



## 18 **Abstract**

19           Dysbiosis is one of the major changes in aging that leads to an accumulation of toxic microbial  
20 metabolites. The aim of this study was to evaluate the effect of a test food containing components of  
21 citrus, carrot, spinach and tomato on gut microbiota and age-related metabolites in senior dogs. The  
22 study was conducted on 36 dogs between 8 and 13 years of age. All dogs were maintained on a control  
23 food (control 1), which used corn as major source of fiber. After 30 days, the dogs were divided into  
24 two groups of 18 dogs. One of the groups received the test food for 30 days while the other group  
25 received the control 2 food, containing multiple whole grains as the test food but without the above  
26 added sources of fiber present in the test food. After a washout period on the control 1 food for 30  
27 days, a crossover was performed so that the test or the control 2 food was fed for 30 days to those dogs  
28 which had not yet been fed that food. Samples from feces and blood were collected after each 30 days  
29 period to analyze changes in gut microbial composition and metabolites. The consumption of the test  
30 food led to increased proportions of *Adlercreutzia*, *Oscillospira*, *Phascolarcobacteria*,  
31 *Faecalibacterium* and *Ruminococcus*, *Christensenellaceae*, *Ruminococcaceae*, *Cyanobacteria* and  
32 *Acidobacteria* and decreased proportions of *Megamonas*, *Salmonella*, *Enterobacteriaceae* and  
33 *Fusobacterium*. Pets had higher levels of glycerol and fatty acids and lower levels of pyrrolidine and  
34 mucin amino acids in feces. The test food also reduced circulating levels of pyrrolidine, symmetric  
35 dimethylarginine and phenolic uremic toxins, including the microbial brain toxin, 4-ethylphenyl  
36 sulfate. *Christensenellaceae* abundance was strongly associated with the observed health benefits.  
37 Fermentable fibers from fruits and vegetables enhance health in senior dogs by modulating the gut  
38 bacteria and metabolites involved in aging, kidney, brain and gut health.

39

## 40 **Introduction**

41           Aging is associated with shifts in the composition of gut microbiota. An example of this is the  
42 increase in the number of facultative anaerobes and a decline in the proportion of beneficial bacteria  
43 associated with aging (1, 2). This shift in the microbial composition leads to the accumulation of toxic  
44 microbial metabolites in the body causing inflammation, oxidative stress and contributing to various  
45 diseases prominent in the aging condition (3). The reduction in the proportion of beneficial bacteria  
46 may lead to constipation, mal-absorption and longer colonic transit time. Decreased absorption of  
47 dietary protein in the upper intestine and longer colonic transit times encourage increased abundance  
48 of proteolytic bacteria, whose fermentation products deteriorate intestinal barrier integrity (4).

49  
50           Foods containing fermentable fibers are known to benefit dogs by increasing nutrient  
51 absorption and reducing enteric infection (5). In an *in vitro* study, Swanson et al. (6) confirmed the  
52 fermentability of fruits and vegetables by canine fecal microflora with the resulting production of short  
53 chain fatty acids. This study evaluates the effect of a test food containing components of citrus, carrot,  
54 spinach and tomato on the microbial composition as well as metabolites associated with aging, kidney,  
55 brain and gut health in senior dogs. A recent study by Hall et al (7) showed that the consumption of a  
56 food with similar composition as the test food employed in the current study led to improvement of  
57 markers of kidney health in geriatric dogs with early stage kidney disease. This study was designed to  
58 evaluate changes in fecal microbial composition and age-related markers of health attributed to the  
59 consumption of the test food by healthy senior dogs.

60

## 61 **Materials and methods**

### 62 **Dogs**

63 All study protocols were reviewed and approved by the Institutional Animal Care and Use  
64 Committee, Hill's Pet Nutrition, Inc., Topeka, KS, USA. Criteria for inclusion were healthy dogs  
65 above the age of 7 years. Dogs having chronic disease conditions such as inflammatory bowel disease,  
66 dermatitis, food allergy, cancer, tumor, kidney disease, liver disease and chronic urinary tract  
67 infections were excluded from the study. A total of 36 dogs between the ages of 8 and 13 years were  
68 grouped into a two groups of 18 each. Each group contained equal number of female and male dogs.  
69 All dogs were spayed or neutered. A summary of the description of the dogs included in this study is  
70 shown in Table 1.

71 **Table 1: Description of dogs used in the study**

Species	Dogs
Age	Group 1: $10.6 \pm 1.3$ ; Group 2: $10.2 \pm 1.1$
Sex	Control: 9M, 9F, Test: 9M, 9F
Breed	Beagles
Initial body weight	Control: $11.2 \pm 2.1$ Kg, Test: $11.5 \pm 1.8$ Kg
Reproductive status	All dogs were spayed or neutered
Health status	Healthy

## 72 **Foods**

73           The study used a test food and two control foods; all in dry form. All foods were produced by  
74 Hill's Pet Nutrition, Inc. Topeka, KS and were essentially isocaloric with respect to metabolizable  
75 energy (control 1 = 3674 kcal/kg; control 2 = 3666 kcal/kg; test = 3684 kcal/kg). The foods were  
76 formulated to meet similar nutrient profiles (Table 2) and contained grain sources such as rice, millet,  
77 oat groats, corn, wheat and/or barley. The test food contained added fiber sources from citrus, carrot,  
78 tomato and spinach in addition to the multiple grains. Unlike the test food, the first control (control 1)  
79 and the second control (control 2) foods did not have the unique fiber sources from fruit and  
80 vegetables. The first control food (control 1) used corn as major source of grain fiber and did not have  
81 multiple grain sources as the test or the control 2 food. The composition of the foods expressed as  
82 percentage of food as fed is shown in Table 2. Food analytical measurements were determined by  
83 Eurofins Scientific Inc. (Des Moines, IA) using Association of Analytical Communities (AOAC)  
84 methods.

