Supplementary Material

Title: Components of the Gut Microbiome that Influence Bone Strength

Authors: Marysol Luna[1], Jason D Guss[1], Laura S. Vasquez-Bolanos[1], Macy Castaneda[1], Manuela Vargas Rojas[1], Jasmin M. Strong[1], Denise A. Alabi[1], Sophie D. Dornevil[1], Jacob C. Nixon[1], Erik A. Taylor[1], Eve Donnelly[4, 5], Xueyan Fu[3], M. Kyla Shea[3], Sarah L. Booth[3], Rodrigo Bicalho[2], Christopher J. Hernandez[1, 5]

Contents

Supplementary Table S1: The seven treatment groups are shown. Antibiotics were used individually or as a cocktail. Zero-calorie sweetener was added to the antibiotic cocktail and as a control. The table shows the antibiotic classification, type of bacteria targeted by the antibiotic, and primary function of the antibiotic (removal of taxa directly targeted by an antibiotic may also change the abundance of other organisms that are dependent on or compete with the removed taxa).

Antibiotic	**Antibiotics Classification**	**Primary Target**	**Primary Function**
Ampicillin	Beta-lactam	Gram +/-	Inhibits peptidoglycan synthesis
Neomycin	Aminoglycoside	Gram -	Inhibits bacterial protein synthesis
Vancomycin	Glycopeptide	Gram +	Inhibits peptidoglycan synthesis
Metronidazole	Nitroimidazole	Anaerobes	Destabilizes bacterial DNA
Ampicillin, Neomycin, Vancomycin, Metronidazole + Sweetener	Beta-lactam, Aminoglycoside, Glycopeptide, Nitroimidazole	Gram +/- & Anaerobes	Combined effects from functions above + zero calorie sweetener to ensure adequate water intake
Untreated Control	n/a	n/a	n/a
Sweetener Only	n/a	n/a	Aspartame-based zero calorie sweetener

Supplementary Table S2. ANCOVA was implemented with a the generalized linear model (GLM) of the form:

$$PeakBendingMoment = a_0 + a_1 Group(Amp) + a_2 Group(Cocktail) + a_3 Group(Untreated) + a_4 Group(Metro) + a_5 Group(Neo) + a_6 Group(Swt) + a_7 \times Section\ Modulus$$

The model $R^2_{adj} = 0.78$ and the analysis of variance F test $p<0.001$. The slope of lines in Figure 2D is determined from this analysis and the intercept is either the intercept shown (does not differ among Untreated, Vanco, Amp, Metro and Cocktail) or the intercept altered by significant parameters (Neomycin and Ampcillin). Analyses with cross-terms ($Group \times SectionModulus$) were not significant ($p > 0.10$).

Parameter	Estimate	Standard Error	p value
a_0, Intercept (Vancomycin)	15.27	3.57	<0.001
a_1, Group[Ampicillin]	-1.65	1.12	0.147
a_2, Group[Cocktail]	-0.53	1.15	0.646
a_3, Group[Untreated]	0.06	1.45	0.968
a_4, Group[Metronidazole]	1.72	1.23	0.168
a_5, Group[Neomycin]	-4.43	1.11	<0.001
a_6, Group[Sweetener]	6.13	1.21	<0.001
a_7, Slope (Section Modulus)	114.58	20.46	<0.001

Supplementary Table S3: Whole-bone mechanical properties derived from 3-point bending.

