SUPPLEMENTARY MATERIALS

Individual and Combined Effects of a Direct-fed Microbial and Calcium Butyrate on Growth Performance, Intestinal Histology and Gut Microbiota of Broiler Chickens

Bishnu Adhikari[1], Alyson G. Myers[1], Chuanmin Ruan[1], Young Min Kwon[1]

and Samuel J. Rochell[1,2,*]

[1]Department of Poultry Science, University of Arkansas, Fayetteville, AR

*Correspondence: rochesj@auburn.edu; Current affiliation: Department of Poultry Science, Auburn University, Auburn, AL, USA

Table S1. Summary of the jejunal samples used for 16S microbiota analysis.

	NC	PC	CS	BP	CS+BP	Total
Non-spiking	9	9	8	9	9	44
Spiking	10	10	9	10	10	49
Total	19	19	17	19	19	93

Table S2. Summary of the taxonomic groups in jejunal samples that showed significant difference among the treatment groups based on different datasets.

Dataset	Phylum	Class	Order	Family	Genus
RMP-93[a]	OD1			OD1_;f_	P_OD1; f_;g_
		Thermomicrobia	JG30-KF-CM45		
			Rhodocyclales		
				Micrococcaceae	
					f_ Tissierellaceae; g_Sporanaerobacter
					f_ Clostridiaceae; g_SMB53
					f_Bacillaceae;_
RMP-49[b]					*Propionibacterium*
					f_Leuconostocaceae
					P_OD1; f_;g_
QMP-49[c]					*Propionibacterium*
					f_Leuconostocaceae_

[a]RMP-93: RMP dataset from all 93 samples, including both 44 non-spiked and 49 spiked samples
[b]RMP-49: RMP dataset from 49 spiked samples
[c]QMP-49: QMP dataset from 49 spiked samples

Effects of spiking of the jejunal samples with *L. xyli* CTM on the structure of microbiotas

We randomly selected 8 jejunum samples across the treatment groups for additional evaluation of the spiking method using *L. xyli*. For the 8 samples, each sample was processed directly (no spiking) or processed after spiking of *L. xyli* (10^6 CFU) to 50, 100, and 200 mg of the sample for DNA extraction, followed by PCR, and MiSeq sequencing. Therefore, a total of 32 samples (8 samples × 4 spiking levels, including no-spiking controls) were processed for microbiota analysis. All procedures for preparation of PCR amplicon library, sequencing and data analysis were performed essentially as described in *Materials and Methods*.

In the resulting OTU table, the mean read counts per sample was 41,383 ± SE 3,123 (ranging from 14,185 to 87,254). First, statistical analysis was performed to test the hypothesis that spiking does not change the microbiota profiles. Comparisons were performed after filtering out all sequence reads matching to *L. xyli*. Statistical analysis using the ANCOM method showed that there were no significance differences among the 4 groups in any taxa at all levels of classification ($p < 0.05$). It also showed that there was no difference among the groups in both alpha and beta diversities (using all metrics available from QIIME2) at $p < 0.05$. The results indicate that spiking of the jejunal samples with *L. xyli* at different levels do not change the structure of the microbiota, providing important evidence to validate the use of spiking with *L. xyli* for quantitative microbiota profiling.

Construction of quantitative microbiota profiles using *L. xyli* spiking

Figure S1A shows the genus-level composition of the 8 samples for which *L. xyli* was spiked into 200 mg sample based on relative abundance of different genera (relative microbiota profiles; RMP). The data was then calibrated by setting the level of *L. xyli* of each sample to $5x10^6$ CFU/g. The new bar graph in **Figure S1B** shows quantitative microbiota profiles (QMP) in which the y-axis shows CFU/g sample of each genus.