85

86

87 **Table 2. Comparison of the three different foods as fed (g/100g)**

<b>Nutrient</b>	<b>Control 1</b>	<b>Control 2</b>	<b>Test Food</b>
Moisture	7.6	8.91	9
Ash	4.52	4.8	4.41
Crude Fiber	1.2	2	2.5
Crude Protein	19.6	17.77	19.89
Carbohydrates*	54.11	53.64	48.92
Soluble Fiber	0.5	1.8	2.7
Insoluble Fiber	6.6	7.2	5.8
Crude Fat	12.67	12.98	15.18
C18:2 Omega 6 (Linoleic)	3.43	3.29	3.32
C18:3 omega 3 (alpha-Linolenic)	0.36	0.41	0.75
C20:4 Omega 6	0.05	0.05	0.09
C20:5 EPA Omega 3	0.01	0.01	0.08
C22:6 DHA Omega 3	0.01	0.01	0.06
Omega 3 Sum	0.4	0.43	0.92
Omega 6 Sum	3.54	3.41	3.49
C16:1 Palmitoleic	0.28	0.26	0.26
C18:0 Stearic	0.93	0.91	0.91
Lysine	0.92	0.84	1.27
Threonine	0.7	0.62	0.76
Tryptophan	0.24	0.19	0.29

88 \*Carbohydrate (Nitrogen-free extract) =100% - (%Protein + %Fat + %Fiber + %Ash + %Moisture)

## 89 Study design and sample collections

90 All dogs were maintained on control 1 food for 30 days and were divided into two groups. At  
91 the beginning of the test food feeding period, one of the groups received the test food while the other  
92 group received control 2 food for 30 days. Both groups were then fed the control 1 food for the next  
93 30 days after which a cross-over was performed so that the test or the control 2 food were fed for 30  
94 days to dogs which did not eat them during the first assignment to test foods. Water was available *ad*  
95 *libitum*. All dogs were meal fed from electronic feeders, where fresh food was offered daily with  
96 amounts calculated to maintain body weight. Exposure to food was allowed for up to 30 minutes to  
97 complete diet consumption. Daily food intake (g/d) was recorded for each dog. Body weights were  
98 measured weekly. Blood and fecal samples were collected at the end of each 30 days period to  
99 compare the effect of food on the abundance of various bacterial genera and various metabolites  
100 (Table 3).

101 **Table 3. Sample analyses and measurement**

Sample/measurement	Analysis	Phase	Days
Blood	Blood chemistry, SDMA, inflammatory cytokines, metabolomics	Pre-feed	25
		Treatment	25, 55
Feces	Microbiome sequencing, metabolomics	Pre-feed	23, 24
		Treatment	23, 24, 53, 54

## 103 **Sequencing of the 16S rRNA gene**

104 Fecal samples were collected within 30 minutes of defecation and stored at -80°C until  
105 processed. Approximately 25mg of frozen stool homogenate was used for DNA isolation with MoBio  
106 PowerFecal® Kit (MoBio, Carlsbad, CA). Instructions provided by the manufacturer were followed  
107 except that a sonication step was added before vortexing the bead tubes with feces samples for 15  
108 minutes. The DNA extracts were stored at -20°C until further processed. One microliter of each DNA  
109 sample was used to amplify the V3V4 region of the 16S rRNA gene using primers 347F and 803R  
110 containing Illumina adapters (8). Amplification was performed on BioRad C1000 Touch Thermal  
111 Cyclor under the following conditions: 25 cycles of denaturation at 95°C for 30 seconds, annealing  
112 at 55°C for 30 seconds extension at 72°C for 45 seconds, and a final elongation step at 72°C for  
113 5 minutes. An internal normalized mock community DNA and PCR-grade water were used as positive  
114 and negative controls, respectively. The mock community was formed by mixing genomic DNA of 28  
115 bacterial species representing 25 genera obtained from the American Type Culture Collection (ATCC,  
116 Rockville, MD). The mock community represented equal copy numbers of the 16S rRNA gene of each  
117 species as described by Diaz et al. (9).

118 PCR amplicons (25µl) were purified by using Agencourt AmPure XP beads (Beckman  
119 Coulter) and concentrations were measured by using Qubit fluorometer 3.0 (Life Technologies). The  
120 quality of the amplicon was assessed by using Agilent 2100 Bioanalyzer. Index PCR, library  
121 quantification, normalization and pooling were performed following the Illumina's 16S metagenomic  
122 sequencing library preparation protocol (Part # 15044223 Rev. A, Illumina, CA). Libraries were  
123 mixed with Illumina generated PhiX control library and denatured using fresh NaOH. Final  
124 sequencing libraries were then loaded onto the Illumina Miseq v3 reagent cartridge and 251-base  
125 paired-end reads were generated using Miseq Control Software (MCS) 2.4., RTA 1.18.54 and Miseq



126 Reporter 2.4. For every Miseq run, a mock community sample and water were run as a positive and a  
127 negative control, respectively.

128 The reads were de-multiplexed using Miseq built-in workflow to obtain FASTQ files  
129 processed using Mothur, version 1.32 (10). Sequences were retained based on criteria such as having  
130 reads between 431 and 458 base pairs, maximum ambiguous bases of 0 and maximum homopolymer  
131 length of 6. The remaining sequences were chimera detected using the UCHIME algorithm  
132 implemented in MOTHUR and excluded from further processing (11). All retained sequences were  
133 aligned to the GreenGenes 16S rRNA gene reference database of (gg.13.5.99). The database was used  
134 for taxonomical assignment of operational taxonomic units (OTUs) at an 80% confidence threshold by  
135 using the naïve Bayesian algorithm (12) implemented in MOTHUR.