	Untreated	Sweetener	Ampicillin	Vancomycin	Neomycin	Metronidazole	Cocktail
Maximum Load (N)	26.36 ± 2.59 $^{\$,\#}$ (21.76, 30.36)	28.80 ± 2.89 $^{\$,\#}$ (23.32, 33.62)	20.06 ± 2.77 (16.35, 25.40)	21.04 ± 3.68 (17.20, 28.21)	17.99 ± 1.71 (15.72, 21.56)	21.78 ± 2.37 (18.94, 24.63)	21.20 ± 2.16 (18.99, 24.79)
Ultimate Stress (MPa)	183.26 ± 18.35 $^{\%,\wedge}$ (147.21, 200.88)	219.45 ± 17.22 (193.86, 249.48)	206.88 ± 38.45 (156.83, 269.64)	199.62 ± 11.59 (186.76, 214.05)	187.63 ± 18.23 (160.05, 214.32)	231.21 ± 32.84 (175.54, 282.40)	206.81 ± 13.33 (190.52, 229.20)
Stiffness (N/mm)	194.31 ± 18.14 $^{\$,\#}$ (168.68, 225.93)	188.09 ± 16.53 $^{\$,\#}$ (160.31, 207.95)	146.86 ± 15.40 (127.57, 164.28)	146.84 ± 27.32 (115.64, 201.52)	118.56 ± 10.67 $^{\&}$ (103.29, 134.27)	132.62 ± 18.83 (119.88, 171.66)	132.86 ± 16.12 (110.05, 151.84)
Young's Modulus (GPa)	6.69 ± 1.25 (5.27, 9.35)	6.68 ± 1.00 (5.21, 8.02)	7.76 ± 1.28 (6.49, 9.87)	7.28 ± 0.94 (6.48, 9.31)	6.44 ± 0.83 (5.26, 8.14)	6.97 ± 0.93 (5.99, 8.61)	6.31 ± 0.60 (5.17, 6.88)
Work to Failure (mJ)	8.97 ± 3.96 (3.59, 17.62)	8.61 ± 1.86 (5.61, 11.86)	5.33 ± 1.90 (1.31, 7.02)	7.56 ± 2.87 (3.92, 12.55)	4.96 ± 2.82 (2.07, 11.37)	7.03 ± 1.52 (4.83, 8.95)	5.62 ± 1.20 (3.82, 6.94)

Values are mean ± SD and (min, max).
$^{\$}$ Untreated v. Ampicillin, Vancomycin, Neomycin, Metronidazole, Cocktail, $p<0.05$
$^{\#}$ Sweetener v. Ampicillin, Vancomycin, Neomycin, Metronidazole, Cocktail, $p<0.05$
$^{\%}$ Untreated v. Sweetener, $p<0.05$
$^{\wedge}$ Metronidazole v. Untreated, Neomycin, $p<0.05$
$^{\&}$ Neomycin v. Ampicillin, Vancomycin, $p<0.05$

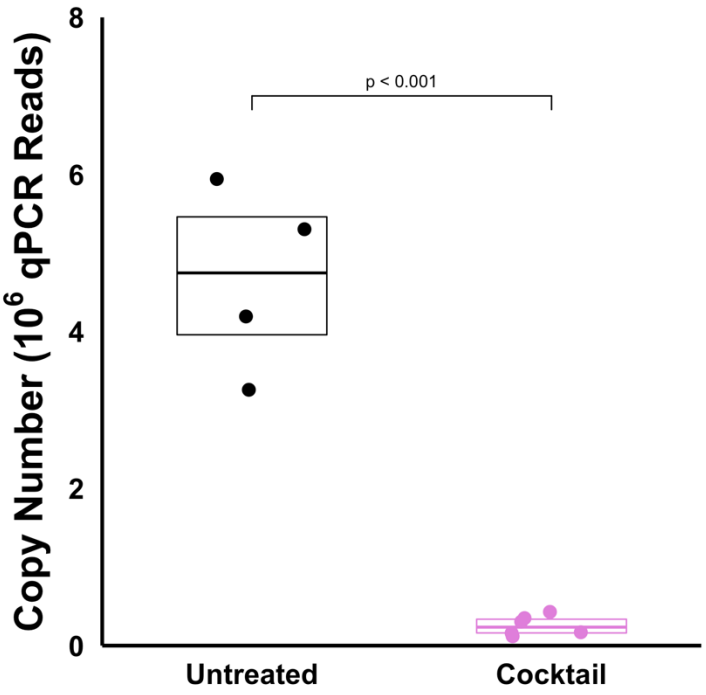

Figure S1: The bacterial load in the feces, as measured by qPCR reads, is shown for the untreated group and the cocktail group. The number of qPCR reads in the cocktail group is 95% less than that of the untreated group.