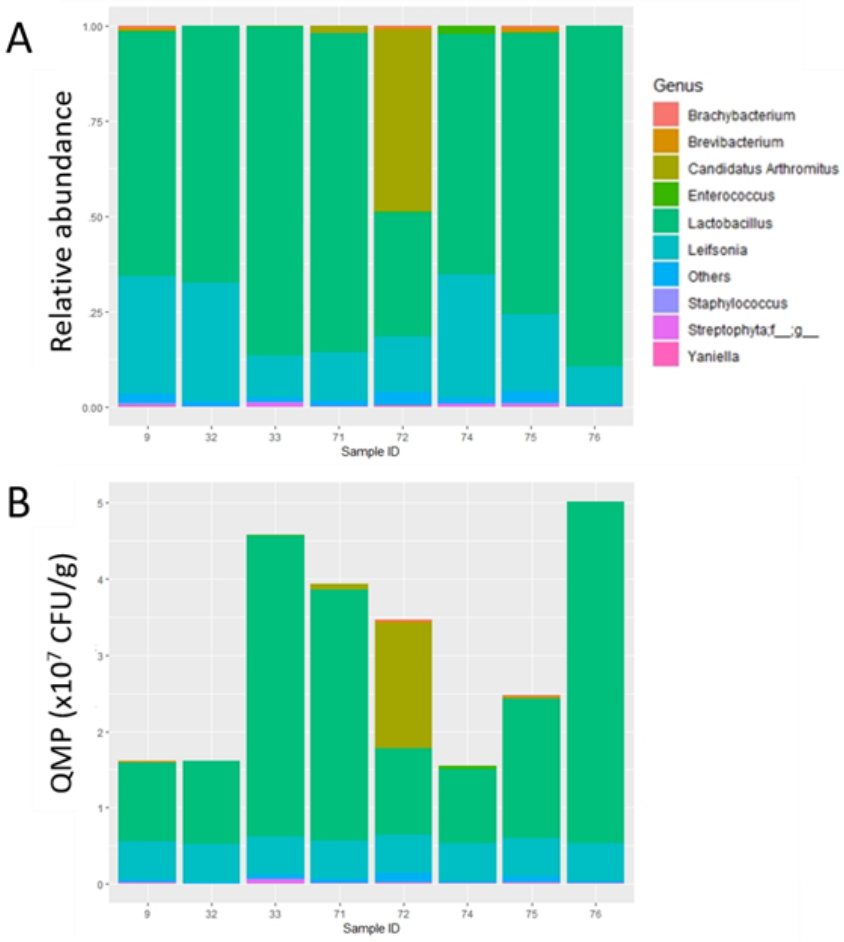


Figure S1. Microbiota profiles of the 8 jejunal samples spiked with *L. xyli* (10^6 CFU per 200 mg) presented in (A) relative microbiota profiles (RMP) and (B) quantitative microbiota profiles (QMP).

In order to assess the quantitative trends associated with varying levels of *L. xyli* spiking, a bar graph was made for the 8 samples spiked at 3 different levels (**Figure S2A**). In this RMP, the relative abundance of *L. xyli* decreased with increasing amounts of the sample to which *L. xyli* (same number CFU) was spiked, except for sample #32. The QMP bar graph in **Figure S2B** also shows the trends for all 8 samples that is expected from the way the spiking was performed. These results indicate that the quantitative information inferred by the spiked *L. xyli* as a spike-in control was consistent and reliable, and the resulting QMP can provide accurate numbers of the bacterial cells representing different genera (or taxa at different levels of classification) in the samples.