136

## 137 **Blood and fecal metabolites**

138 Metabolomic profiles of blood and fecal samples were determined by Metabolon (Durham,  
139 NC). The methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and  
140 a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated  
141 electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass  
142 resolution. Different aliquots of sample extracts were analyzed under different chromatographic  
143 conditions optimized for hydrophilic or hydrophobic compounds (13). Standards present in each  
144 aliquot were used to ensure injection and chromatography consistency. Peaks were identified and  
145 processed using proprietary hardware and software. The relative quantification of the metabolites was  
146 performed by using area-under-the curve.

147

148 Symmetric dimethylarginine (SDMA) concentrations in blood samples were determined using  
149 liquid chromatography-mass spectroscopy (LC-MS) as described by Hall et al. (14).

150

## 151 **Statistical analysis**

152 Matched-pair analyses were performed with JMP version 12 (SAS Institute, Carry, NC) to  
153 compare differences between means of the microbial abundances and relative levels of metabolites on  
154 samples collected from the same dog after the consumption of the test or the control 2 food. P values  
155 were calculated for differences between means and false discovery rate (FDR) corrections were made  
156 on each group of markers. FDR-P values less than 0.05 were considered significant. A bivariate  
157 regression analysis was performed to evaluate correlations between the changes in the microbial  
158 abundance and fecal metabolites.

159

## 160 **Results**

### 161 **Food intake and body weight**

162 All dogs completed the study successfully and there was no adverse health report. There was a  
163 trend (P=0.06) in the body weights of the dogs consuming the test food (10.96 Kg) to increase when  
164 compared to the control 2 food (10.74 Kg). There was very little difference in intakes of the test  
165 (113.41 Kcal/Body weight<sup>0.75</sup>, SE=3.89) or the control 2 food (113.9 Kcal/Body weight<sup>0.75</sup>,  
166 SE=4.15).

167

## 168 **Changes in the gut microbial composition**

169           The test food led to significant changes in the proportions of bacteria at various taxa levels. Fig  
170 1 summarizes the log (base 2) fold changes of the taxa after the consumption of the test food compared  
171 to the control 2 food. At the phylum level, *Acidobacteria* and *Cyanobacteria* increased by 0.91 and  
172 0.72 log fold changes, respectively. This was accompanied by a -0.65 log fold reduction in the phylum  
173 *Fusobacteria*. This was equivalent to 87% and 65% increase in *Acidobacteria* and *Cyanobacteria*,  
174 respectively, and a 66% reduction in *Fusobacteria*. At the family level, *Christensenellaceae* and  
175 *Ruminococcaceae* increased by 1.76 and 0.68 log fold changes, respectively. These were equivalent to  
176 a 138% and 60% increase in the proportions of *Christensenellaceae* and *Ruminococcaceae*,  
177 respectively, compared to their levels on the Control 2 food. On the contrary, the test food led to 1.65  
178 log fold reduction (68%) in *Enterobacteriaceae*. At the genus level, *Adlercreutzia* and  
179 *Phascolarctobacterium* increased by 77% and 68%, respectively.

180

181 **Fig 1. Log fold-changes (base 2) of the different taxa after the consumption of the test food.** The  
182 test food led to a significant reduction in the proportion of bacteria belonging to the genus  
183 *Megamonas*, an unclassified genus in family *Enterobacteriaceae*, *Salmonella* and *Peptostreptococcus*.  
184 The consumption of the test food significantly increased the proportions of the genera *Adlercreutzia*,  
185 *Oscillospira*, *Phascolarctobacterium*, *Faecalibacterium* and *Ruminococcus*. At the family level, the  
186 test food led to a significant increase in *Christensenellaceae* and *Ruminococcaceae* and a significant  
187 reduction in *Enterobacteriaceae*. At the phylum level, the test food increased the phyla *Acidobacteria*  
188 and *Cyanobacteria* and led to a significant reduction in *Fusobacteria*.

189           Although they did not meet the statistical significance, *Oscillospira* and *Ruminococcus*  
190 increased by 73% and 39%, respectively, after the consumption of the test food. This was  
191 accompanied by reductions in the proportions of the genera *Salmonella*, *Megamonas*,  
192 *Peptostreptococcus* and an unknown genus (OTU\_10001 in the family *Enterobacteriaceae*) by 58%,  
193 81%, 32%, 80%, respectively. S1 Table provides the means and standard errors of the proportions of  
194 the above taxa on the test and the control 2 foods.

195

## 196 **Levels of fatty acids and glycerol**

197           The levels of fecal and circulating unsaturated fatty acids increased after the consumption of  
198 the test food (Table 4). In plasma, levels of docosahexaenoate (DHA; 24:6n3), docosapentaenoate  
199 (DPA; 22:5n6), eicosapentaenoate (EPA; 22:5n3), linolenate (18:3n3) and stearidonate (18:4n3) were  
200 increased. In feces, in addition to these fatty acids, docosapentaenoate (DPA; 22:5n3) and palmitoleate  
201 (16:1n7) were increased on the test food. Despite the similar levels of circulating glycerol on both  
202 foods, fecal levels of glycerol increased by 24% when the pets consumed the test food compared to  
203 the control 2 food (P=0.001) (Table 4).

