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Research Article
Efficacy of a Novel, Native Microbial Feed Supplement on the Mitigation of Necrotic Enteritis in Cobb 500 Broiler Chickens

Andrew Izzo, Grant Gogul, Josh Lefler, Allison Wells, James Gaffney, Jennifer Cao, Norman Pitt, Brian Dirks and Mallory Embree

Native Microbials, Inc., San Diego, CA 92121, United States of America

Abstract

Background and Objectives: Necrotic enteritis, a potentially fatal intestinal disease, has been a recurring issue in the poultry industry. This problem has been exacerbated due to the phasing out of widespread prophylactic antibiotic usage. This study assessed the efficacy of a microbial feed supplement (MFS) (Native Microbials, San Diego, CA)comprised of bacteria native to the chicken gastrointestinal tractas an antibiotic alternative that prevents both clinical and subclinical necrotic enteritis. This MFS is composed of two chicken gastrointestinal microbiome members, *Hungatella xylanolytica* ASCUSBR21 and *Clostridium beijerinckii* ASCUSBR67. **Materials and Methods:** The effect of this MFS was tested in two trials by challenging broiler chickens with direct administration of *Clostridium perfringens*, the causative agent of necrotic enteritis. Methods were similar between Trial 1 and Trial 2, with the main variance being the number of birds used (Trial 1, n = 540; Trial 2, n = 936). Production and mortality data were collected over the entire study period (42-days). **Results:** Trial 1 revealed no statistically significant effects on production metrics (bird gain, feed intake, feed conversion ratio) but there was a positive improvement in feed conversion ratio in the group receiving the MFS (FCR: 1.61$\pm$0.01; p>0.05). Additionally, in Trial 1 there was a significant reduction in mortality from necrotic enteritis in the treated groups (2.89$\pm$2.68%; p = 0.05) and reduced severity of intestinal lesions (0.73$\pm$0.25; p<0.01). Trial 2 showed a 4-point improvement in feed conversion ratio compared to the positive control (FCR: 1.64$\pm$0.02; p < 0.05). **Conclusion:** The diet supplemented with MFS reduced mortality attributable to necrotic enteritis and improved feed conversion ratio. Further testing is needed to better understand the response and variability of these factors as they relate to the MFS.

Key words: Broiler, necrotic enteritis, *Clostridium perfringens*, microbial feed supplement, mortality

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Corresponding Author: Mallory Embree, Native Microbials, Inc., 10255 Science Center Drive, San Diego, CA 92121 Tel: 877-696-8945

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Necrotic enteritis (NE) is an intestinal infection of broiler chickens associated with increased rates of morbidity and mortality[1]. It is generally accepted that the primary etiological agent of NE is *Clostridium perfringens*, a gram-positive, spore-forming, anaerobic bacterium[2,3]. Considered to be a multifactorial disease, various circumstances predispose broilers to the development of NE, including housing conditions, management and intestinal co-infection by *Eimeria* species[4-6]. Clinical cases of NE often present with intestinal lesions and can cause abnormally high flock mortality, with rates known to exceed 10%[4]. A less severe variant of this disease can lead to impaired growth performance and feed efficiency[1,7]. As one of the most common diseases affecting broiler chickens, the economic and animal welfare impact of NE on global poultry producers is significant. It has been estimated that the global cost of the disease may approach $6 billion, highlighting the extreme importance of controlling NE from an economic perspective[8]. Historically, the use of in-feed antibiotics was the primary method employed for treating NE in commercial broiler flocks[9]. Antibiotics such as bacitracin methylene disalicylate effectively treat intestinal infection by *C. perfringens*, lowering flock mortality and improving growth performance during an NE outbreak[10,11]. However, the practice of feeding antibiotics to production animals has come under increased scrutiny in recent years from consumers and the scientific community. The public has expressed concern over the use of antibiotics in production animals and recent research demonstrates that an increasing number of consumers prefer to consume meat from animals raised without antibiotics[9,12]. Concern is also growing in the scientific community that continual use of in-feed antibiotics may intensify the development of antibiotic-resistant bacteria[13-15]. The United States government has acted as well, with the FDA's implementation of the Veterinary Feed Directive in 2017 that limited the extent to which food animal producers can use antibiotics[16]. Due to these and other factors, many large poultry producers have voluntarily removed antibiotics from their broiler chicken feeding programs[17]. The removal of antibiotics has increased the incidence and severity of NE in broilers[12,18]. This has created an urgent need for alternative methods to control NE.