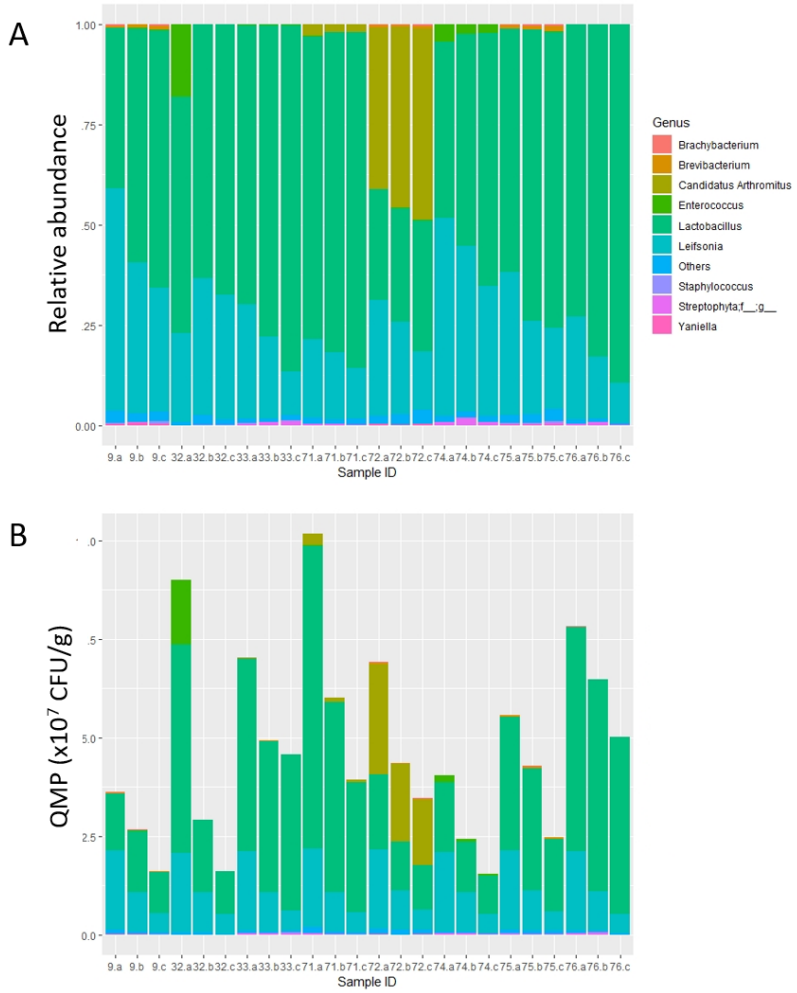
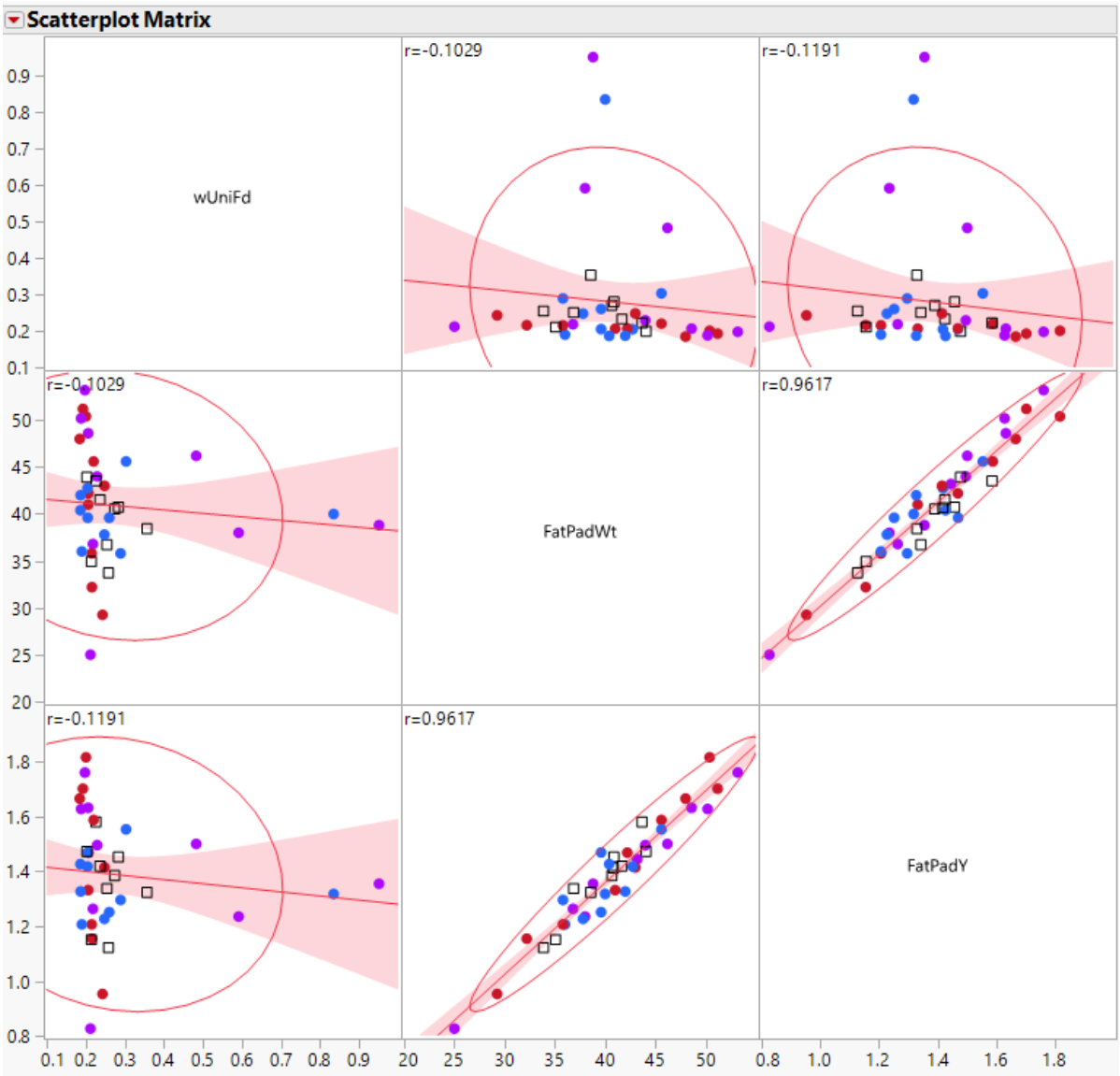