204

205

206

207 **Table 4. Change in levels of fatty acids and glycerol in feces and plasma of the senior dogs**  
 208 **during the consumption of the Test versus the Control 2 food.**

Metabolite in feces	Feces				Plasma			
	% change on Test	P Value (FDR)	Mean difference (test-Control 2)	SE	% change on Test	P Value (FDR)	Mean difference (test-Control 2)	SE
arachidonate (20:4n6)	-16.24	1.49	-0.2	0.12	10.51	2.46	0.11	.08
docosahexaenoate (DHA; 22:6n3)	256.47	3.52E-11	2.17	0.21	177.33	1.02E-10	1.44	0.14
docosapentaenoate (DPA; 22:5n3)	43.85	0.02	0.45	0.13	35.53	0.11	0.36	0.12
docosapentaenoate (n6 DPA; 22:5n6)	120.98	1.84E-07	1.1	0.15	57.13	0.0003	0.57	0.11
eicosapentaenoate (EPA; 20:5n3)	434.21	1.22E-13	3.97	0.3	191.50	4.83E-08	1.61	0.21
glycerol	31.70	0.01	0.36	0.1	3.33	1.0*	0.04	0.09
laurate (12:0)	-4.52	1.0*	-0.05	0.06	2.45	1.0*	0.03	0.08
linoleate (18:2n6)	-4.84	1.0*	-0.05	0.07	7.86	1.0*	.081	0.08
linolenate (18:3n3 or 3n6)	45.47	0.004	0.44	0.1	40.36	0.02	0.42	0.12
myristate (14:0)	10.73	1.0*	0.11	0.07	5.10	1.0*	0.06	0.1
oleate/vaccenate (18:1)	8.80	1.0*	0.1	0.08	9.49	1.0*	.096	0.07
palmitate (16:0)	1.01	1.0*	0.01	0.09	7.64	1.0*	0.08	0.06
palmitoleate (16:1n7)	121.28	2.01E-09	1.23	0.1	26.21	0.51	0.28	0.13
stearate (18:0)	-22.59	0.08	-0.26	0.09	3.03	1.0*	0.03	0.06
stearidonate (18:4n3)	776.56	1.33E-14	8.1	0.6	132.47	7.68E-06	1.29	0.21

209 Changes that were statistically significant ( $P < 0.05$ ) are marked grey.

210 1.0\*: FDR P values greater than 1 are referred as 1.

211

## 212 **Fecal levels of mucin amino acids**

213 The relative fecal levels of amino acids that make up the mucin layer, such as aspartate,  
 214 proline, serine and threonine were significantly affected by the type of food consumed by the senior  
 215 dogs. Compared to the control 2 food, the consumption of the test food led to a 28 – 61% reduction in

216 levels of these amino acids in the feces (Table 5).

217 **Table 5. Relative levels of mucin amino acids in feces**

218

Amino acid in feces	% change on Test	P Value (FDR)
Asparagine	-61.03	0.007
Proline	-28.5	0.007
Serine	-36.2	0.004
Threonine	-35.6	0.001

219

220

221

222

**Advanced glycation**

223 **end products (AGE)**

224 High levels of circulating advanced glycation end products (AGE) are associated with aging  
225 and various age-related diseases. The test food led to about 70% reductions in both circulating  
226 (P=1.15E-07 and fecal (P=5.29E-13) levels of one of the AGE, pyrrolidine. The circulating level of  
227 another AGE, N6-carboxymethyllysine (CML), was not affected by the different diets; but the fecal  
228 levels were higher on the test food. The third AGE, N6-carboxyethyllysine (CEL), was detected only  
229 in feces and did not change during the consumption of the different diets (Table 6).

230

231

232 **Table 6. Changes in levels of advanced glycation end products (AGE)**

Matrix	AGE	% change on Test	P Value (FDR)	Mean difference (Test-Control 2)	SE
Feces	Pyrraline	-69.65	5.29E-13	-1.74	0.15
Plasma	Pyrraline	-68.82	1.15E-07	-1.56	.23
Feces	N6-carboxymethyllysine	48.86	0.0006	0.44	0.11
Plasma	N6-carboxymethyllysine	4.25	0.89	0.05	.07
Feces	N6-carboxyethyllysine	9.15	1.0	0.08	.09

233 Changes that were statistically significant are marked grey.

## 234 **Changes in circulating uremic toxins**

235 Uremic toxins are among the major toxic metabolites that lead to renal and associated diseases  
 236 in aging. Some uremic toxins originate from protein fermentation in the colon by proteolytic bacteria.  
 237 Products of the putrefaction process are absorbed and converted to toxic derivatives causing an  
 238 increased burden on kidney function. We detected a total of 14 phenolic and indolic uremic toxins in  
 239 plasma (Fig 2). The phenolic uremic toxins, 3-methyl catechol sulfate (P=0.0015, SE=0.19), 4-  
 240 ethylphenyl sulfate (P=2.38E-09, SE=0.05), 3-methoxycatechol sulfate (P=0.02, SE=0.11) and 4-  
 241 vinylphenol sulfate (P=0.05, SE=0.05) declined by 175%, 73%, 67% and 23%, respectively after the  
 242 consumption of the test food. On the contrary, the indolic uremic toxins 5-hydroxyindole sulfate  
 243 (P=2.23E-06, SE=0.05) and 7-hydroxyindole sulfate (P=2.97E-10, SE=0.06) increased by 29% and  
 244 43%, respectively, after the consumption of the test food. None of the other uremic toxins were  
 245 significantly influenced by the different foods.

246

247 **Fig 2. Changes in circulating levels of 9 phenolic (black bars) and 5 indolic (grey bars) uremic**  
248 **toxins after the consumption of the test food.** The test food led to significant (\*: False discovery rate  
249 corrected (FDR) P-value <0.05) reductions in levels of phenolic uremic toxins such as 3-methyl  
250 catechol sulfate, 4-ethylphenyl sulfate, 3-methoxycatechol sulfate and 4-vinylphenol sulfate. Two of  
251 the indolic uremic toxins, namely 5-hydroxyindole sulfate and 7-hydroxyindole sulfate, increased after  
252 the consumption of the test food. There were no significant changes in the typical uremic toxins such  
253 as 3-indoxyl sulfate or P-cresol sulfate.

