

Effects of oral *Akkermansia muciniphila* supplementation in healthy dogs following antimicrobial administration

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The mucin layer of the GIT provides a physical and biochemical barrier between luminal contents and GIT epithelium. Mucus-secreting GIT epithelial cells produce the major glycoprotein elements, with additional components (eg, immunoglobulins and antimicrobial peptides) derived from the innate and adaptive subparts of the immune system.^{1,2} The microbiota of the mucin layer is distinct from the predominant GIT phyla and consists of mucin-degrading microorganisms.¹ Mucin-degrading commensals and

ABBREVIATIONS

BCS	Body condition score
CK	Cytokeratin
CV	Coefficient of variation
GIT	Gastrointestinal tract
GLP	Glucagon-like peptide
IBD	Inflammatory bowel disease
LPS	Lipopolysaccharide

OBJECTIVE

To measure effects of oral *Akkermansia muciniphila* administration on systemic markers of gastrointestinal permeability and epithelial damage following antimicrobial administration in dogs.

ANIMALS

8 healthy adult dogs.

PROCEDURES

Dogs were randomly assigned to receive either *A muciniphila* (10^9 cells/kg; $n = 4$) or vehicle (PBS solution; 4) for 6 days following metronidazole administration (12.5 mg/kg, PO, q 12 h for 7 d). After a 20-day washout period, the same dogs received the alternate treatment. After another washout period, experiments were repeated with amoxicillin-clavulanate (13.5 mg/kg, PO, q 12 h) instead of metronidazole. Fecal consistency was scored, a quantitative real-time PCR assay for *A muciniphila* in feces was performed, and plasma concentrations of cytokeratin-18, lipopolysaccharide, and glucagon-like peptides were measured by ELISA before (T0) and after (T1) antimicrobial administration and after administration of *A muciniphila* or vehicle (T2).

RESULTS

A muciniphila was detected in feces in 7 of 8 dogs after *A muciniphila* treatment at T2 (3/4 experiments) but not at T0 or T1. After metronidazole administration, mean change in plasma cytokeratin-18 concentration from T1 to T2 was significantly lower with vehicle than with *A muciniphila* treatment (-0.27 vs 2.4 ng/mL). Mean cytokeratin-18 concentration was lower at T1 than at T0 with amoxicillin-clavulanate. No other significant biomarker concentration changes were detected. Probiotic administration was not associated with changes in fecal scores. No adverse effects were attributed to *A muciniphila* treatment.

CONCLUSIONS AND CLINICAL RELEVANCE

Detection of *A muciniphila* in feces suggested successful gastrointestinal transit following oral supplementation in dogs. Plasma cytokeratin-18 alterations suggested an effect on gastrointestinal epithelium. Further study is needed to investigate effects in dogs with naturally occurring gastrointestinal disease. (*Am J Vet Res* 2018;79:884–892)

pathogenic bacteria express specialized mucolytic enzymes (glycosidases), allowing the use of mucin glycoproteins as a primary energy source and generating by-products that are consumed as substrates by other microorganisms.^{1,3} Bacterial structural components and degradation products provide additional stimulation for mucus production, resulting in a continuous breakdown-production cycle to help maintain the mucin barrier.⁴

Lipopolysaccharide and CK-18 serve as systemic markers of GIT permeability and epithelial damage, respectively. The LPS comprises a portion of the outer membrane of gram-negative bacteria, and systemic absorption occurs secondary to decreased GIT barrier function. Endotoxemia stimulates an abnormal inflammatory response to typically innocuous antigens,⁵⁻⁷ and serum concentrations of LPS correlate with disease severity in people with IBD.⁶⁻⁸ Cytokera-

tin-18 is a cytoplasmic filament of epithelial cells, and increased serum concentrations have been detected in human patients with dose-limiting chemotherapy-induced GIT epithelial cell toxicosis.⁹⁻¹¹

Akkermansia muciniphila is an anaerobic gram-negative bacterium, and it is the predominant mucin-degrading microorganism in the GIT of people.³ Decreased numbers of *A muciniphila* have been detected in people with ulcerative colitis and Crohn disease.^{3,4,12} Treatment with *A muciniphila* in rodents used to study metabolic syndrome improved systemic markers of GIT barrier function, including decreased serum concentrations of LPS.¹³ Indirect increases in *A muciniphila* counts following administration of prebiotic agents led to decreased serum LPS concentrations and increased numbers of L cells in rodents.¹⁴ Increased L-cell number is likely key to *A muciniphila*-mediated systemic influences, as these specialized enteroendocrine cells secrete GLP-2, which augments the GIT barrier through increases in intestinal weight, crypt depth, and villus height.¹⁵

The specific type or types of mucin-degrading bacteria in the GIT of dogs are unknown. In addition, it is unknown whether *A muciniphila* supplementation has an effect in dogs. Therefore, the purpose of the study reported here was to measure the effects of oral *A muciniphila* administration on markers of GIT permeability and epithelial damage in healthy dogs following administration of 2 commonly used antimicrobials. An additional aim was to determine whether *A muciniphila* could be detected in fecal samples of healthy dogs prior to its administration. We hypothesized that antimicrobial administration would result in increased circulating concentrations of LPS and CK-18, indicative of decreased GIT mucosal barrier function, and that *A muciniphila* administration would stimulate GLP-1 and GLP-2 secretion and result in decreased plasma LPS and CK-18 concentrations.