Figure S2. Microbiota profiles of 8 selected jejunal samples to which *L. xyli* (10^6 CFU) was spiked into 50, 100 or 200 mg sample. (A) Relative microbiota profile (RMP) and (B) Quantitative microbiota profile (QMP)

Figure S3. The scatterplot matrices created by JMP Genomics that show the significant correlations between the parameters in growth performance (0-15 d) and carcass characteristics, and microbiota parameters (selected alpha diversity indices, observed OTUs and Shannon index, and beta diversity index based on weighted UniFrac distance in reference to NC). Nonparametric correlation analyses were conducted using (A) Spearman, (B) Kendall and (C) Hoeffding correlation tests. Symbols for the treatment groups: NC (open square), PC (closed square), CS (red circle), BP (blue circle), and CS+BP (purple circle). * indicates significant difference at $p < 0.05$.

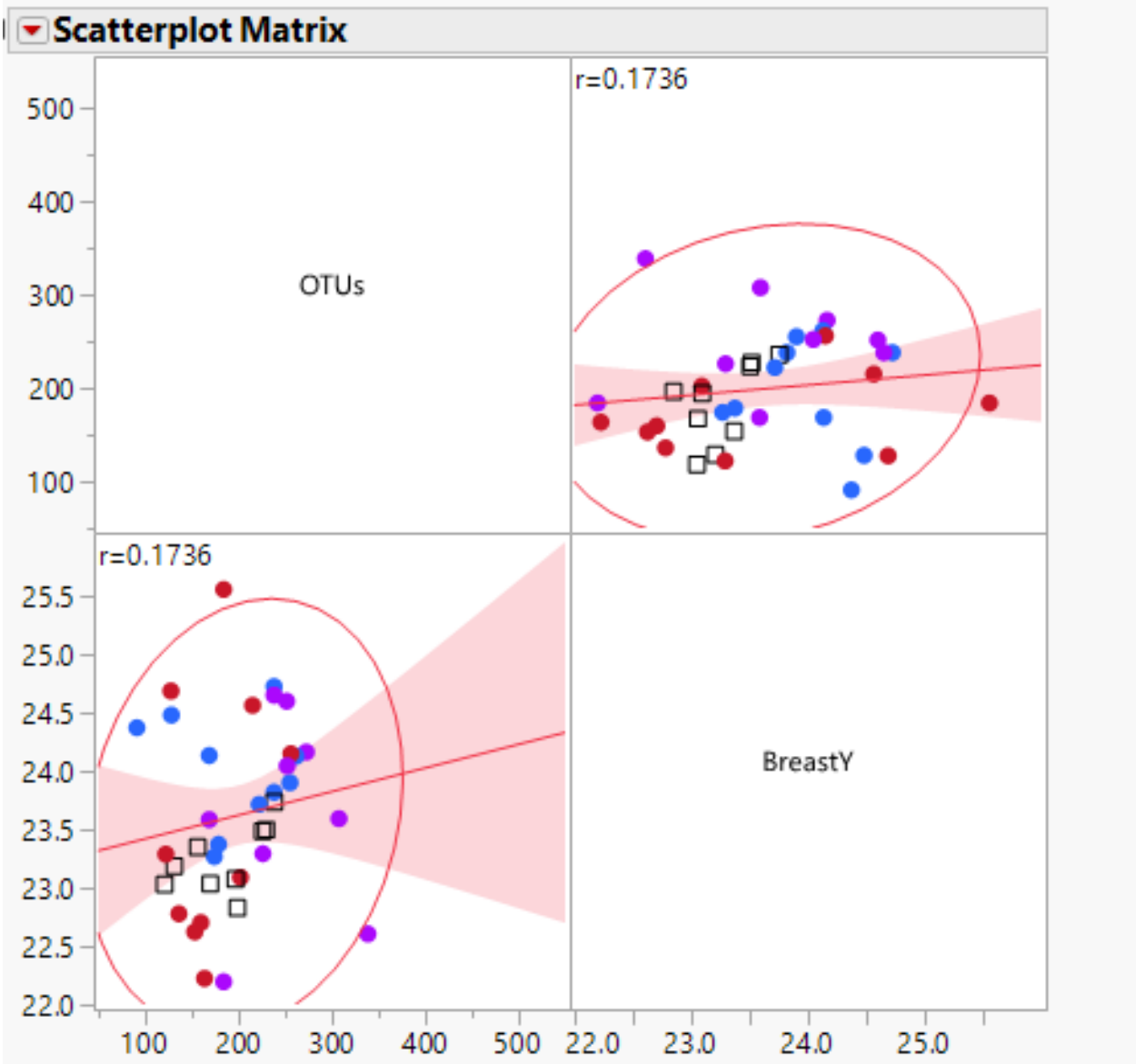
A



Nonparametric: Spearman's Rho

Variable	by Variable	Spearman Rho	Prob>|Rho|
FatPadWt	wUniFd	-0.3332	0.0410*
FatPadY	wUniFd	-0.3240	0.0472*
FatPadY	FatPadWt	0.9402	<.0001*

