fig. 2). By the end of the incubation period, measurement of the amount of remaining L-dopa in the media indicated that 99.9 % of all supplemented L-dopa had been converted to dopamine. These results satisfy a key requirement of any administered probiotic, namely that its mode of action within the gut occurs quickly enough to account for gastrointestinal transit before it would be excreted out from the animal. Given that transit times in poultry can be as short as 4 hours depending on the viscosity of the digesta (Sacranie, et al., 2012), the data presented indicate that incorporation of this probiotic into feed should meet that requirement.

In order to induce sub-clinical, chronic gut inflammation, we utilized a validated dietary model to employ a NSP diet (30 % rice bran) (Dal Pont, et al., 2021). Rice bran is an alternative ingredient for energy used in animal nutrition. Compared to other alternatives, such as wheat, rice has higher metabolizable energy and lower fiber content. Although rice bran is not considered a soluble ingredient, it still has a considerable amount of fiber, one of the highest phytate content in vegetable ingredients (1.37 %), and a large amount of lipids (14.2 %) that may undergo peroxidation (Dal Pont, et al., 2021). As shown in Fig.s 3A and B, chickens fed the NSP diet had a pronounced increase in cecal norepinephrine concentrations as compared to corn-soy fed group. Norepinephrine values in the corn-soy diet group were within the same range as previously reported in chickens also fed a corn-soy diet (Lyte et al., 2022). As previously shown (Dal Pont, et al., 2021; Kogut, et al., 2024), the NSP-group experienced decreased growth performance (data not shown). Norepinephrine is known as a “fight-or-flight” catecholamine neurochemical and is normally produced locally within the gut (Laverty, 1978; Lyte, et al., 2011). Interestingly, the production of norepinephrine in the gut has been shown, in mammals, to be affected by the microbiome (Strandwitz, 2018). As a gut neurochemical, norepinephrine plays a critical role in immune cell activation (Stolk, et al., 2020). Increases in norepinephrine beyond what is normally required for immune regulation can result in immune dysfunction and promotion of an inflammatory state in the gut (Kogut, et al., 2024). Dopamine, which is also secreted by the elements of the enteric nervous system as well as immune regulatory cells and dendritic cells in the mucosal layer ameliorates the development of inflammation in the gut (Channer, et al., 2023). Importantly, increased production of dopamine has not been linked to immune dysfunction but instead to play a positive role in regulation of immune function not only in preventing inflammation but also increasing other immune responses such as the increased response to oral vaccines (Channer, et al., 2023).

As shown in the replicate trials (Fig.s 3A and 3B), the inclusion of the *E. faecium* dopamine-producing probiotic in combination with the prebiotic L-dopa abrogated ($p < 0.05$) the NSP-inflammation diet-induced increase in norepinephrine. Interestingly, in both of the trials, the inclusion of only the prebiotic, L-dopa, in the feed also resulted ($p < 0.05$) in a suppression of the NSP-induced production of norepinephrine within the gut. This was not an unexpected result given that poultry are colonized to a variable degree by *Enterococci* and related genera in the gut that would possess the necessary enzymes to convert L-dopa into dopamine. Further, it should be borne in mind that the number of commensal enterococci within the gut, which are responsible for the conversion of the prebiotic L-dopa to dopamine, can vary greatly between birds intra- and inter-flock (Shang, et al., 2018; Yue, et al., 2024). This is also most likely why in the second trial that although we observed the ability of the L-dopa prebiotic alone or in combination with the *E. faecium* dopamine-converting probiotic to reduce the NSP-induced rise in norepinephrine in the gut, we did not concurrently note as large a difference between the groups (L-dopa prebiotic alone or in combination with the probiotic) as we did in the first trial . This result between trials highlights that in order to achieve flock to flock and year-to-year consistent and reproducible suppression of inflammation-induced increases in norepinephrine, the inclusion of the *E. faecium* dopamine-converting probiotic will be required. An additional benefit would be that such inclusion would also presumably result in benefitting growth performance through the mitigation of the negative consequences of gut inflammation (Morgan, 2017; Shehata, et al., 2022).

The proposal that the use of a Microbial Endocrinology-designed dopamine-producing probiotic can effectively control the development of inflammation within the gut by modulating the neuroimmune dependent mechanisms governing inflammation is based upon a number of avenues of research (Lyte, 2011). Dopamine, which is secreted by the elements of the enteric nervous system as well as immune regulatory cells and dendritic cells in the mucosal layer has been shown to be capable of ameliorating the development of inflammation in the gut (Villageliu and Lyte, 2018). A major mechanism by which dopamine can affect the immune system occurs through the down-regulation of innate immune cell activity such as involving the secretion of proinflammatory cytokines and inflammasome activity (Villageliu and Lyte, 2018). However, due to a number of factors such as low chemical stability during processing and as well as high economic cost, dopamine has not been previously utilized to control inflammation. Instead, a number of dopamine agonists to ameliorate gut inflammation have been evaluated as therapeutic drugs which have met with limited success in vertebrate models (Oehlers, et al., 2017) but would be cost and regulatory prohibitive to use in poultry feed.

We therefore propose that the use of a dopamine-producing probiotic can effectively control the development of inflammation within the gut by modulating the neuroimmune mechanisms that are involved in the pathogenesis of inflammation. Future research is directed towards examining the role of neurochemicals in the pathogenesis of enteric infections and the ability of the Microbial Endocrinology designed dopamine-producing probiotic, as well as future neurochemical-producing probiotics, to mitigate disease progression and inflammation.

Conflict of interest

One of the authors, M.L., declares that The Iowa State University Research Foundation has filed a number of patents surrounding the discovery and utilization of the dopamine-producing probiotic described in this report.

None of the other co-authors has any declared conflict of interest.

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Disclosures

The Iowa State University Research Foundation and one of the

authors (M.L.) has filed a number of patents surrounding the discovery and utilization of the dopamine-producing probiotic described in this report.

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