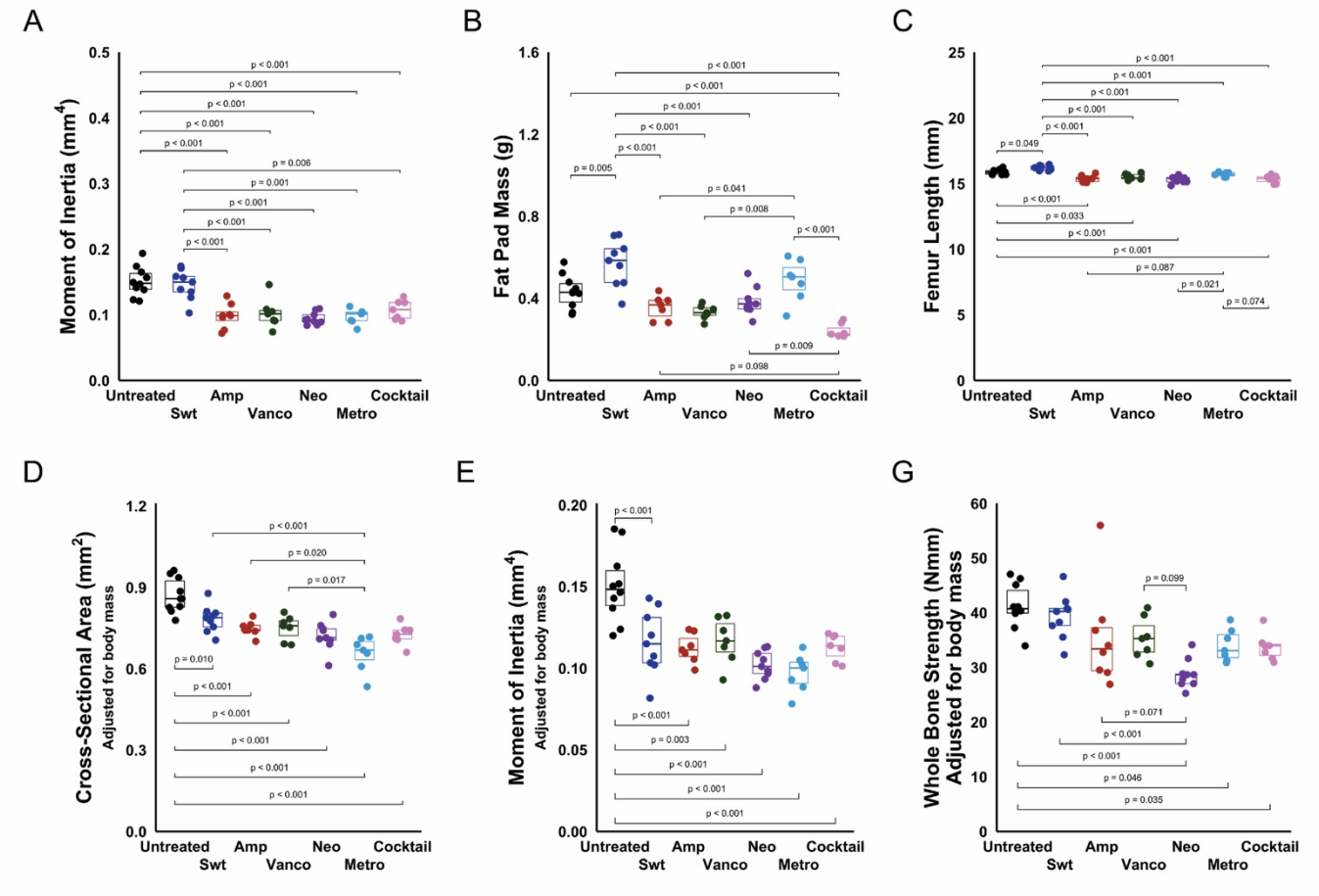

Figure S2: Removal of components of the gut microbiota using narrow spectrum antibiotics decreased A) bacteria load in the cocktail group compared to untreated as measured by qPCR reads. (B) moment of inertia compared to untreated animals. (C) Epididymal fat pad mass was decreased in animals receiving an antibiotic cocktail and increased in animals receiving only sweetener as compared to untreated animals. (D) Femur length was increased in animals receiving sweetener, and decreased in animals receiving ampicillin, vancomycin, neomycin or cocktail. Measures of bone geometry properties adjusted for body mass using the GLM approach (Jepsen et al J Bone Miner Res 2015) show reduced whole bone geometrical properties: (E) Cross-sectional area. (F) Moment of inertia. (G) Femur length, (H) whole bone strength.

