COMPANION ANIMAL EQUINE LAB REPORT 2022

EFFECT OF DIAMOND V™ TRUEQUINE™ C ON *IN VITRO* VOLATILE FATTY ACID (VFA) PRODUCTION AND FECAL METAGENOMIC PROFILES

Diamond V TruEquine C, a *Saccharomyces cerevisiae* fermentation postbiotic was evaluated at two levels to characterize its influence on VFA production and fecal metagenomic outcomes.

Study Background

Horses rely on volatile fatty acids (VFA) to provide a source of energy and fermentative end-products for their health and well-being. An improvement in VFA production, has been associated in various species with improved feed conversion and availability of nutrients necessary to maintain epithelial cell growth, blood flow, normal secretory and absorptive functions of the intestine, and positive changes in the microbial ecology of the gut and expression of markers associated with possible functions such as carbohydrate utilisation potential and ability to minimise development of antibiotic resistance antibiotic resistance.

Experimental Overview

The IAMM and Treatment Array (Figure 1). An *in vitro* equine intestinal model (IAMM) was utilised to simulate the microbiological activity occurring in the horse hindgut to characterize VFA and metagenomic outcomes. Treatments evaluated included TruEquine C at 150 mg (TEC) and 225 mg (TECP) to mimic common supplementation regimens *in vivo* of 14 g/d and 21 g/d. A negative control containing inoculated medium, and a positive control containing the prebiotic inulin were included with the full array of treatments and replicates repeated in duplicate and incubated anaerobically at 37.2 C for 24 hours.

Sample Analysis. VFA concentrations were analysed by gas chromatography and data was analysed using the GLM model of JMP (SAS Institute Inc) and two-way ANOVA GraphPad (v 9.2) for the effect of treatments on intestinal microbial fermentation. Samples for microbial metagenomics were collected and frozen after 24 hours of incubation. DNA extraction was performed using ZymoBIOMICS 96 MagBead DNA kit (Zymo Research Corporation, Irvine CA) using a Biomek i7 workstation (Beckman Coulter, Indianapolis, USA). Shotgun metagenomic sequencing was performed using R9.4.1 FLO-MIN 106 flow cells on the GridION platform (ONT, Oxford, UK). Fastq files obtained from the MinKNOW ONT workflow were used for microbial taxonomic classification Taxonomic assignation was performed using Kraken2 with the POWER3-All database. Genomes from microbial species identified with Kraken2 were annotated using PROKKA, followed by additional assessment of gene function using EggNOG-mapper v2. After the annotation process was completed, an in-house python script was used to compile the Carbohydrate-activate enzymes (CAZy) for each genome. Resistome data was obtained by mapping shotgun sequences for each sample against the MEGARes – Antimicrobial Database.

Statistical Analysis. Diversity metrics were calculated in R using the Phyloseq package with the species count table from Bracken as input using rarefaction. Statistical analysis for diversity metrics was performed with the lme4 package from R, using the fit linear mixed-effect model function (lm). Package emmeans from R was used to determine the pairwise statistical significance. Differential abundance analysis was performed using the R package LinDA using Negative Control samples as the intercept. Differential abundance analysis was

performed for multiple taxonomic levels; species, genus, family, phylum, and for functional potential; CAZy and Resistome.

Results

VFA Production (Figure 2). Both levels of TruEquine™ C supported higher ($P < 0.05$) acetate, propionate, butyrate, and total VFA production versus the untreated sample (negative control). Versus inulin (positive control), the higher level of TruEquine C produced more acetate, propionate, butyrate and total VFA ($P<0.05$). The 1.5-fold higher level of TruEquine C further elevated acetate, propionate and total VFA ($P < 0.01$), and 18.3% more butyrate (ns) versus the base level.

Species Richness (Figure 3). Both levels of TruEquine C produced an enrichment of observed species versus the negative and positive controls.