Materials and Methods

Animals

Eight healthy adult dogs (6 spayed mixed-breed females and 2 sexually intact male Beagles) from a research colony at The Ohio State University were studied. Dogs were considered healthy on the basis of history and the results of physical examination and routine laboratory tests, including a CBC, serum biochemical analysis, urinalysis, and fecal examination. Dogs were weighed and BCS (scale of 1 to 9)¹⁶ was assessed prior to study initiation and every 2 weeks thereafter, corresponding with changes in study treatments. At study initiation, median age of the dogs was 25 months (range, 15 to 32 months), median weight was 24.7 kg (range, 11.2 to 31.9 kg), and median BCS was 6 (range, 5 to 8).

Dogs were housed in individual, adjacent runs, with visual interaction with other dogs, at an AAALAC-accredited facility for the duration of the study. Runs were cleaned individually, and although dogs did not have direct access to other runs, fecal

contamination between runs and coprophagy were possible. All dogs were housed in 1 room for the first half of the study and then separated into 2 rooms (arbitrary assignment; 4 dogs each) for the second half of the study, with 2 weeks remaining in the washout period. Housing changes were made for facility considerations that were unrelated to the study.

Dogs were fed once daily in the morning. Dogs received the same diet^a for 3 months prior to study initiation, and no orally administered supplements, medications, or treats were given from 1 week prior to study initiation through study completion. No changes in caloric allocation were made during the study, regardless of BCS when the study was started. The study was approved by The Ohio State University Institutional Animal Care and Use Committee (protocol No. 2014A00000129).

Study design

A double-crossover, placebo-controlled, prospective study design was used to assess the effects of oral *A muciniphila* administration in dogs following administration of the 2 selected antimicrobials (**Figure 1**). Baseline fecal samples were collected daily for 7 days and stored for future analysis (not evaluated in the current study) before all dogs were administered metronidazole^b (12.5 mg/kg, PO, q 12 h) for 7 days. Food was withheld for ≥ 12 hours after the final antimicrobial administration (T1); then dogs received omeprazole^c (1 mg/kg, PO, q 24 h) followed by either *A muciniphila* (10^9 cells/kg, PO; $n = 4$) or the study treatment vehicle (an equal volume of PBS solution, PO; 4) for a total of 6 days. Random assignment for these study treatments was performed by simple randomization of female dogs to reach 3/group; once the group size was reached, remaining dogs were assigned to the alternate group. Simple randomization of male dogs was used to reach 1/group. The *A muciniphila* (or vehicle) was administered 1 hour after omeprazole to increase the likelihood of successful transit of live bacteria through the stomach. After a 20-day washout period, the same procedures were repeated, with dogs receiving the alternate study treatment (ie, *A muciniphila* vs vehicle). After another 20-day washout period, the same experimental procedures were repeated, with amoxicillin-clavulanate^d (13.5 mg/kg, PO, q 12 h) given instead of metronidazole.

Blood and fecal samples were collected at the end of the 7-day baseline or each 20-day washout period, prior to antimicrobial administration (T0), after each 7-day antimicrobial administration period (T1), and after each 6-day study treatment (*A muciniphila* or vehicle) period (T2). Only fecal samples collected on the day of venipuncture were analyzed; remaining samples were stored for future analysis.

Probiotic culture, testing, and preparation

The *A muciniphila* was obtained from an independent source.^e The probiotic bacteria were cultured under anaerobic conditions in hog gastric mu-