Many researchers have explored gut-derived microbes in a variety of contexts relating to production animals. Several studies have examined shifting gut microbial populations in response to a disease or treatment, in attempt to determine the interplay between microbiome composition and the physiology of the host animal[19-21]. Others have attempted to isolate new species and utilize them to treat symptoms of various diseases affecting production animals, including NE[22,23]. Direct-fed microbial products have also been tested for their potential to ameliorate the effects of NE[24-26]. However, a commonality among these studies is that existing direct-fed microbial products often display limited or inconsistent efficacy[27,28]. As a result of this inconsistency and the ever-present issue of potential bacterial infection, antibiotic-alternative products are still a high-interest area among poultry producers.

The purpose of these studies was to investigate the effectiveness of a novel microbial feed supplement comprised of native GI chicken microorganisms on broilers under a NE challenge. Two strains of bacteria, *Hungatella xylanolytica* ASUCSBR21 and *Clostridium beijerinckii* ASCUSBR67, were selected and isolated to axenicity from the gut microbiome of healthy broiler chickens. These strains were associated with high growth performance and survivability in broilers as well as with displaying a capacity to interact with the host intestinal tract and preexisting gastrointestinal microbiome. When administered together as a live microbial feed supplement (MFS), they may reduce the incidence of NE and improve performance parameters. An *in vivo* study was designed to investigate the effects of this MFS on mortality and performance of broiler chickens housed in floor pens on a large-scale.

MATERIALS AND METHODS

Experimental design: In Trial 1, a total of 540 Cobb 500 male broiler chickens were divided into three treatment groups (180 birds/treatment) and placed in concrete floor pens on the day of hatch. In Trial 1,each treatment group consisted of six pens, each containing 30 birds. In Trial 2, a total of 936 Cobb 500 male broiler chickens were divided into three treatment groups (312 birds/treatment) and placed in concrete floor pens on the day of hatch. Each Trial 2 treatment group consisted of 12 pens, each containing 26 birds. All other aspects of the experimental design were consistent between Trial 1 and Trial 2.

Chicks were placed in floor pens that had dimensions of 4'×4' and contained fresh pine shavings top-dressed with used pine shavings. All broilers were fed the same standard, corn/soy-based diet. Additionally, the MFS treatment group (MFS) was supplemented with an MFS containing 1×10^6 CFU of both *H. xylanolytica* ASCUSBR21 and *C. beijerinckii* ASCUSBR67 (Native Microbials, San Diego, CA). The treatment was mixed as a lyophilized powder with a base mash of

corn and soy feed to create treatment feed at the test concentrations. The remaining groups received calcium carbonate without the MFS.

All birds were vaccinated for Marek's disease at the hatchery. Additionally, all birds were vaccinated for Infectious Bronchitis and Newcastle disease upon receipt at the facility, as well as for coccidiosis via spray cabinet before placement into study pens. Feed was provided *ad libitum* via a single hanging tube feeder in each pen. Birds were fed a three-phase commercial-type corn/soy mash diet consisting of a starter (day 0-16), grower (day 17-35) and finisher (day 36-42). All feed was sampled and tested at each change in feed to ensure that the feed met or exceeded nutritional requirements as designated by the National Research Council[29]. Water was provided *ad libitum* via a bell drinker. Pens were housed within an environmentally controlled building. The building temperature was regulated based on the breed recommendation for Cobb 500 broiler chickens[30]. A commercial lighting program was used based on the breed recommendation for Cobb 500 broilers[30].

All procedures were approved by the Colorado Quality Research Institutional Animal Care and Use Committee and overseen by onsite veterinarians.

Clostridium perfringens challenge: The MFS treatment group (MFS) and positive control group (POS) received an oral challenge of *Clostridium perfringens* (CL-15, Type A, α and β2 toxins). The strain used, referred to as CL-15, was received from Microbial Research, Inc. and was isolated from a NE outbreak on a commercial broiler farm in Colorado, USA. This strain of *C. perfringens* is known to produce Net B, a toxin with direct links to causing severe avian NE[31].