254 Symmetric dimethylarginine (SDMA) is a uremic toxin originating from the host metabolism  
255 and methylation of arginine (15, 16). The test food resulted in a significant reduction in blood  
256 concentrations of SDMA (P=0.035, SE=0.2) in the senior dogs compared to the control 2 food (Fig 3).  
257

258 **Fig 3. Changes in circulating levels of the renal health marker symmetric dimethylarginine**  
259 **(SDMA).** Matched pair analyses of each dog on the test food versus the control 2 food showed  
260 significant reduction in plasma concentrations of symmetric dimethylarginine (SDMA) (P=0.035,  
261 SE=0.2) after the consumption of the test food.

262

## 263 **Correlations of microbial taxa with changes in metabolites**

264 The genus *Adlercreutzia* and the family *Christensenellaceae* were strong positive predictors of  
265 glycerol levels in feces (**Table 7**). *Faecalibacterium prausnitzii*, family *Ruminococcaceae*, genus  
266 *Phascolarctobacterium* and phylum *Actinobacteria* also correlated positively with fecal levels of  
267 glycerol. On the contrary, phylum *Fusobacterium* and genus *Salmonella* negatively correlated with  
268 glycerol levels in feces (Table 7). The genera *Oscillospira* and *Adlercreutzia* also had a negative



269 correlation with levels of pyrrolidine in feces. The phylum *Fusobacterium* correlated positively with  
270 fecal levels of pyrrolidine and threonine. *Salmonella* also had a positive correlation with pyrrolidine levels  
271 in feces (Table 7).

272

273 **Table 7. Correlations of different taxa with fecal levels of glycerol, pyrrolidine and mucin amino**  
 274 **acids**

<b>Correlations with glycerol in feces</b>	<b>FDR P-value</b>	<b>R</b>
Genus <i>Adlercreutzii</i>	3.95E-09	0.51
Family <i>Christensenellaceae</i>	0.0001	0.42
<i>Faecalibacterium prausnitzii</i>	0.02	0.39
Family <i>Ruminococcaceae</i>	0.02	0.39
Genus <i>Phascolarctobacterium</i>	0.003	0.33
Phylum <i>Actinobacteria</i>	0.0005	0.32
Phylum <i>Fusobacterium</i>	0.002	-0.4
Genus <i>Salmonella</i>	0.005	-0.25
<b>Correlations with pyrrolidine in feces</b>		
Family <i>Christensenellaceae</i>	1.05E-12	-0.52
Genus <i>Oscillospira</i>	0.002	-0.48
Genus <i>Adlercreutzia</i>	1.52E-14	-0.44
Genus <i>Salmonella</i>	0.026	0.48
Phylum <i>Fusobacterium</i>	0.017	0.41
<b>Correlations with mucin amino acids in feces</b>		
Family <i>Christensenellaceae</i> and threonine	0.0004	-0.32
Family <i>Christensenellaceae</i> and serine	0.0007	-0.27
Phylum <i>Fusobacterium</i> and threonine	0.00015	0.33
Phylum <i>Fusobacterium</i> and serine	0.02	0.25

## 275 Discussion

276 The test food increased the relative abundance of health-promoting bacteria belonging to the  
277 genera *Adlercreutzia* and *Phascolarctobacterium*. The abundance of the short-chain fatty acids  
278 producers *Phascolarctobacterium* was reported to have a positive association with positive mood and  
279 their number declines in elderly humans (17). Both *Adlercreutzia* and *Phascolarctobacterium* have the  
280 capacity to metabolize isoflavones to equol, which has been implicated to have antioxidative  
281 properties and prevent various age-related diseases including diabetes and obesity (18-20). Equol is  
282 also associated with a decreased risk of certain types of cancer; therefore increasing the abundance of  
283 equol-producing gut microbiota has been recommended to reduce this risk (21). In this study, although  
284 it did not reach statistical significance ( $P=0.17$ ), the fecal level of equol increased by 59.6% after the  
285 consumption of the test food. Polyphenols bound to fruits and vegetables present in the test diet may  
286 have led to the increased abundance of these bacteria in the senior dogs.

287 The consumption of the test food led to an increase in the proportion of the butyrate producer  
288 *Faecalibacterium prausnitzii*. *F. prausnitzii* has been reported to have anti-inflammatory effects (22)  
289 and the abundance of *Faecalibacterium* species declines during active inflammatory bowel disease  
290 (23). The family *Christensenellaceae* also increased after the consumption of the test food. Our  
291 correlation analysis showed *Christensenellaceae* abundance was a strong positive predictor of fecal  
292 levels of glycerol. In a study that compared the microbiome of 416 twin-pairs, *Christensenellaceae*  
293 were in a greater abundance in lean individuals compared to obese (24). *Christensenellaceae* have also  
294 been reported to have the capability to produce short-chain fatty acids (SCF) (25). SCF are known to  
295 improve the intestinal barrier integrity, which is in line with the result of our correlation analysis  
296 showing a negative association between fecal levels of mucin amino acids and the proportion of  
297 *Christensenellaceae*. Their increased abundance may have reduced the degradation of the mucin layer,

298 which in turn would decrease inflammation attributed to the translocation of bacteria and their  
299 secretions through the gut barrier.