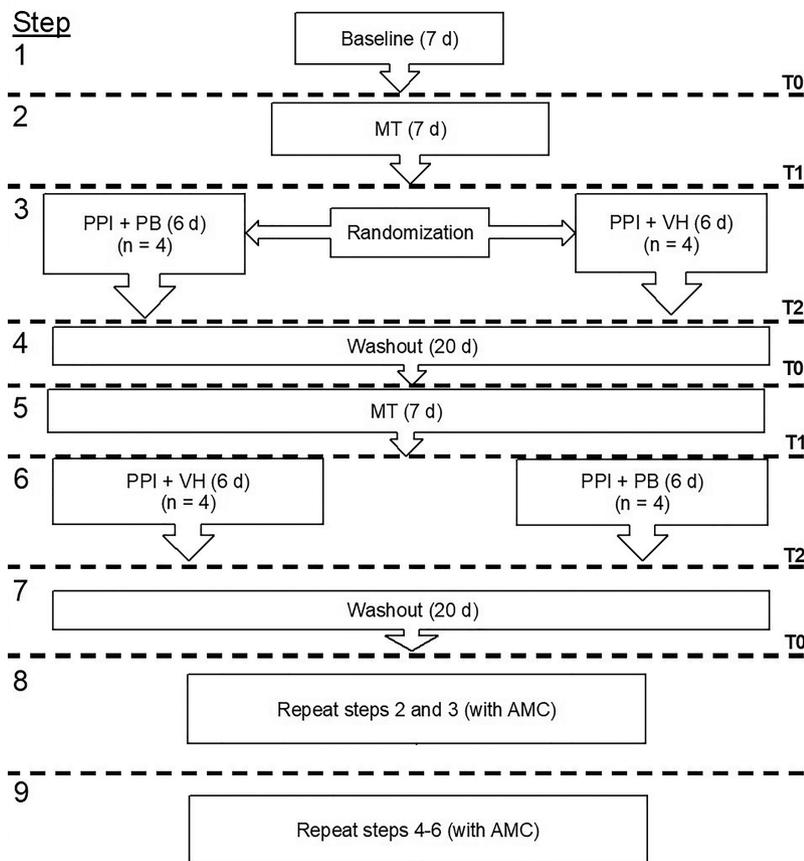


Figure 1—Timeline of treatments and sample collection points (T0, T1, and T2; each at the end of the step shown) in a blinded, placebo-controlled, double cross-over–design study to measure effects of oral *Akkermansia muciniphila* (probiotic) administration on systemic biomarkers of gastrointestinal permeability and epithelial damage as well as fecal consistency following administration of metronidazole (12.5 mg/kg, PO, q 12 h for 6 days) or amoxicillin-clavulanate (13.5 mg/kg, PO, q 12 h for 6 days) in 8 healthy adult dogs. Food was withheld for ≥ 12 hours after the last antimicrobial administration (at each T1), and a proton pump inhibitor (omeprazole) was administered 1 hour prior to administration of *A muciniphila* or the treatment vehicle (PBS solution) to increase the likelihood of successful transit of live bacteria through the stomach. AMC = Amoxicillin-clavulanate. MT = Metronidazole. PB = Probiotic. PPI = Proton pump inhibitor. VH = Vehicle.

cin medium as previously described.¹⁷ Environmental modifications (75% nitrogen, 20% carbon dioxide, and 5% hydrogen atmosphere) were made to increase the microorganism growth rate. Total DNA was extracted^f from a 50-mg sample of the cultured organism, amplified via PCR assay, and purified.⁸ Purified DNA product was shipped unfrozen in DNase- and RNase-free water to be sequenced,^h and the sequence was then submitted for strain confirmation.ⁱ The product was determined to have a 99% sequence match to *A muciniphila* strain GP28 16S rRNA. Bacteria were diluted to a density of 3.5×10^{10} cells/mL to 6×10^{10} cells/mL on the basis of counting by hemocytometer (1×10^{10} CFUs/mL to 5×10^{10} CFUs/mL, 1:4 in PBS solution) and frozen at -80°C in individual cryogenic vials until used for experiments.

To determine oxygen tolerance of *A muciniphila*, the anaerobically cultured bacteria were placed in cryogenic vials on a shaker and exposed to ambi-

ent air at room temperature (approx 23°C). Samples were collected after 0, 2, 5, 10, 20, 60, and 120 minutes. Aliquots from samples at the same time points were incubated at room temperature at a concentration of 1×10^6 cells/mL with a fluorescent nucleic acids stain^j ($5 \mu\text{M}$) and propidium iodide ($45 \mu\text{M}$) to label live and dead cells, respectively. Flow cytometry^k was used to measure cell fluorescence with 488-nm excitation. Optical filters for fluorescence intensity channels 1 and 3 were set at 530 to 533 nm and 610 to 620 nm, respectively. The fraction of live cells was determined as follows:

$$\text{Count}_F / (\text{count}_F + \text{count}_{PI})$$

where F and PI represent cells labeled with the fluorescent nucleic acids stain and propidium iodide, respectively. The standard curve of live to dead cells was used to gate live and dead cell populations.

One batch of the cultured *A muciniphila* per antimicrobial was used for administration to all dogs. Individual vials of *A muciniphila* and vehicle were thawed at room temperature for 30 minutes prior to administration. A new aliquot from the same batch was used daily to avoid freeze-thaw changes.

Blood collection

Blood was collected through jugular vascular access ports that were previously placed for another study ($n = 6$) or by lateral saphenous venipuncture (2). Blood was collected after food was withheld for 24 hours, just prior to the dog's morning feeding. Dogs were then fed half of their daily meal, and 3-hour postprandial blood samples were collected before the other half of the meal was given. Blood was collected into EDTA-containing glass tubes and centrifuged for 20 minutes at $1,000 \times g$ and $4^\circ\text{C} \leq 60$ minutes after the samples were obtained. Samples with gross hemolysis or lipemia underwent a second centrifugation cycle. Plasma was collected and stored at -80°C until testing. Samples were analyzed in 1 batch at the end of the study.

Fecal sample collection and evaluation

Samples were collected daily from the first morning feces. Three samples per dog were collected into individual vials and immediately frozen at -80°C . Fecal scores were performed daily by 1 observer (MCJ) through use of a previously described scale.¹⁸ Scores were applied on the basis of gross consistency as fol-

lows: 1 = hard and dry; 2 = well formed, leaving no mark when picked up; 3 = beginning to lose form, leaving a mark when picked up; 4 = poor consistency with most form lost; and 5 = watery consistency with no form.¹⁸

Sample analysis

Probiotic—The cultured *A muciniphila* had 76% viability after exposure to ambient air for 2 hours at room temperature. Quantitation of the probiotic bacteria was performed by a quantitative real-time PCR assay as previously described on DNA coding for the rRNA sequences.¹⁹ Briefly, primers were chosen for 16S rRNA sequences¹ (S-St-Muc-1129-a-a-20 [sequence, 5'-CAG-CAC-GTG-AAG-GTG-GGG-AC-3'] and S-St-Muc-1437-a-A-20 [sequence, 5'-CCT-TGC-GGT-TGG-CTT-CAG-AT-3']). Calculation of copy number (molar copies/ μ L) was performed by use of nanodrop spectrometry on the basis of the purified PCR product's optical density at 260 nm. The PCR^m assay with melting curve analysis measuring fluorescence of double-stranded DNA-binding dyeⁿ was performed to generate a standard curve ($y = -3.3x + 39.7$; $r^2 = 0.99$).