On day 17, each pen in the MFS and POS groups received an oral *C. perfringens* challenge. Approximately 25 g of feed mixed with 1.5 mL of inoculum was administered to the POS and MFS pens per bird. In Trial 1, the *C. perfringens* inoculum had a concentration of 7.7×10^{7} CFU mL^{-1}. In Trial 2, the *C. perfringens* inoculum had a concentration of 5.6×10^{8} CFU mL^{-1}. All other aspects of the *Clostridium perfringens* challenge were consistent between Trial 1 and Trial 2.

All feeder trays in the POS and MFS pens were removed after the inoculated feed was consumed. The negative control group (NEG) also had their feeder trays removed when the trays for the challenged groups were removed. The concentration of liquid inoculum of *C. perfringens* was determined pre-administration using spectrophotometry at a wavelength of 600 nm using a standard curve. *Clostridium perfringens* concentrations were subsequently confirmed post-administration by plating the inoculum onto tryptose-sulfite-cycloserine agar, incubating anaerobically for 18 h at 37°C. Black colonies were presumed to be *C. perfringens* and counted to confirm the colony forming unit (cfu) concentration of inoculum.

Data collection: Facility conditions and bird health were observed twice daily by trained facility staff for the duration of both studies. Throughout each study, any bird that was removed from the study was necropsied and had sex, weight and probable cause of death recorded. Birds were weighed by pen on days 0,17, 28, 35 and 42. The remaining feed in each pen was weighed and recorded prior to feed change on days 17, 28, 35 and 42. This data was used to calculate feed intakes for the following time periods: days 0-17, 0-28, 17-28, 0-35, 28-35, 0-42 and 35-42.

Necrotic enteritis lesion scoring: On study days 21 and 28, 5 birds were removed from each pen at random, weighed and euthanized using cervical dislocation. The number of birds removed per pen was decreased and replaced by the number of birds that died from NE prior to lesion scoring up to 5 birds per pen. Portions of their intestines were then scored for lesions resulting from NE. Lesions were scored using the following criteria: (0) Normal: no NE lesions, small intestine has normal elasticity (rolls back on itself after being opened); (1) Mild: small intestinal wall is thin and flaccid (remains flat when opened and does not roll back into normal positions after being opened), excess mucus covering mucus membrane, (2) Moderate: noticeable reddening and swelling of the intestinal wall, minor ulceration and necrosis of the intestine membrane, excess mucus, (3) Severe: extensive necrosis and ulceration of the small intestine membrane, significant hemorrhage, a layer of fibrin and necrotic debris on the mucous membrane, (4) Dead or moribund: dead bird (or bird that would likely die within 24 h) with NE gross lesions scored 2 or more. This was consistent between Trial 1 and Trial 2.

Statistical analysis: Data from each trial were analyzed using R software (version 3.5.1)[32]. One-way Analysis of Variance tests (ANOVA) were used to compare the performance data between treatment groups. A significant ANOVA result ($p\leq0.05$) warranted further analysis via Fisher's Least Significant Difference (LSD) test (package agricolae version 1.3.3), in order to determine significance when comparing individual treatment groups. Pairwise comparisons generated via LSD were corrected using the Bonferroni procedure. p-values generated via both ANOVA and LSD were considered

trending if p≤0.10 and significant if p≤0.05. ANOVA generated p-values are presented in tables and the LSD pairwise p-values are indicated by superscripts in tables.

RESULTS AND DISCUSSION

Mortality and lesion scores: In Trial 1, there were no significant differences between any groups regarding mortality over the entire study period (day 0-42, p = 0.24, Table 1). Although not significant, the MFS group showed a reduction that was trending when compared to the POS (p = 0.10, Table 1) and numerically lower than the NEG control group (p = 0.26, Table 1). This result can be expected as the birds were vaccinated with only a 1× dose of coccidiosis vaccine and no other challenge conditions were applied to instigate a response until day 17 when *C. perfringens* was applied to the MFS and POS group. However, in the post-challenge period (day 17-28), MFS supplementation reduced necrotic mortality rate in Trial 1 (p = 0.05, Table 2).