300 In a study that compared the microbial composition of Japanese people ranging from infants to  
301 the elderly (26), the relative proportions of bacteria in the genera *Fusobacterium* and *Megamonas*  
302 increased with age. The positive correlation of *Fusobacterium* with fecal levels of mucin amino acids  
303 supports a previous report that showed the capacity of *Fusobacterium* species to degrade mucin (27).  
304 Odamaki et al. (26) showed a negative correlation between *Enterobacteriaceae* and a cluster of  
305 butyrate producing bacteria including *Faecalibacterium*. The decline in the proportions of  
306 *Enterobacteriaceae* in senior dogs after the consumption of the test food may have benefited the  
307 senior dogs as some of these bacteria are endotoxin producers, which compromise intestinal barrier  
308 integrity leading to inflammation. *Salmonella* belonging to *Enterobacteriaceae* also declined due to  
309 the test food consumption. Some species of *Salmonella* are major public health concerns causing  
310 salmonellosis. Although dogs are subclinical carriers of *Salmonella*, the intimate relationship between  
311 dogs and humans may lead to the risk of human exposure to *Salmonella*. The test food led to a  
312 significant relative reduction in *Salmonella* shedding by increasing the proportion of other bacteria  
313 that may have an anti-pathogenic effect.

314 Although not statistically significant, the relative proportions of the genera *Ruminococcus* and  
315 *Oscillospira* increased by 43% and 73%, respectively, after the consumption of the test food. Both  
316 genera belong to the family *Ruminococcaceae*, which increased significantly after the consumption of  
317 the test food. These bacteria are known to produce short-chain fatty acids (SCF) that are beneficial to  
318 the host mainly due to their anti-inflammatory effects (28, 29). They also serve as an energy source for  
319 enterocytes, regulate intestinal motility and ameliorating leaky gut syndrome (30). Bacteria in the  
320 genus *Ruminococcus* are fiber degraders and major producers of butyrate, which serves as an energy

321 source for intestinal epithelial cells and has anti-inflammatory effects (31). Members of the genus also  
322 produce bacteriocins, which have anti-microbial effects against a wide variety of pathogenic bacteria  
323 (32).

324 *Oscillospira* are known to produce butyrate by relying on fermentation products secreted by  
325 other bacterial species (33). In humans, *Oscillospira* have been associated with leanness or lower  
326 body mass index in both infants and adults (34, 35). A meta-analysis by Kaakoush et al. (36), showed  
327 a negative association of the abundance of *Oscillospira* with pediatric inflammatory bowel disease. In  
328 ruminants, the abundance of *Oscillospira* is increased during the consumption of fresh green leaves  
329 and decreases upon consumption of grain containing diets (37). The presence of increased fruit and  
330 vegetable fiber in the test food may have encouraged the increase in the abundance of *Oscillospira* in  
331 the senior dogs. Conley et al. (2) showed the genus that declines the most in aged mice compared to  
332 young is *Oscillospira*. The decline in *Oscillospira* was accompanied by an increase in the marker of  
333 inflammation, monocyte chemoattractant protein-1 (MCP-1). A similar reduction in the abundance of  
334 *Oscillospira* was also associated with paracellular permeability and a decline in the anti-inflammatory  
335 cytokine, IL-10 as reported by Hamilton et al. (38).

336 Despite dietary levels of threonine being higher in the test food, fecal threonine declined when  
337 the dogs consumed the test food. This suggests that the increased fecal excretion of threonine is  
338 associated with the degradation of the mucin layer as reported by Weir et al, (39). The composition of  
339 the gut microbiota is a key factor in maintaining intestinal barrier integrity. The reduction in the  
340 proportion of beneficial bacteria may lead to constipation, mal-absorption and longer colonic transit  
341 time. This encourages increased presence of proteolytic bacteria, whose products of fermentation  
342 deteriorate the intestinal barrier.

343 The consumption of the test food reduced levels of the advanced glycation end product,

344 pyrraline. AGE are a complex group of compounds derived from the non-enzymatic glycation of  
345 proteins, lipids, and nucleic acids. They can also be acquired from food; thus restriction of foods with  
346 high levels of AGE has been recommended to decrease circulating AGE in the body (40). AGE are  
347 known to accelerate the process of aging and they are linked to a number of age-related diseases such  
348 as diabetes, vascular and renal diseases mainly by inducing inflammation and oxidative stress (41, 42).  
349 Fecal microorganisms have been shown to be capable of degrading various AGE including pyrraline  
350 (43). The negative correlation of pyrraline with *Oscillospira*, *Christensenellaceae* and *Adlercreutzia*  
351 suggests the capability of these bacteria to degrade pyrraline or prevent its formation. The  
352 consumption of diets rich in AGE has been shown to shift the microbiota towards a more detrimental  
353 composition (44). This is in line with our correlation analyses that showed a positive association of  
354 pyrraline with *Salmonella* and *Fusobacterium*. After the consumption of the test food, the level of  
355 pyrraline in blood declined by almost the same amount (70%) as in feces. This implies induced  
356 microbial degradation of pyrraline or its precursors by the above microbes as a more likely mechanism  
357 than the test food influencing absorption of pyrraline from the GI tract. Interestingly, one of the AGE,  
358 N6-carboxymethyllysine (CML), increased in feces after the consumption of the test food. However,  
359 the level of CML in the blood did not change. This may suggest that fecal levels of CML may not be  
360 biologically significant.

361         The level of glycerol in the feces of the senior dogs was higher when they were fed the test  
362 food. Glycerol is known to increase water retention in the colon and thus it is used to treat constipation  
363 (45). Prolonged transit times are risks to develop various diseases due to the exposure to toxic  
364 products accumulating due to putrefaction (46). A shorter transit time leads to a limited accumulation  
365 of such products that may cause various diseases (46). People with functional constipation have been  
366 shown to contain bacteria with more abundant genes to degrade glycerol (47). The strong negative