Fecal samples—Fecal bacterial DNA extraction was performed for samples collected according to the protocol during the study period. Total DNA was extracted from 220 mg of thawed fecal material by use of a commercial kit^o per the manufacturer's directions. The previously described quantitative real-time PCR assay for *A muciniphila* was performed on the collected samples with dilution concentrations prepared according to the described standard curve. To verify that initial negative PCR assay results at 1 postsupplementation time point were not spurious, fecal DNA extraction and PCR assay analysis were repeated on samples from that day.

Biomarkers—Plasma CK-18 concentrations were measured by use of a canine-specific ELISA^p; the intra-assay and interassay CVs were 18.6% and 12.6%, respectively. Lipopolysaccharide concentrations were measured with a canine-specific ELISA^q for which the intra-assay CV was 2.6% and interassay CV was 14.5%. The CK-18 assay used was previously validated with canine CK-18 standards by the manufacturer.^p The LPS assay used was validated with bovine serum albumin standards by the manufacturer.^q Pre- and postprandial total GLP-1 concentrations were measured with an ELISA, which was previously validated for use with human, rat, and mouse samples.^f The DNA sequence of GLP-1 has 100% homology in mammals.^{20,21} The assay for human GLP-1 has previously been validated and used for samples from dogs.^{22,23} The intra-assay CV for this assay was 12.2%, and the interassay CV was 27%. Pre- and postprandial total GLP-2 concentrations were measured with an ELISA, which was previously validated in human, rat, and mouse samples.⁵ Canine GLP-2 has 88% DNA sequence homology to human GLP-2, with 100% homology to the N-terminal sequence, which is responsible for metabolic effects.¹⁵ There were too few samples with concentrations above background to calculate

inter- and intra-assay CVs of GLP-2 in the present study. Concurrent validation of GLP-2 could not be performed because of this lack of significant biomarker detection. All samples were run in duplicate. All assays per antimicrobial for an individual dog were run on a single plate.

Statistical analysis

Data were analyzed by use of statistical software.^{24,t-v} Data were assessed for normality with the Shapiro-Wilk test. Normally distributed data were presented as mean \pm SD; nonnormally distributed data were presented as median and range. Significance was set at $P \leq 0.05$. To evaluate for carryover effects, a mixed model incorporating treatment sequence (ie, *A muciniphila*-vehicle or vehicle-*A muciniphila*), treatment (ie, *A muciniphila* vs vehicle), and time was designed. As there were no effects of treatment sequence or evidence of residual effects ($P > 0.05$ for all comparisons), fecal score changes between time points (ie, T0 vs T1 and T1 vs T2) were analyzed. For each antimicrobial administration period, absolute biomarker (CK-18, LPS, GLP-1, and GLP-2) concentrations for T0 and T1 were compared with a paired Student *t* test (for normally distributed data) or Wilcoxon signed rank test (for nonnormally distributed data). There was no difference between the T0 values for the first (end of baseline) and second (postwashout) metronidazole administration periods (Figure 1; steps 1 and 4). There was also no difference between T1 values following the first and second metronidazole administration periods (steps 2 and 5). Therefore, all T0 values and all T1 values for metronidazole administration were combined for statistical analysis. The same analysis was performed for amoxicillin-clavulanate administration, and no differences between T0 or T1 values were found for the first and second amoxicillin-clavulanate periods, respectively; all T0 and T1 values for amoxicillin-clavulanate were combined for statistical analysis. Changes in fecal scores were calculated from T0 to T1 and T1 to T2 and compared between *A muciniphila* and the vehicle with paired Student *t* tests (for normally distributed data) or Wilcoxon signed rank tests (for nonnormally distributed data). Absolute biomarker concentrations were compared between T0 and T1 and T1 and T2 by means of paired *t* tests or Wilcoxon signed rank tests. Pre- and postprandial plasma concentrations of GLP-1 were compared at the same time points with a paired Student *t* test or Wilcoxon signed rank test as appropriate. Descriptive statistics were calculated for *A muciniphila* fecal PCR assay results and plasma GLP-2 concentrations. For results that did not meet significance criteria but had values of $P \leq 0.1$, post hoc power was calculated.

Results

Fecal PCR assay for *A muciniphila*

No *A muciniphila* was detected in any fecal sample at T0 (baseline or the end of washout) or T1 (end

of antimicrobial administration), regardless of the treatments. *Akkermansia muciniphila* was detected by fecal DNA PCR assay in 7 of 8 dogs after supplementation. Fecal PCR assays detected the probiotic at 3 of the 4 T2 sampling time points in the study, with positive results in 4 of 4 (concentration range, 4,545 to 45,454 molar copies/g), 4 of 4 (455 to 45,454 molar copies/g), and 3 of 4 dogs (4,545 to 45,454 molar copies/g) following administration of the probiotic in steps 3, 8, and 9, respectively (Figure 1). No quantifiable amount of *A muciniphila* was identified in samples from any dog at 1 T2 (that for step 6), including the 4 dogs that were given *A muciniphila* in the preceding time period. This sampling time followed a period of metronidazole administration. Presence of *A muciniphila* (concentration, 4,545 molar copies/g) was detected in feces from 2 dogs following treatment with the vehicle during step 3, prior to any direct *A muciniphila* supplementation in these dogs.