Evenness Among Species (Figure 4). There was a trend for reduced evenness in microbial species for TruEquine C compared to the negative control. This same general pattern of change in the microbiome was observed between the positive control and the higher level of TruEquine C but with an even greater number of microbes affected—59 in total vs 39 ($P < 0.01$).

Species and Genus Diversity (Figure 5). Compared to the positive control (inulin), the base level of TruEquine C produced a more diverse set of microbes (39 in total; $P < 0.01$) that included lactic acid producing bacteria (*Veillonella*), butyrate producing bacteria (*Butyricicoccus*, *Anaerobutyricum*), and increase of multiple Bacteroides species – known for their richness in carbohydrate degrading enzymes. Interestingly, multiple *Streptococcus* species showed decreased presence with TruEquine C – indicating a distinct effect of TruEquine C in the microbiome compared to a singular prebiotic, inulin.

This same general pattern of change in the microbiome was observed between the positive control and the higher level of TruEquine C but with an even greater number of microbes affected—59 in total vs 39 ($P < 0.01$) (Figure 6).

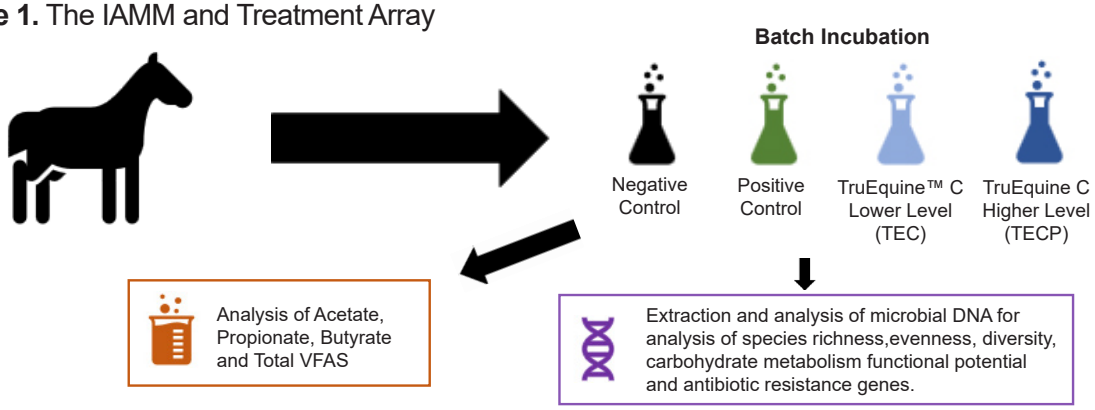
Carbohydrase Enzyme Potential Function (Figure 7). Carbohydrates are the main energy source for gut bacteria. To understand how treatments tested affected carbohydrate metabolism functions of these microbial populations, quantified carbohydrate-active enzymes (CAZymes) were annotated and quantified. The base level of TruEquine C increased PL21 – a polysaccharide lyase and decreased 4 carbohydrate binding enzymes, 5 glycosyl hydrolases, and 4 glycosyl transferases (left panel). A 1.5-fold increase in TruEquine C resulted in even more changes in the CAZy potential with a significant increase of 2 polysaccharide lyases, 4 glycosyl hydrolases, and 7 glycosyl transferases versus the negative control (right panel).

Antibiotic Resistance Genes (Figure 8). Both levels of TruEquine C had a beneficial effect decreasing the presence of tetracycline resistance genes whereas inulin did not have an effect on any resistome genes when compared to the negative control.

Summary

In this study, TruEquine C stimulated important beneficial changes in VFA production and the microbial community versus no postbiotic or a singular prebiotic (inulin). A further enhancement of VFA and metagenomic outcomes were observed with a 1.5-fold increase of TruEquine C (simulating a 46 mg/kg BW; 21 g/d *in vivo* level) vs the base level (simulating a 31 mg/kg BW *in vivo* level).