There was also a trending reduction in NE specific mortality throughout the entire study (day 0-42) (p = 0.07, Table 1). There was a significant difference between the POS and NEG control groups (p≤0.05, Table 1). While not significantly different from the POS or NEG control groups, there was a numerical reduction between the MFS group and the POS control group suggesting that the MFS may be exerting a beneficial effect on the *C. perfringens* challenged birds (Table1). The reduction being non-significant may be related to the number of pens, as a sample size of six pens is small compared to similar studies[22,33,34]. Additionally, the necrotic mortality data across the whole trial may be influenced by the lack of differences seen in either total mortality or NE associated mortality between groups after day 28. This pattern was expected, however, as most cases of NE in broilers are seen between 10 and 28 days of age[35,36]. Finally, broilers supplemented with the MFS had significantly lower lesion scores compared to the POS group on Day 21 (p<0.05, Table 5). On Day 28, the MFS group displayed numerically reduced intestinal lesions when compared to the POS control (Table 5).

In Trial 2, there was a significant difference in mortality between groups, largely driven by the low mortality in the non-challenged NEG group (p< 0.01, Table 3). This difference in mortality was driven by NE, as the NE specific mortality rate was much higher in the MFS and POS group than the NEG group, accounting for approximately 80% of total mortality in the groups that received the *C. perfringens* challenge (p<0.01, Table 3). MFS supplementation numerically reduced the necrotic mortality rate in the MFS group when compared to the POS group during the post-challenge period (day 17-28),

Table 1: Trial 1: data for all groups for whole trial (days 0-42)

	Negative control[1]	Positive control	MFS	Treatment p-value
Bird gain (kg)	2.22±0.05[a]	2.19±0.05[a]	2.23±0.09[a]	0.79
Intake per bird (kg)	4.62±0.28[a]	4.57±0.27[a]	4.46±0.14[a]	0.70
Adjusted feed conversion ratio	1.62±0.01[a]	1.63±0.03[a]	1.61±0.01[a]	0.38
Mortality rate (%)	13.89±7.50[a]	20.00±7.5[a]	11.11±2.78[a]	0.24
Necrotic mortality[2] rate (%)	1.11±1.11[b]	10.00±6.67[a]	5.00±3.33[a]	0.07

[ab]Means within a row not sharing a common superscript differ (post-hoc analysis p≤0.05). [1]The Negative Control group did not receive a *C. perfringens* challenge. [2]Total mortality due to necrotic enteritis

Table 2: Trial 1: Data for all groups post-challenge period (days 17-28)

	Negative control[1]	Positive control	MFS	Treatment p-value
Bird gain (kg)	0.60±0.04[a]	0.59±0.02[a]	0.58±0.03[a]	0.52
Intake per bird (kg)	1.04±0.11[a]	1.01±0.14[a]	0.87±0.12[a]	0.22
Adjusted feed conversion ratio	1.62±0.18[a]	1.59±0.20[a]	1.42±0.14[a]	0.34
Mortality rate (%)	10.36±7.45[a]	15.89±6.61[a]	6.59±4.17[a]	0.24
Necrotic mortality[2] rate (%)	0.60±0.89[b]	9.75±6.67[a]	2.89±2.68[a]	0.05

[ab]Means within a row not sharing a common superscript differ (post-hoc analysis p≤0.05). [1]The negative control group did not receive a *C. perfringens* challenge. [2]Total mortality due to necrotic enteritis

Table 3: Trial 2: data for all groups for whole trial (days 0-42)

	Negative control[1]	Positive control	MFS	Treatment p-value
Bird gain (kg)	2.55±0.08[a]	2.50±0.08[a]	2.52±0.05[a]	0.75
Intake per bird (kg)	6.24±0.25[a]	6.16±0.11[a]	6.00±0.25[a]	0.36
Adjusted feed conversion ratio	1.63±0.02[b]	1.68±0.04[a]	1.64±0.02[b]	0.01
Mortality rate (%)	5.77±3.21[b]	23.40±6.09[a]	21.47±7.38[a]	<0.01
Necrotic mortality[2] rate (%)	0.96±1.28[b]	18.20±5.61[a]	17.63±6.89[a]	<0.01

[ab]Means within a row not sharing a common superscript differ (post-hoc analysis p≤0.05). [1]The Negative Control group did not receive a *C. perfringens* challenge. [2]Total mortality due to necrotic enteritis

Table 4: Trial 2: data for all groups post-challenge period (days 17-28)