Fecal score

The T0 fecal scores were higher (worse) prior to amoxicillin-clavulanate (median, 2.65 [scale of 1 to 5]; range, 2 to 4) than metronidazole (median, 2.60; range, 2 to 4) administration ($P = 0.04$). Mean fecal score at T1 was higher after metronidazole (2.96 ± 0.31) than after amoxicillin-clavulanate (2.67 ± 0.20 ; $P < 0.05$) administration. The mean change in fecal score between T0 and T1 was an increase (0.3 ± 0.14) with metronidazole and a decrease (-0.11 ± 0.13 ; $P < 0.01$) with amoxicillin-clavulanate administration.

Mean fecal score increased significantly ($P < 0.01$) from T0 (2.66 ± 0.12) to T1 (2.96 ± 0.31) with metronidazole administration. After use of this antimicrobial, the mean fecal score at T2 did not differ between dogs that received *A muciniphila* (2.81 ± 0.31) versus the vehicle (2.69 ± 0.29 ; $P = 0.32$). There was an apparent but nonsignificant ($P = 0.09$) improvement in mean fecal score change from T1 to T2 when metronidazole administration was followed by *A muciniphila* treatment (-0.4 ± 0.30), compared with that observed when metronidazole was followed by vehicle administration (-0.17 ± 0.23). Post hoc power analysis revealed that power to detect a significant difference was 0.69.

Fecal scores decreased from T0 (median, 2.65; range, 2.55 to 3.4) to T1 (median, 2.58; range, 2.45 to 3; $P = 0.03$) with amoxicillin-clavulanate administration. Following administration of this drug, the mean fecal score at T2 did not differ between dogs that were given *A muciniphila* (2.69 ± 0.21) and those that received the vehicle (2.65 ± 0.20 ; $P = 0.46$), and the mean change in fecal score from T1 to T2 did not differ

between the vehicle (-0.03 ± 0.15) and *A muciniphila* (0.03 ± 0.21 ; $P = 0.51$) treatment.

Plasma biomarker concentrations

Absolute biomarker concentrations and concentration differences were compared among time points after metronidazole administration (Figure 2). Administration of metronidazole did not significantly affect concentrations of any biomarkers at T1 relative to T0. Mean CK-18 concentration decreased from T1 to T2 when the vehicle was administered after metronidazole administration (change of -0.27 ng/mL) and increased from T1 to T2 when *A muciniphila* was administered (change of 2.4 ng/mL); this difference was significant ($P = 0.03$). Mean postprandial GLP-1 concentrations (38.17 ± 10.01 pmol/L) were higher than preprandial concentrations (15.54 ± 9.59 pmol/L; $P < 0.05$) across all time points (T0, T1, and T2). There were no differences in mean absolute concentrations of LPS or pre- or postprandial GLP-1 among metronidazole, vehicle, or *A muciniphila* treatments at T1 or T2, and the change in concentrations of these biomarkers from T0 to T1 or T1 to T2 did not differ among treatments. Presence of GLP-2 was detected once in plasma from 1 dog (1.05 ng/mL) following vehicle administration.

Absolute biomarker concentrations and concentration differences were compared between time points after amoxicillin-clavulanate administration (Figure 3). Mean CK-18 concentration decreased from T0 (20.18 ± 8.39 ng/mL) to T1 (18.03 ± 9.46 ng/mL; $P = 0.05$) with this antimicrobial, but significant effects were not observed for LPS or GLP-1. Postpran-

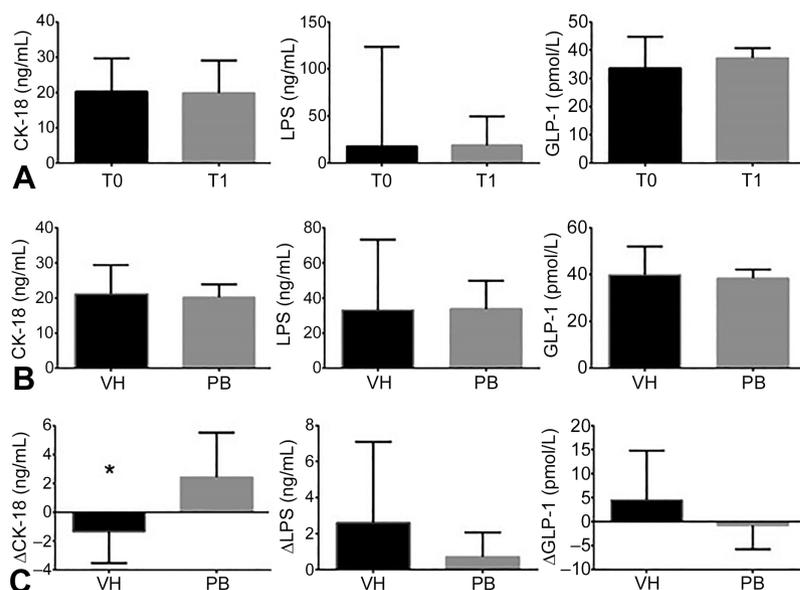


Figure 2—Results of biomarker (CK-18, LPS, and postprandial GLP-1) analysis of plasma samples for the same 8 dogs as in Figure 1 (steps 1 through 3 and 4 through 6; data combined for analysis). A—Median and range biomarker concentrations at baseline or the end of the washout period (T0) and after metronidazole administration for 1 week (T1). B—Mean \pm SD biomarker concentrations at T2 following vehicle or *A muciniphila* administration for 6 days. C—Change in biomarker concentrations (Δ) from T1 to T2. In panel C, CK-18 and GLP-1 data are mean \pm SD; LPS data are median and range. *Difference between treatments is significant ($P < 0.05$). See Figure 1 for remainder of key.