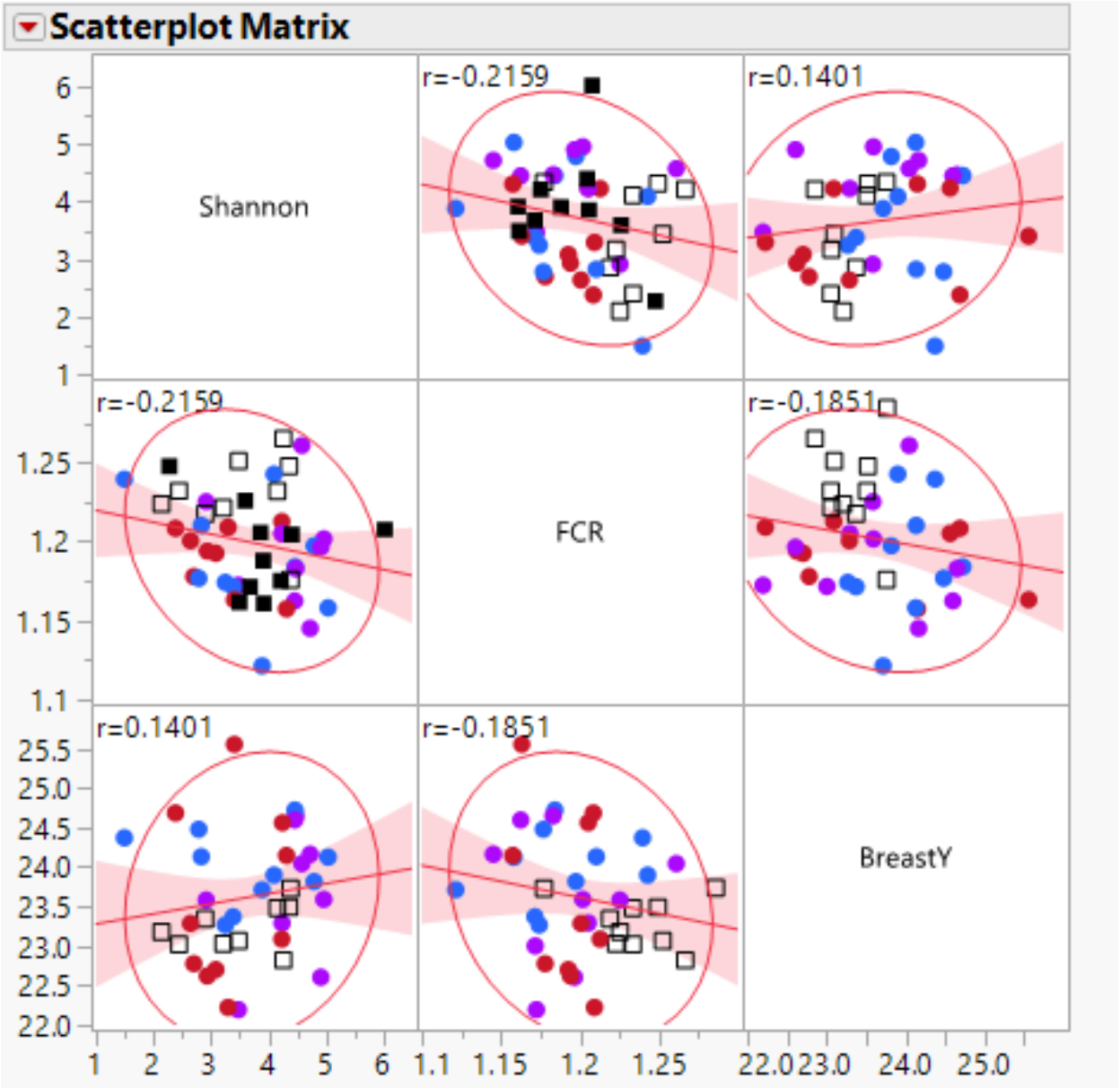
B



Nonparametric: Kendall's Tau

Variable	by Variable	Kendall Tau	Prob>|Tau|	-.8 -.6 -.4 -.2 0 .2 .4 .6 .8
BreastY	OTUs	0.2287	0.0441*	