	Negative control[1]	Positive control	MFS	Treatment p-value
Bird gain (kg)	0.76 ± 0.03^{a}	0.71 ± 0.03^{ab}	0.70 ± 0.03^{b}	0.08
Intake per bird (kg)	1.90 ± 0.06^{a}	1.78 ± 0.04^{b}	1.74 ± 0.08^{b}	<0.01
Adjusted feed conversion ratio	1.59 ± 0.03^{b}	1.78 ± 0.08^{a}	1.74 ± 0.05^{a}	<0.01
Mortality rate (%)	1.06 ± 1.08^{v}	20.62 ± 6.96^{a}	18.91 ± 7.27^{a}	<0.01
Necrotic mortality[2] rate (%)	0.71 ± 0.90^{b}	18.93 ± 5.99^{a}	17.58 ± 7.39^{a}	<0.01

[ab]Means within a row not sharing a common superscript differ (post-hoc analysis p≤0.05). [1]The Negative Control group did not receive a *C. perfringens* challenge. [2]Total mortality due to necrotic enteritis

Table 5: Average lesion scoring for trial 1 and trial 2

Trial	Day	Negative control[1]	Positive control	MFS	Treatment p-value
Trial 1	Day 21	0.13 ± 0.20^{b}	1.53 ± 0.63^{a}	0.73 ± 0.25^{b}	<0.01
	Day 28	0.30 ± 0.22^{b}	1.60 ± 0.72^{a}	0.97 ± 0.42^{a}	0.02
Trial 2	Day 21	0.07 ± 0.10^{b}	2.02 ± 0.68^{a}	1.92 ± 0.51^{a}	<0.01
	Day 28	0.07 ± 0.10^{b}	1.95 ± 0.75^{a}	1.87 ± 0.98^{a}	<0.01

[ab]Means within a row not sharing a common superscript differ (post-hoc analysis p≤0.05). [1]The negative control group did not receive a *C. perfringens* challenge

Table 6: Adjusted feed conversion rate across all time periods for trial 1 and trial 2

Trial	Period	Negative control[1]	Positive control	MFS	Treatment p-value
Trial 1	Day 0-17	1.42 ± 0.06^{a}	1.46 ± 0.07^{a}	1.41 ± 0.09^{a}	0.71
	Day 17-28	1.62 ± 0.18^{a}	1.59 ± 0.2^{a}	1.42 ± 0.14^{a}	0.34
	Day 28-35	1.68 ± 0.22^{a}	1.50 ± 0.24^{a}	1.35 ± 0.15^{a}	0.19
	Day 35-42	1.83 ± 0.04^{a}	1.85 ± 0.07^{a}	1.77 ± 0.03^{a}	0.12
	Day 0-42	1.62 ± 0.01^{a}	1.63 ± 0.03^{a}	1.61 ± 0.01^{a}	0.38
Trial 2	Day 0-17	1.41 ± 0.04^{a}	1.41 ± 0.07^{a}	1.34 ± 0.02^{a}	0.09
	Day 17-28	1.59 ± 0.03^{a}	1.78 ± 0.08^{b}	1.74 ± 0.05^{b}	<0.01
	Day 28-35	1.71 ± 0.08^{a}	1.70 ± 0.04^{a}	1.68 ± 0.03^{a}	0.84
	Day 35-42	1.96 ± 0.07^{a}	1.93 ± 0.05^{a}	1.87 ± 0.05^{a}	0.22
	Day 0-42	1.63 ± 0.02^{b}	1.68 ± 0.04^{a}	1.64 ± 0.02^{b}	0.01

[ab]Means within a row not sharing a common superscript differ (post-hoc analysis p≤0.05). [1]The negative control group did not receive a *C. perfringens* challenge

despite the NE outbreak being much more intense than that in Trial 1 (Table 4). Additionally, lesion scores were higher in Trial 2 for challenged birds than in Trial 1 due to the intensity of the NE outbreak. There were no significant differences in lesion scoring between the POS and MFS groups in Trial 2 but the MFS lesion scores were numerically lower than the POS group on both sampling days (Table 5).

In both the POS and MFS groups of Trial 2, mortality due to NE was approximately 18%, suggesting that the outbreak of NE was much more intense in Trial 2 than in Trial 1 which had necrotic mortality rates of approximately 10 and 3% for POS and MFS, respectively (Table 1 and 3). While many factors may influence the severity of a NE outbreak, the likely source of this severity difference between trials is the *C. perfringens* inoculum used for the challenge[37,38]. The inoculum used in Trial 1 was at a concentration of 7.7×10^{7} CFU mL^{-1}, while the concentration of the inoculum used for Trial 2 was approximately seven times higher at 5.6×10^{8} CFU mL^{-1}. As *C. perfringens* infection is a major factor in the development of NE, it is very likely that the increased concentration of the inoculum was a driving factor in the intensity of the outbreak in Trial 2[2,26].