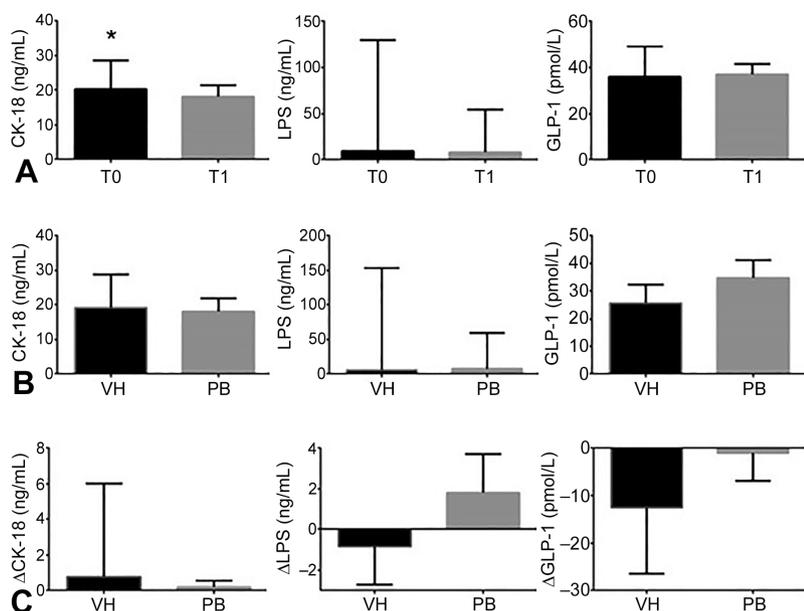


Figure 3—Results of biomarker analysis of plasma samples for the same 8 dogs as in Figure 1 (steps 7 through 9; data combined for analysis). A—Measurements at T0 and after amoxicillin-clavulanate administration for 1 week (T1). B—Measurements at T2 following vehicle or *A muciniphila* administration for 6 days. C—Change in biomarker concentrations from T1 to T2. In panels A and C, LPS data are median and range; all other graphics show mean \pm SD. See Figures 1 and 2 for remainder of key.

dial GLP-1 concentrations (33.59 ± 12.44 pmol/L) were higher than preprandial concentrations (18.48 ± 7.54 pmol/L; $P < 0.05$), with no impact of either *A muciniphila* or vehicle administration on this variable. Minimal GLP-2 concentrations were detected in 3 dogs across 4 time points (range, 1.11 to 2.67 ng/mL). There were no differences in mean absolute concentrations of LPS or pre- or postprandial GLP-1 among amoxicillin-clavulanate, vehicle, or *A muciniphila* treatments at T1 or T2, and the change in concentrations of these biomarkers from T0 to T1 or T1 to T2 did not differ between treatments.

Adverse events

No adverse effects directly attributable to *A muciniphila* administration were noted. There were 3 episodes of vomiting noted (2 during the vehicle administration period and 1 during the metronidazole administration period) in 3 dogs. One dog ate only approximately 25% of its daily meal for 7 days during the washout period following vehicle administration. One dog received an oral NSAID (meloxicam, 0.2 mg/kg, once, and then 0.1 mg/kg, q 24 h) for 3 days as treatment for interdigital cysts during the washout period following vehicle administration.

Discussion

Antimicrobial therapy is associated with substantial morbidity in human medicine, with serious implications resulting from GIT hyperpermeability.²⁵ Mucin-degrading bacteria, specifically *A muciniphila*, and their metabolic products reduce local and

systemic signs of GIT hyperpermeability in people and rodents.^{3,13,14,26} In the present study, we evaluated the effects of oral *A muciniphila* on systemic measures of GIT permeability and epithelial damage in dogs following antimicrobial therapy.

Presence of *A muciniphila* was not detected in the fecal samples of any dog in the study before experiments that included supplementation with *A muciniphila* began. One explanation for the lack of detection at T0 could be the absence of natural *A muciniphila* colonization in the canine GIT. Although this cannot be proven without analysis of samples obtained directly from the GIT, in other species *A muciniphila* is readily detectable in naturally voided fecal samples.¹⁷ The *A muciniphila* DNA in our study was detected, however, in fecal samples of almost all dogs following supplementation in 3 of 4 instances, suggesting effective passage of identifiable organisms through the GIT. Low numbers of DNA copies in these samples, relative to the amounts administered, sug-

gested that colonization of the GIT by *A muciniphila* did not occur. However, given a narrow pH range for growth in culture,^{19,27} it was also possible that DNA degradation occurred in the environment or in the GIT, reducing the number of detectable DNA copies, regardless of colonization. Levels of colonization below detectable PCR assay quantification are an additional explanation. As *A muciniphila* cells had $> 75\%$ viability with exposure to ambient air in our study, we expect that environmental survival was possible; however, decreased growth may have occurred with prolonged exposure. Furthermore, microbiome analysis by direct collection from the GIT, rather than fecal assessment, may yield substantially different results and could help to clarify whether GIT colonization of *A muciniphila* occurs.²⁸ The reason for lack of detection at 1 postsupplementation time point was unclear. Assessment of housekeeping genes could have helped distinguish between low-cellularity samples, inhibition of the PCR in the assay, and lack of *A muciniphila*. In contrast, *A muciniphila* was detected prior to direct *A muciniphila* administration in 2 dogs. This could have resulted from environmental exposure to fecal material from other dogs. Although the dogs were housed in individual pens, indirect exposure was possible. This transmission would require survival of *A muciniphila* in aerobic conditions. Previously thought to be an obligate anaerobe, a recent investigation found low-level *A muciniphila* growth in room air for up to 24 hours at 37° to 40°C,²⁹ and we found that viability was good after 2 hours in room air at 23°C.