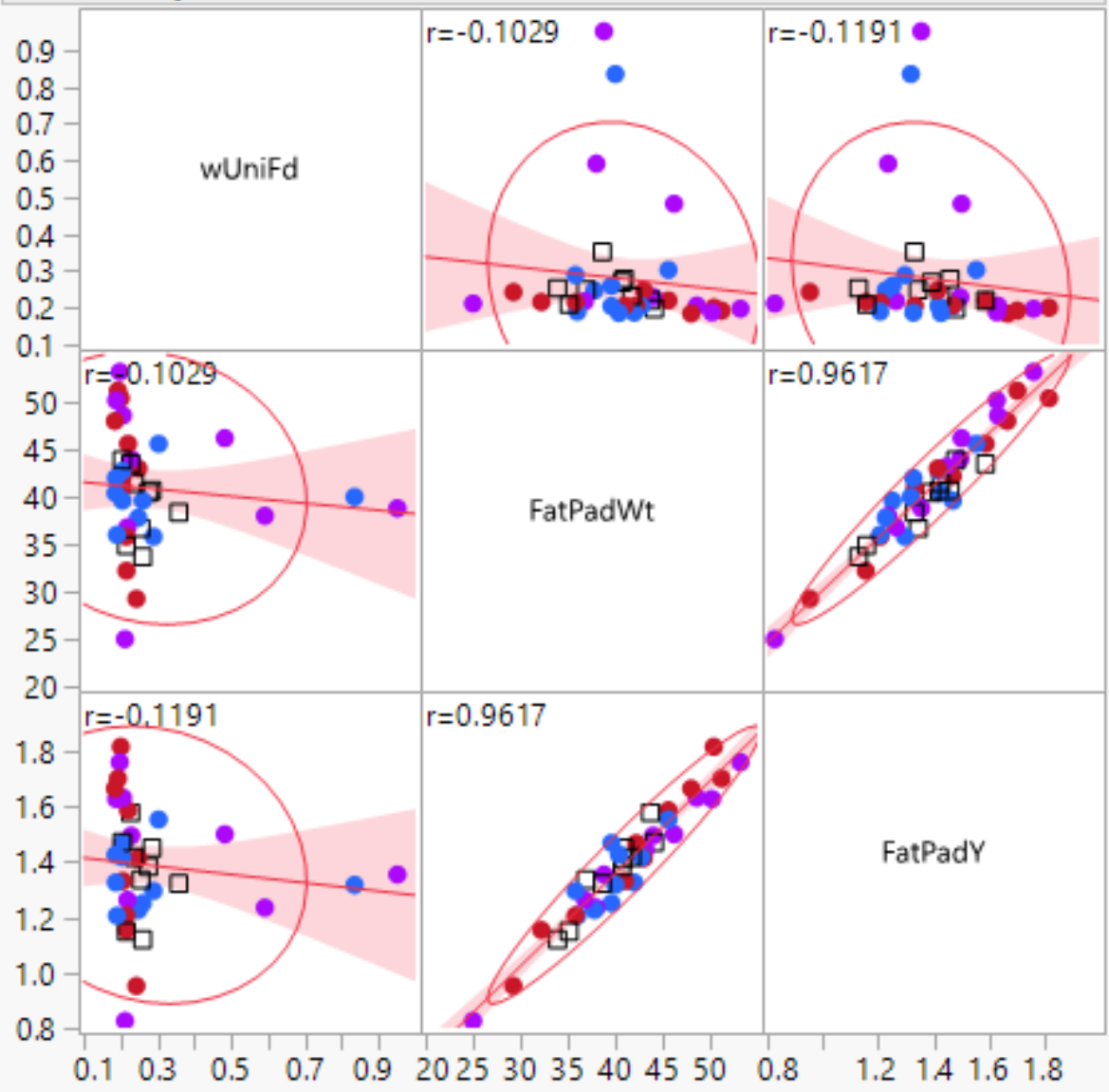
C



Nonparametric: Hoeffding's D

Variable	by Variable	Hoeffding D	Prob> D
FCR	Shannon	0.0196	0.0496*
BreastY	Shannon	0.0388	0.0186*
BreastY	FCR	-0.0035	0.5062