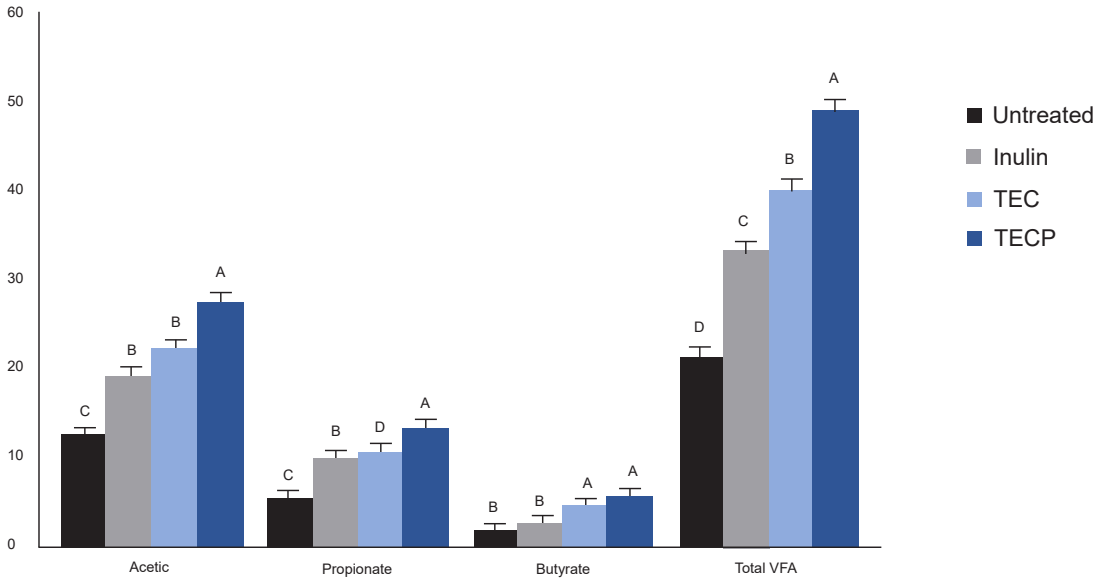
Figure 1. The IAMM and Treatment Array



Treatment Description	Substrate[1]	Fecal Media	Inulin	TruEquine C
Negative Control	•	•		
Negative Control	•	•	34.92 mg	
TEC	•	•	4.09	150 mg
TECP	•	•	0.60	225 Mg

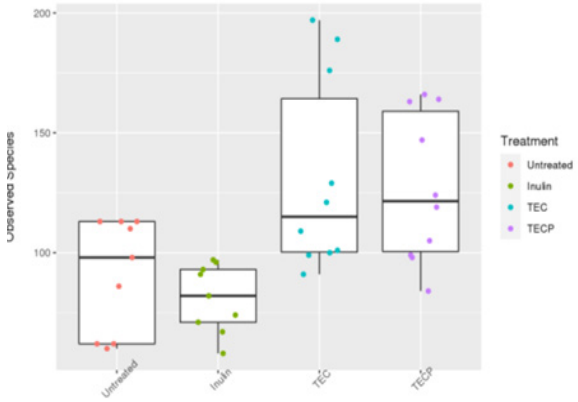
[1.] 30 ml of an inoculated medium containing pre-digested blend of alfalfa and timothy hay substrate and a dilution of fresh equine excreta

Figure 2. VFA Production



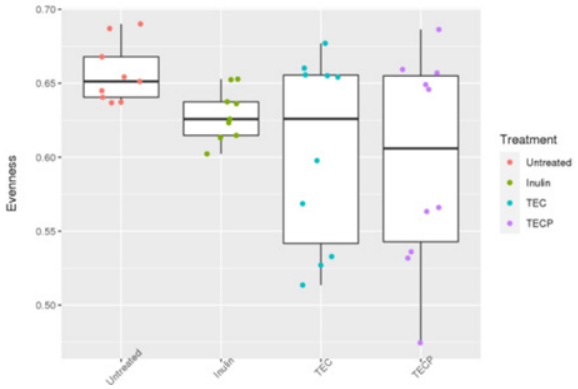
[abcd] Responses within each block of bars without a common superscript differ significantly ($P \leq 0.05$)

Figure 3. Species Richness
Observed Species Richness: Left panel shows the observed species distribution in boxplots and the right table shows pairwise comparison among treatments.