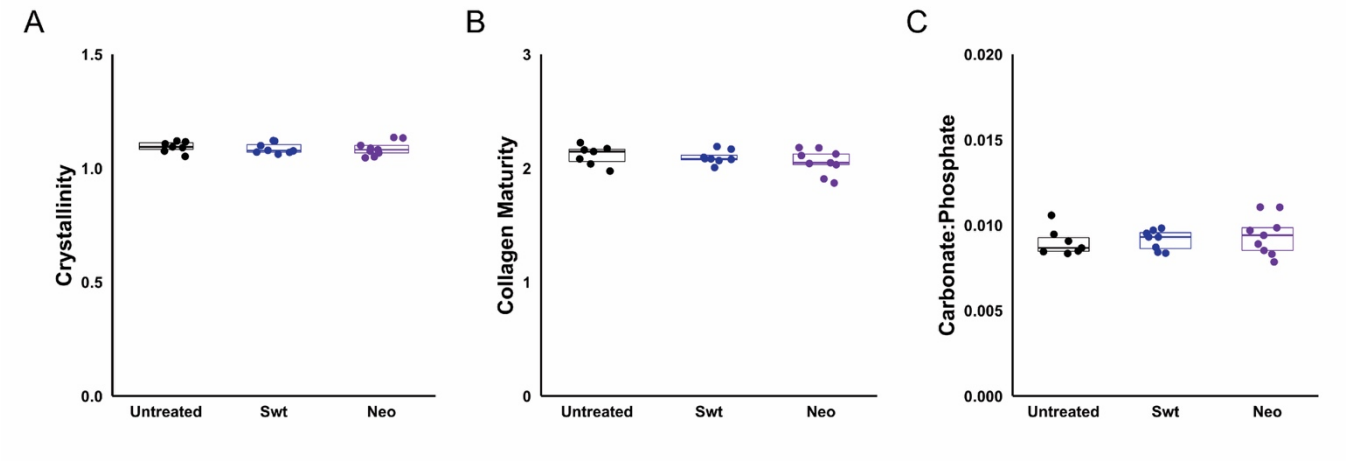

Figure S3: Bone material properties were analyzed in groups with altered tissue strength (sweetener and neomycin groups). A) crystallinity, B) collagen maturity, C) carbonate:phosphate did not differ among groups.