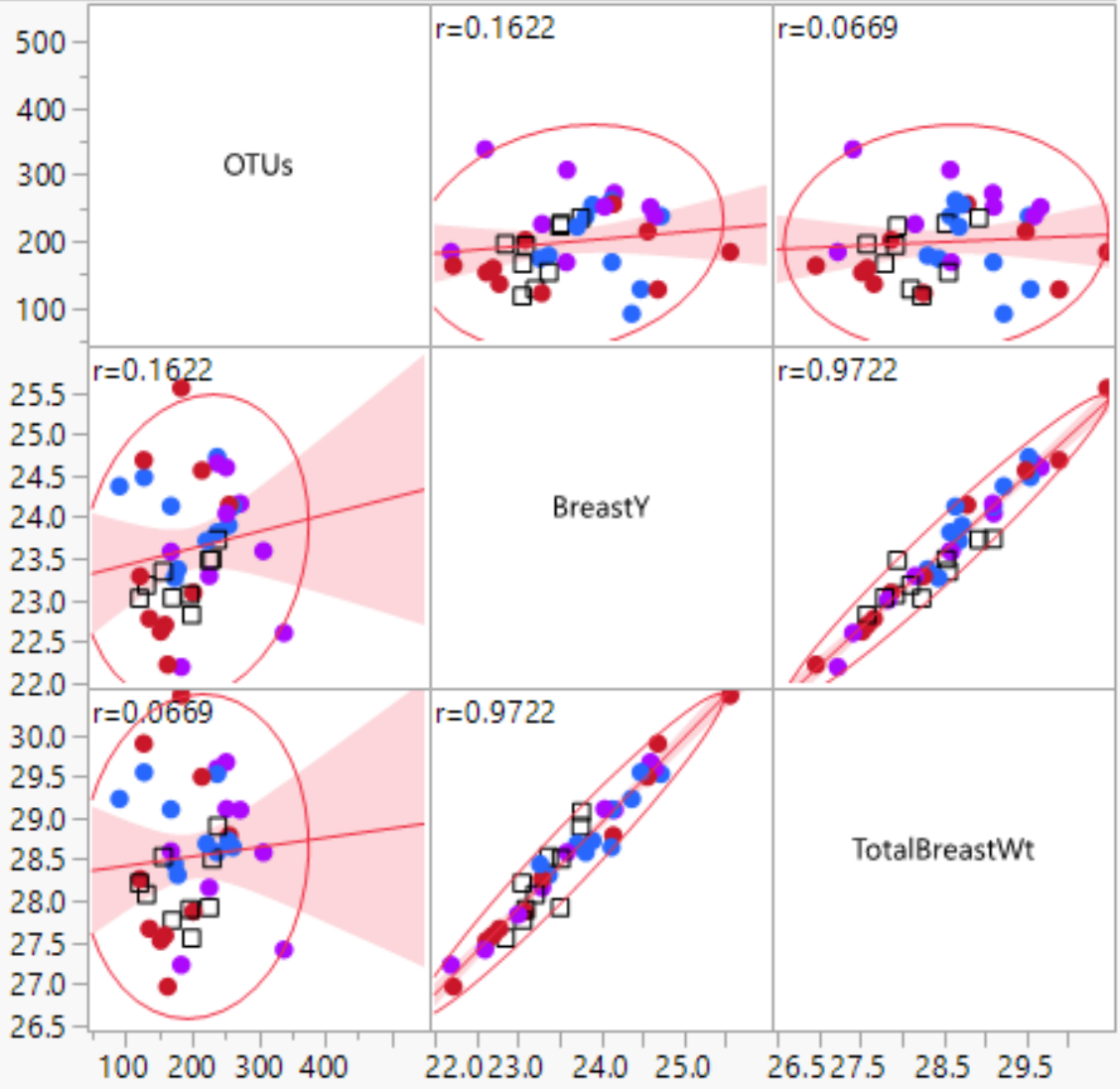
Scatterplot Matrix



Nonparametric: Hoeffding's D

Variable	by Variable	Hoeffding D	Prob>D
FatPadWt	wUniFd	0.0340	0.0260*
FatPadY	wUniFd	0.0429	0.0141*
FatPadY	FatPadWt	0.5231	<.0001*

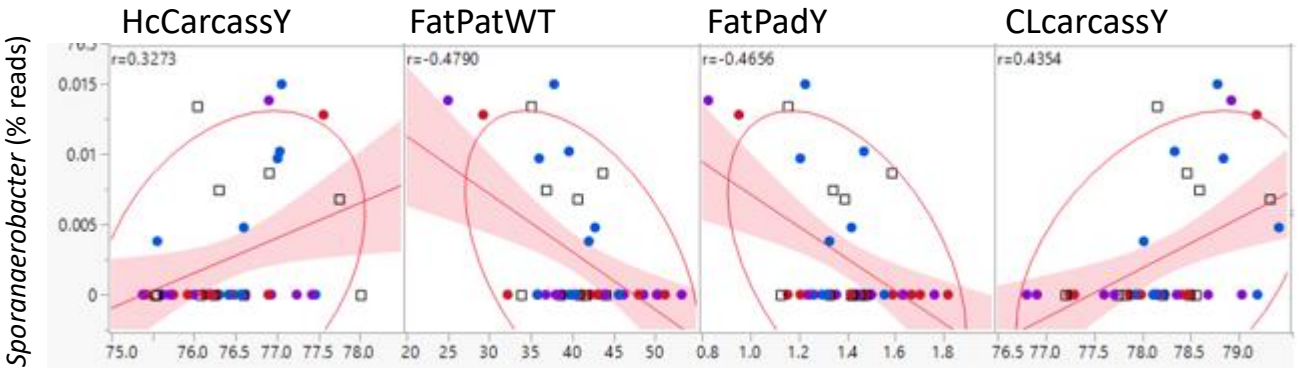
Scatterplot Matrix



Nonparametric: Hoeffding's D

Variable	by Variable	Hoeffding D	Prob>D
BreastY	OTUs	0.0804	0.0012*
TotalBreastWt	OTUs	0.0324	0.0291*
TotalBreastWt	BreastY	0.6849	<.0001*

Figure S4. The scatterplot matrices created by JMP Genomics that show the significant correlations between the parameters in carcass characteristics and the relative abundance of the genus *Sporanaerobacter*. Nonparametric correlation analyses were conducted using Spearman test. Symbols for the treatment groups: NC (open square), PC (closed square), CS (red circle), BP (blue circle), and CS+BP (purple circle). * indicates significant difference at $p < 0.05$.



Genus	Carcass	Spearman Rho	P
g__Sporanaerobacter	HCarcassY	0.3646	0.0207*
g__Sporanaerobacter	FatPadWt	-0.4163	0.0075*
g__Sporanaerobacter	FatPadY	-0.3837	0.0145*
g__Sporanaerobacter	ClCarcassY	0.5088	0.0008*