The NE mortality reductions seen in this study may be due to a combination of several possible modes of action of the supplemented strains. Supplementation with live bacterial strains has been previously shown to affect both hosts and their microbiomes[39-41]. The possible avenues the supplemented microorganisms took to ameliorate NE may include enhancing host immunity[42-45], improving intestinal barrier function[27,46,47] and promoting a better intestinal balance of beneficial microorganisms[39,48,49]. In birds with a balanced microbiome, the microbiome contributes to protecting the bird from potentially pathogenic organisms[50,51]. Bacterial supplementation to both healthy and challenged intestinal microbiomes has been associated with competitive exclusion, reducing the likelihood of a pathogenic bacteria attaching to the epithelial cells[52,53]. These supplemented strains are also acting antagonistically with pathogens, in addition to competing for resources and space, by producing inhibitory metabolites, such as organic acids, to reduce the pathogen load in the gastrointestinal tract and prevent dysbiosis by promoting a more balanced gut microbiome[34,37,54]. The microorganisms used in this trial (*H. xylanolytica* ASUCSBR21 and *C. beijerinckii* ASCUSBR67)

were isolated from the gastrointestinal tracts of healthy broiler chickens that were more resilient to NE, so it is plausible that these organisms are contributing to these positive effects.

Based on these findings, it appears that the MFS had a rapid and impactful effect on NE immediately following the *C. perfringens* challenge. The reduced presence of lesions, in addition to the lower NE mortality rate, strengthens the support for the effectiveness of the bacterial strains in the MFS in reducing the severity of necrotic enteritis infection.

Performance: No significant effects of the MFS on body weight gain, feed intake per bird, or adjusted FCR were observed during Trial 1 (Table 1 and 2). However, adjusted FCR was numerically lower in every time period for the MFS group (Table 6). In Trial 2, the MFS supplemented group displayed an FCR decrease of 4 points when compared to the POS group across the whole trial period ($p = 0.01$, Table 3). In both trials, improvements in the performance of the MFS group began to increase toward the end of the trial. Additional studies utilizing a variety of challenge models, such as diet and nutrition-based challenges, may yield further insights into the efficacy and consistency of the results seen in the trials presented in this study. A future study with increased replicates and an extended trial period may elucidate these findings.

Due to the difference in mortality during the challenge period by the second half of Trial 1, there was a marked difference in pen density between groups. Due to NE mortality in the POS group being used for lesion scoring and the sacrifices in the NEG group for lesion scoring, the densities of POS and NEG pens were similar during the trial. The initial stocking density was 0.45 ft^2/bird for all groups. Prior to the challenge on day 17, all groups had an average density of 0.50 ft^2 $bird^{-1}$. By day 28 the average density of the control groups was 0.72 ft^2 $bird^{-1}$ while the MFS group was 0.65 ft^2 $bird^{-1}$. By the end of the trial (d 42), the control groups were at an average density of 0.98 and 0.97 ft^2 $bird^{-1}$, for POS and NEG respectively, while the MFS group was 0.87 ft^2 $bird^{-1}$. Previous studies have shown that the density of broilers can influence the performance of the birds[55-57]. Although, there was no difference in performance at the end of Trial 1 between the MFS and control groups, the ability of the treatment to maintain performance with a higher bird density may provide evidence indicating that the product may be improving performance. Future studies would be needed with more pens to investigate this hypothesis.

CONCLUSION

The results of this study suggest that *H. xylanolytica* ASCUSBR21 and *C. beijerinckii* ASCUSBR67, when fed in combination, were able to reduce mortality attributable to NE infection as well as improve some performance parameters. This demonstrates the feasibility and potential to isolate new microbial treatments from the gut microbiome of broiler chickens and the ability of these MFS to potentially improve or maintain bird performance even during disease outbreaks. These initial trials provide foundational evidence to continue research into the application of these native microorganisms to improve broiler health and performance when challenged with NE. Further studies are needed to examine the functional potential of these organisms and their benefit in broilers across varying conditions with a larger sample size, with particular attention paid towards deepening the understanding of their ability to mitigate the NE impact on broilers.

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