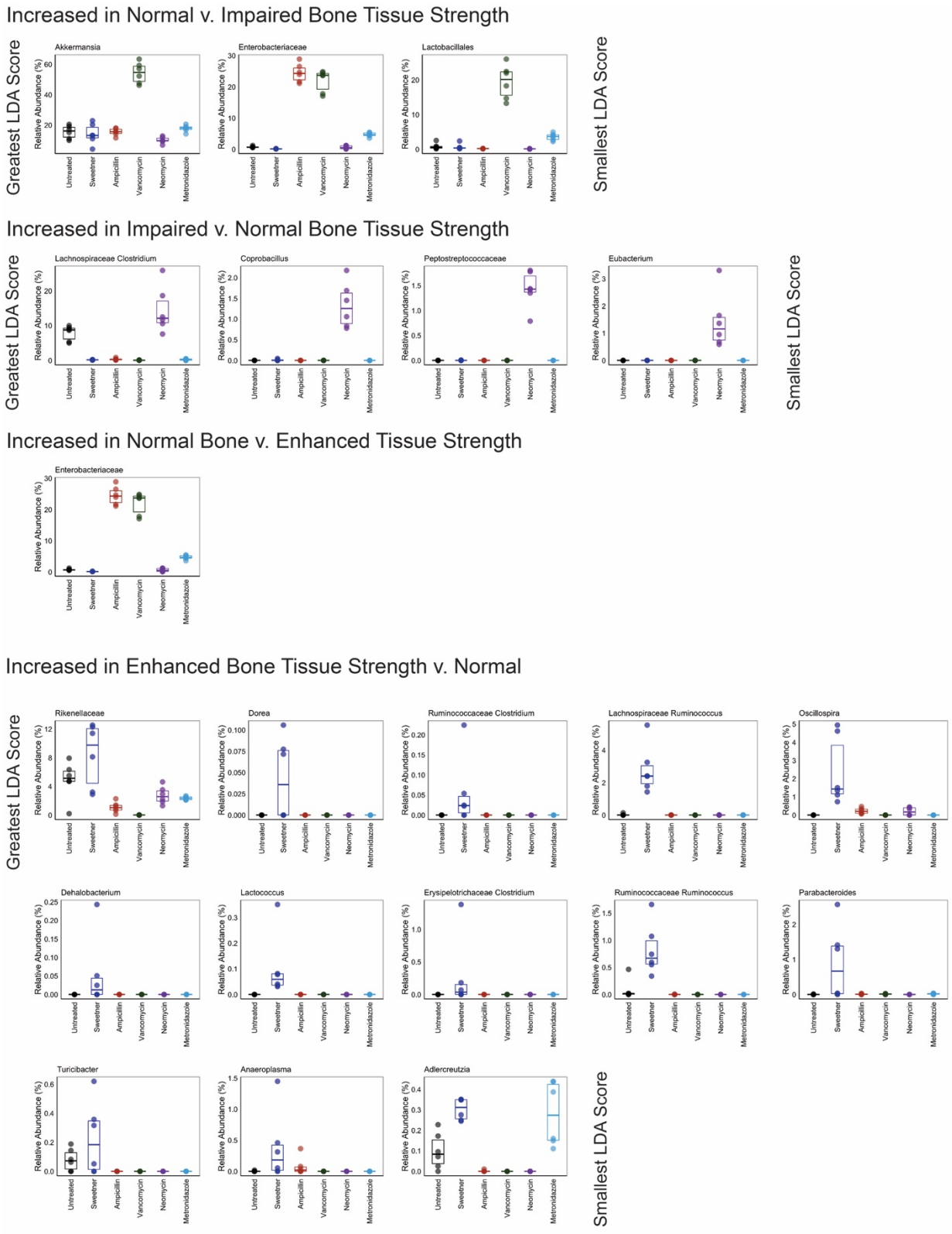

Figure S4: The relative abundance of lower rank taxonomic features with effect size greater than |2| in Fig. 3 are shown in order of LDA score (greatest to least).

Predicted Pathways	Normal Tissue Strength	Increased Tissue Strength	Decreased Tissue Strength
PWY-4722 creatinine degradation II [#,%]			
PWY-6163 chorismate biosynthesis from 3-dehydroquinate [$,%]			
PWY-5121 superpathway of geranylgeranyl diphosphate biosynthesis II (via MEP) [$,%]			
NONMEVIPP-PWY methylerythritol phosphate pathway I [$,%]			
PWY-7560 methylerythritol phosphate pathway II [$,%]			
PWY-6123 inosine-5'-phosphate biosynthesis I [#,$,%]			
TRNA-CHARGING-PWY tRNA charging [$,%]			
PWY-7219 adenosine ribonucleotides de novo biosynthesis [$,%]			
SALVADEHYPOX-PWY adenosine nucleotides degradation II [$,%]			
PWY-4984 urea cycle [#,$,%]			
PWY-6353 purine nucleotides degradation II (aerobic) #,$,%			
PWY-6608 guanosine nucleotides degradation III [$,%]			
PWY-7208 superpathway of pyrimidine nucleobases salvage [$,%]			
FUC-RHAMCAT-PWY superpathway of fucose and rhamnose degradation [#,$,%]			
RUMP-PWY formaldehyde oxidation I [#,%]			
PENTOSE-P-PWY pentose phosphate pathway [#,$,%]			
ANAGLYCOLYSIS-PWY glycolysis III (from glucose) [#,$,%]			
PWY-5265 peptidoglycan biosynthesis II (staphylococci) [#,%]			
PWY-5484 glycolysis II (from fructose 6-phosphate) [#,$,%]			
PWY-5667 CDP-diacylglycerol biosynthesis I [$,%]			

Normal v. Decreased Tissue Strength, p<0.05
$ Normal v. Increased Tissue Strength, p<0.05
% Decreased v. Increased Tissue Strength, p<0.05

Figure S5: Predicted functional pathways from estimated from the taxonomic information using PICRUSt. Comparison of pathways between animals with normal (Untreated), increase (Sweetener), and decreased (Neomycin) tissue strength resulted in 285 differential pathways out of 489 detected pathways. The twenty most differential pathways are listed and ordered by ascending p-value.