367 correlation between fecal levels of glycerol versus *Fusobacteria* and *Salmonella* may suggest the  
368 capacity of these bacteria to degrade glycerol. The reduction in the proportions of these bacteria after  
369 the consumption of the test food may have led to the increased levels of glycerol in feces. On the other  
370 hand, other taxa such as *Adlercreutzii*, *Christensenellaceae*, *Faecalibacterium prausnitzii*,  
371 *Ruminococcaceae*, *Phascolarctobacterium* and *Actinobacteria* correlated positively with glycerol.  
372 Weir et al. (39) found a positive association of a *Rumonococcus* species with fecal levels of glycerol  
373 and free fatty acids. To our knowledge, this study is the first to show the associations of the other taxa  
374 with fecal glycerol concentration. This may suggest the test food altered the microbial composition  
375 towards a population with higher lipase activity. Along with the increased level of glycerol in feces,  
376 the level of both fecal and plasma levels of omega fatty acids, DHA, EPA, DPA also increased in  
377 feces after the consumption of the test food due to added fish oil. Omega-3 fatty acids have health  
378 benefits throughout life by improving cardiovascular, immune, cognitive and other functions (48).  
379 Similarly, the increased level of linoleate while consuming the test food is due to the increased dietary  
380 concentration. Despite the similar levels of C:16:1 and C:18:0 fatty acids measured in the foods (Table  
381 2), the levels of these fatty acids in feces increased after the consumption of the test food. This  
382 supports the possible increased microbial lipase activity attributed to the change in the microbial  
383 composition.

384 Uremic toxins are among the major metabolites that cause age-related complications. There  
385 was a marked improvement in markers of kidney health after the consumption of the test food.  
386 Circulating symmetric dimethylarginine (SDMA) has been shown to be a good biomarker of kidney  
387 function in dogs as it detects reduction in glomerular filtration rate (GFR) much earlier than serum  
388 creatinine (16). The reduction in circulating concentration of SDMA in the senior dogs after the  
389 consumption of the test food may thus indicate an increased GFR and improved kidney function.

390 Furthermore, the test food reduced several phenolic uremic toxins originating from microbial  
391 fermentation of protein (49). One of these metabolites was 4-ethylphenyl sulfate (4-EPS), which is  
392 also known to have a negative impact on brain health by causing anxiety-like symptoms (50, 51). The  
393 role of foods containing fruits and vegetables in improving kidney health has been reported (7, 52).  
394 The significant reduction in such metabolites in the senior dogs after the consumption of the test food  
395 may be due to the changes in the microbial composition. The two indolic uremic toxins, 5-  
396 hydroxyindole sulfate and 7-hydroxyindole sulfate, increased after the consumption of the test food.  
397 Indolic uremic toxins originate from colonic fermentation of the amino acid tryptophan (53). The test  
398 food was formulated to contain 53% higher tryptophan compared to the control 2 food. The presence  
399 of more substrate may have led to increased levels of the two indolic metabolites. However, the typical  
400 indolic uremic toxin, 3-indoxyl sulfate, was not affected by the consumption of the different diets.

401 In conclusion, old dogs fed fiber sources from vegetables and fruits containing high soluble  
402 fiber benefit by having a gut microbial composition promoting healthier metabolic profiles.