Median T0 fecal scores for dogs in the present study were higher (worse) before amoxicillin-clavulanate than before metronidazole administration, and the finding of a higher mean fecal score after metronidazole (vs amoxicillin-clavulanate) administration was unexpected. Metronidazole is a common treatment for chronic enteropathies in dogs,³⁰⁻³³ and our finding was in contrast to results of a previous study³³ that showed improvement with metronidazole. In that study, however, metronidazole was given in combination with prednisone, and controlled studies on metronidazole use for chronic enteropathies are lacking, with investigators of 1 study³² reporting that improvement was questionable in dogs with chronic IBD that received metronidazole treatment.

A slight improvement was observed in fecal scores of *A muciniphila*-treated dogs after metronidazole administration; however, the finding did not differ significantly from the result when the vehicle was given after metronidazole. As no additional signs of illness were noted, the effects of metronidazole that resulted in worsened fecal consistency may have been mild, and thus the degree of improvement with probiotic administration was too small to result in a statistical difference. Likewise, amoxicillin-clavulanate administration may not have caused antimicrobial-induced GIT effects in this population, as no change in fecal score was noted with *A muciniphila* treatment after the drug was given.

To our knowledge, circulating concentrations of CK-18 have not been evaluated in the context of GIT disease in dogs. A noteworthy difference in this study was that plasma CK-18 concentrations increased when *A muciniphila* was administered to metronidazole-treated dogs, compared with results after vehicle administration, but this was not observed in amoxicillin-clavulanate-treated dogs. Because CK-18 is an intracellular component, increased circulating concentrations are considered markers of cellular damage.^{10,11} However, research in rodents has also demonstrated increased CK-18 expression during hepatic regeneration³⁴ and GIT epithelial cell proliferation at the tissue level.³⁵ The net effect of increased CK-18 in dogs that received this treatment in our study could have represented cell damage, but alternatively, it could have resulted from reparative GIT cell turnover following epithelial insult. Also, an increase in CK-18 could have reflected a direct effect of *A muciniphila* on GIT epithelium, which could imply negative effects of *A muciniphila* supplementation. Finally, because an increase in this biomarker was not detected following amoxicillin-clavulanate administration, the results may represent cumulative damage resulting from metronidazole and *A muciniphila*.

The role of GLP-1 and GLP-2 in GIT disease of dogs has not been explored to our knowledge. Glucagon-like peptide-1 and GLP-2 are cosecreted from L cells. Their secretion is largely controlled by intraluminal nutrients but is also affected by other luminal signals, including metabolic products of GIT bacte-

ria.^{15,36} Circulating concentrations of GLP-1 and GLP-2 increase in mice following treatment with prebiotics, *A muciniphila*, and mucin-degradation products of *A muciniphila*.^{13,14,26} Therefore, we expected an increase in plasma concentrations of GLP-1 and GLP-2 in response to *A muciniphila* treatment in the dogs of this study. The lack of *A muciniphila* administration effects on GLP-1 or GLP-2 measurements could have resulted from a lack of *A muciniphila* viability or inability of the live bacteria to colonize the GIT. It was also possible that GLP-1 or GLP-2 secretion were already maximally stimulated in this population of dogs, and antimicrobial administration did not induce changes in the GIT severe enough to allow effects of treatment (antimicrobials, *A muciniphila*, or vehicle) to be observed.

The presence of GLP-2 was not successfully detected in our plasma samples. The GLP-2 ELISA used in the study was designed to measure human and rat GLP-2. Although there is a high degree of homology between human and canine GLP-2,¹⁵ it was possible that canine GLP-2 was not detected by this ELISA or that natural inhibitors existed in our population. Furthermore, sample timing was extrapolated from peak concentrations in pigs,³⁷ and peak postprandial release may occur at different times in dogs.

There were several limitations to the present study, including the small number of dogs, which may have prevented detection of small differences in circulating biomarker concentrations. On the basis of a 2-tailed, matched-pairs *t* test with a group of 8 dogs, an effect size of 1.16 (ie, 1.16 SD difference between means) would be needed to achieve statistical significance with an α of 5% and power ($1 - \beta$) of 80%. For biomarker values that were not significantly different between dogs after receiving *A muciniphila* versus the vehicle in our study, the range of SDs was 27% to 50% of the respective means, yielding effect sizes of 0.1 to 0.58. Although these effect sizes were too low to result in *P* values ≤ 0.05 , they still might have been biologically important. Therefore, these results can be useful for planning future studies in terms of determining minimal sample size.