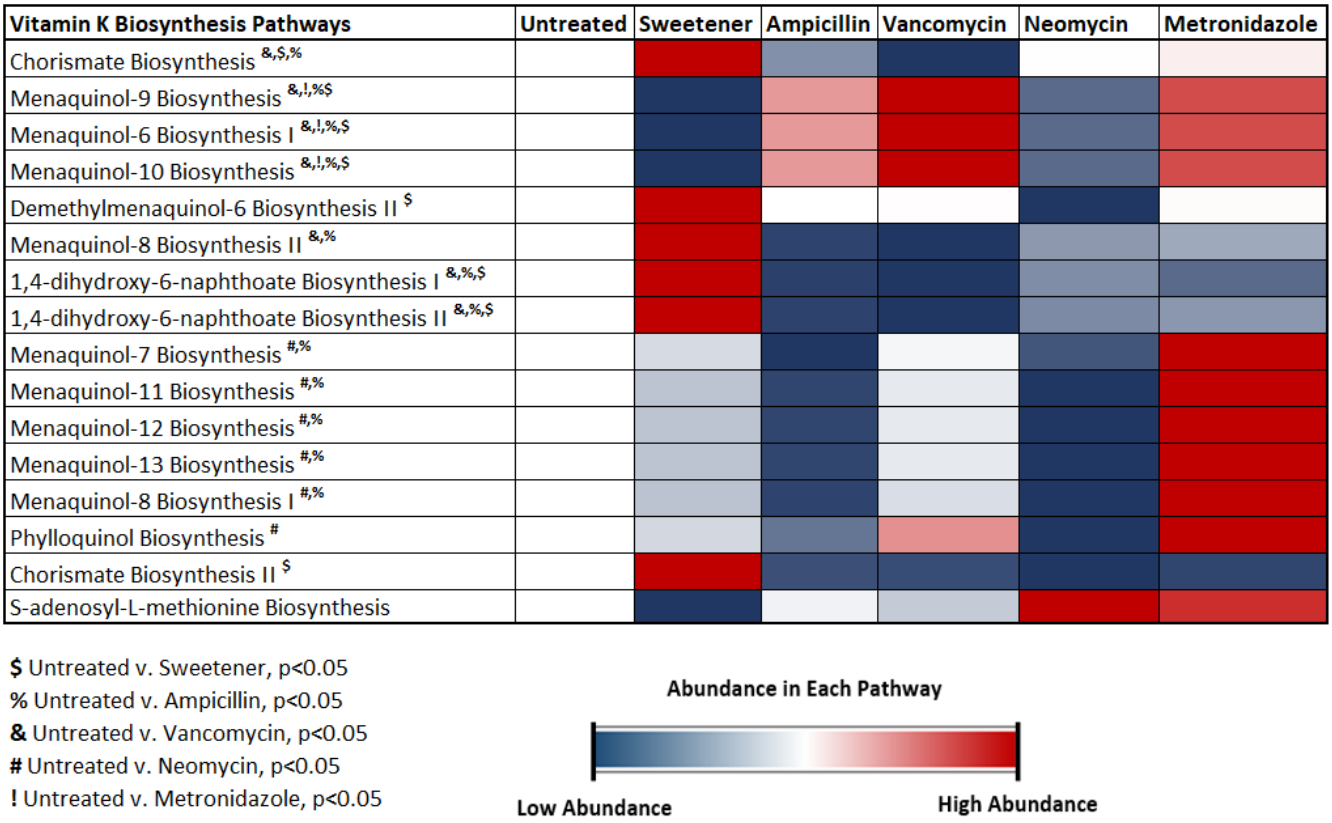

Figure S6: Predicted functional capacity from PICRUSt for vitamin K biosynthesis for all groups.

Pathways are listed and ordered by ascending p-value.