403

404



## 405 **Acknowledgments**

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407 the paper and had primary responsibility for final content. All authors read and approved the final  
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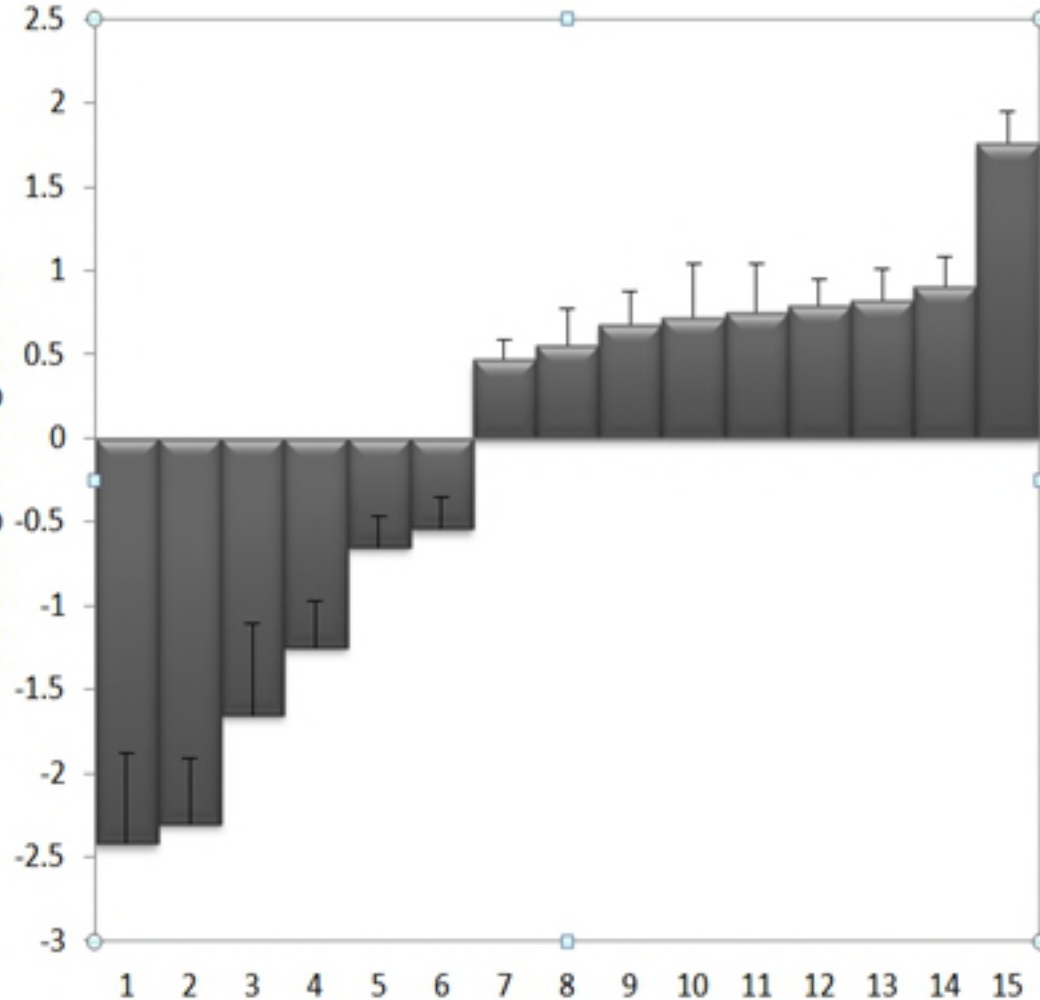
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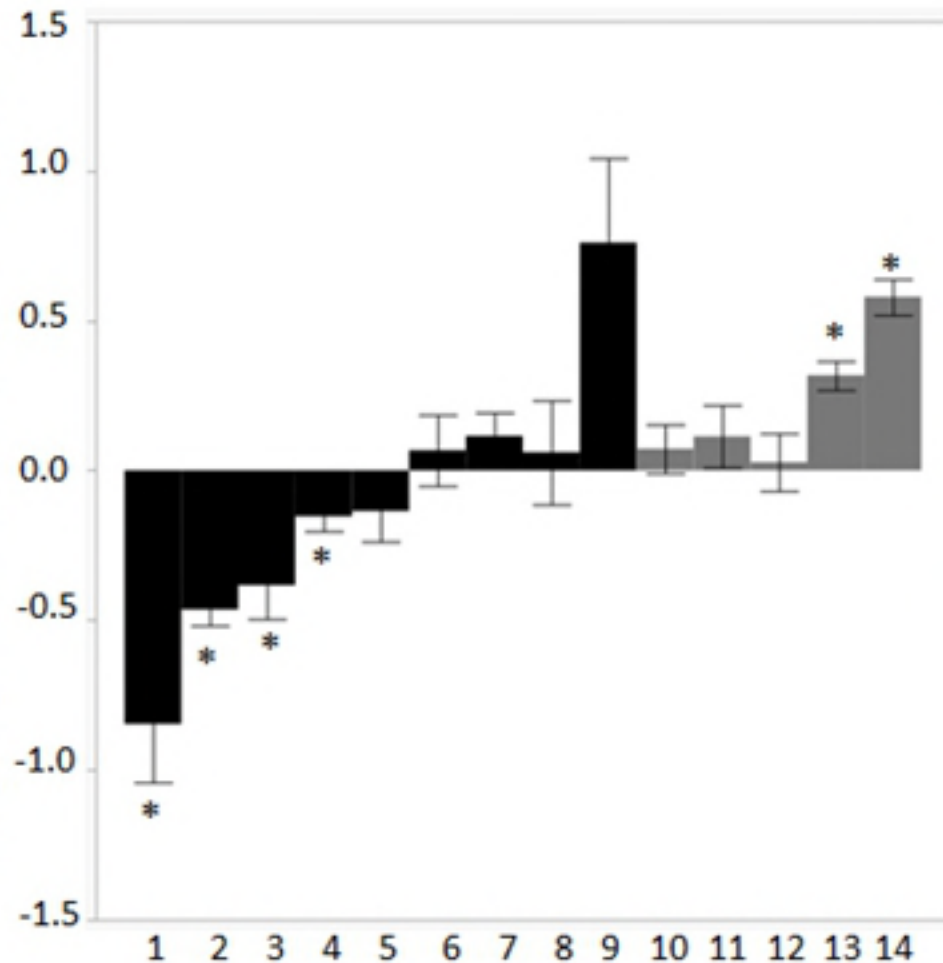
Fold change at log base 2



1. Genus *Megamonas* ( $P=0.004$ ,  $SE=0.54$ )
2. Unclassified genus in family *Enterobacteriaceae* ( $P=0.0002$ ,  $SE=0.39$ )
3. Family *Enterobacteriaceae* ( $P=0.04$ ,  $SE=0.39$ )
4. Genus *Salmonella* ( $P=0.004$ ,  $SE=0.28$ )
5. Phylum *Fusobacteria* ( $P=0.01$ ,  $SE=0.17$ )
6. Genus *Peptostreptococcus* ( $P=0.04$ ,  $SE=0.18$ )
7. Genus *Ruminococcus* ( $P=0.1$ ,  $SE=0.19$ )
8. Genus *Faecalibacterium* ( $P=0.0019$ ,  $SE=0.115$ )
9. Family *Ruminococcaceae* ( $P=0.035$ ,  $SE=0.21$ )
10. Phylum *Cyanobacteria* ( $P=0.01$ ,  $SE=0.19$ )
11. Genus *Phascolarctobacterium* ( $P=0.01$ ,  $SE=0.2$ )
12. Genus *Oscillospira* ( $P=0.09$ ,  $SE=0.32$ )
13. Genus *Adlercreutzia* ( $P=0.06$ ,  $SE=0.29$ )
14. Phylum *Acidobacteria* ( $P=0.002$ ,  $SE=0.19$ )
15. Family *Christensenellaceae* ( $P=5.32E-09$ ,  $SE=0.16$ )



Change in plasma uremic toxins (Test – Control 2)



1. 3-methyl catechol sulfate
2. 4-ethylphenyl sulfate
3. 3-methoxycatechol sulfate
4. 4-vinylphenol sulfate
5. Catechol sulfate
6. O-methylcatechol sulfate
7. P-cresol sulfate
8. Phenol sulfate
9. 4-methylcatechol sulfate
10. 3-Indoxyl sulfate
11. 6-hydroxyindole sulfate
12. 3-hydroxyindolin-2-one sulfate
13. 5-hydroxyindole sulfate
14. 7-hydroxyindole sulfate