The strain of *A muciniphila* used in our study has been naturally detected in people and mice.^{12,13} Although *Akkermansia*-like organisms have been detected in the order Carnivora,¹² the exact species in dogs has not been documented. Some isolates from the genus *Akkermansia* have low structural and functional similarities to human isolates,¹² which could impact the effects of supplementation. In addition, we attempted to preserve viability by avoiding *A muciniphila* freeze-thaw cycles, but we did not reculture samples from the frozen aliquots. Effects of nonviable *A muciniphila* formulations have been documented³⁸; however, previous findings suggest that *A muciniphila* must be viable to exert positive effects.¹⁵

We also could not exclude the possibility of persistent antimicrobial effects on the microbiome dur-

ing the baseline or washout periods or effects of other treatments and systemic conditions altering the microbiome or biomarker measurements. Although a previous study³⁹ investigating the effects of metronidazole on the fecal microbiota in dogs identified a return to baseline microorganism populations ≤ 30 days after treatment ended, a subsequent study^w demonstrated longer effects. There is also recent evidence that *Akkermansia* spp are susceptible to the β -lactam antimicrobial, imipenem, and resistant to metronidazole.⁴⁰ This raises the possibility that the effects of *A muciniphila* in dogs of our study might have been impacted by persistent antimicrobial effects, and supports that the timing of *A muciniphila* administration relative to antimicrobial treatments should be considered carefully if *A muciniphila* is considered as treatment for GIT disease. Omeprazole has been shown to alter counts of specific GIT microbiota in people,⁴¹ and such an effect could impact the results of *A muciniphila* treatment when the products are administered concurrently. However, the aforementioned study⁴¹ did not find an impact on overall microbiome diversity and was performed over a longer duration than was used in our investigation; furthermore, omeprazole was given to dogs with both treatments (vehicle and *A muciniphila*) after antimicrobial administration in our study. Several dogs in the present study were overweight. In people and rodents, obesity can impact enteroendocrine hormone concentrations as well as LPS absorption from the GIT,⁴²⁻⁴⁴ but a similar impact of obesity in dogs has not been described and might have a different magnitude or even a different direction. Importantly, the cross-over study design should have helped to minimize, if not abolish, the effects of individual variation in body condition as well as other dog-specific factors. No fecal microbiome analysis was performed in this study; therefore, we could not exclude the possibility that there were effects on the local GIT environment that were not detected by the systemic (hematologic) assessments.

Short-term oral administration of *A muciniphila* in the present study had no adverse clinical effects in healthy dogs. Administration of metronidazole in this study was associated with worsened fecal consistency, compared with that following amoxicillin-clavulanate administration, and the greater CK-18 concentrations when dogs received the probiotic (vs the vehicle) after metronidazole administration suggested that the treatment had effects on the GIT epithelium that warrant further investigation. Future studies should include reculture of *A muciniphila* after freezing and assessments to determine whether the organism successfully colonizes the GIT in dogs. Further study is needed to determine the optimal species or strain and dosing of *A muciniphila* to achieve positive probiotic effects in healthy dogs and to assess whether the treatment is beneficial in canine patients with clinical GIT disease.

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The authors declare that there were no conflicts of interest.

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Footnotes

- a. Laboratory Canine Diet 5006, LabDiet, St Louis, Mo.
- b. Metronidazole, 250 mg, manufactured for Unichem Pharmaceuticals, Heshbrouck Heights, NJ, by Unichem Laboratories, Ghaziabad, India.
- c. Omeprazole, 20 mg, manufactured for Sandoz Inc, Princeton, NJ, by Lek Pharmaceuticals dd, Ljubljana, Slovenia.
- d. Clavamox, 250 mg, manufactured for Zoetis Inc, Kalamazoo, Mich, by GlaxoSmithKline, Mumbai, India.
- e. ATCC BAA-835, American Type Culture Collection, Manassas, Va.
- f. Quick-DNA Fungal/Bacterial Miniprep Kit, Zymo Research, Freiburg, Germany.
- g. QiaQuick PCR Purification Kit, QIAGEN Inc, Germantown, Md.
- h. Retrogen Inc, San Diego, Calif.
- i. Nucleotide BLAST, National Center for Biotechnology Information, US National Library of Medicine, Bethesda, Md. Available at: blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch. Accessed Jun 26, 2015.
- j. Syto 9, Molecular Probes, Eugene, Ore.
- k. Accuri C6 Flow Cytometer, BD Biosciences, San Jose, Calif.
- l. GenBank, National Center for Biotechnology Information, US National Library of Medicine, Bethesda, Md. Available at: www.ncbi.nlm.nih.gov/genbank/. Accessed Jun 1, 2015.
- m. Applied Biosystems StepOne Real-Time PCR System, Thermo Fisher Scientific, Waltham, Mass.
- n. SYBR Green, Applied Biosystems, Foster City, Calif.
- o. QIAamp DNA stool kit, QIAGEN Inc, Germantown, Md.
- p. Canine CK-18 ELISA, MyBioSource Inc, San Diego, Calif.
- q. Canine LPS ELISA, MyBioSource Inc, San Diego, Calif.
- r. GLP-1 total ELISA, EMD Millipore, St Charles, Mo.
- s. GLP-2 total ELISA, EMD Millipore, St Charles, Mo.
- t. IBM SPSS Statistics, version 24.0, SPSS Inc, Armonk, NY.
- u. GraphPad Prism, GraphPad Software Inc, San Diego, Calif.
- v. G*Power, version 3.1.9.2, Universitat Kiel, Germany.
- w. Suchodolski J, Olson E, Honneffer J, et al. Effects of a hydrolyzed protein diet and metronidazole on the fecal microbiome and metabolome in healthy dogs (abstr). *J Vet Intern Med* 2016;30:1455.

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