Contrast	Estimate	ProbF
Negative vs Positive Control (Inulin)	9.78	0.895
Negative Control vs TEC	-40.42	0.0257
Negative Control VS TECP	-36.12	0.0541
Positive Control (Inulin) vs TEC	-50.2	0.004
Positive Control (Inulin) vs TECP	-45.9	0.0093
TEC vs TECP	4.3	0.9878

Figure 4. Evenness Among Species
Evenness: Left panel shows the evenness metric distribution in boxplots and the right table shows pairwise comparison among treatments.



Contrast	Estimate	ProbF
Negative vs Positive Control (Inulin)	0.02799	0.652
Negative Control vs TEC	0.0525	0.132
Negative Control VS TECP	0.05976	0.0694
Positive Control (Inulin) vs TEC	0.02451	0.723
Positive Control (Inulin) vs TECP	0.03177	0.5339
TEC vs TECP	0.00725	0.9886

Figure 5. Species and Genus Diversity
Diversity: A positive Log_2 fold change means higher presence with TruEquine™ C and a negative Log_2 fold change means lower presence with TruEquine C versus the positive control.

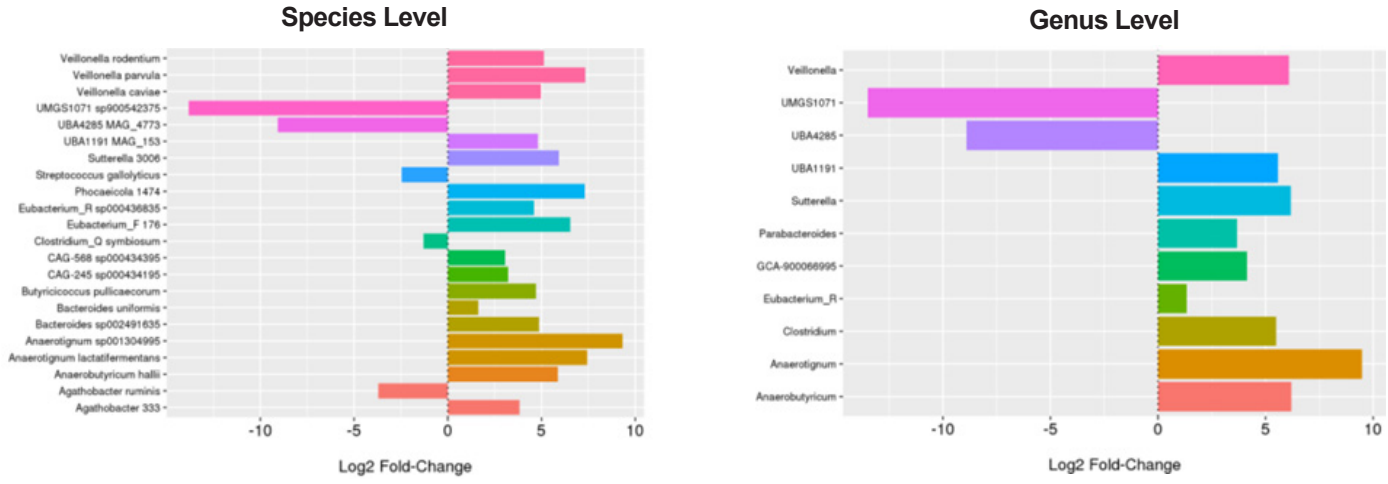


Figure 6. Species Level - Higher Level of TruEquine™ C vs Negative Control
Diversity: A positive Log_2 fold change means higher presence at the higher level of TruEquine C while a negative Log_2 fold change means lower presence.

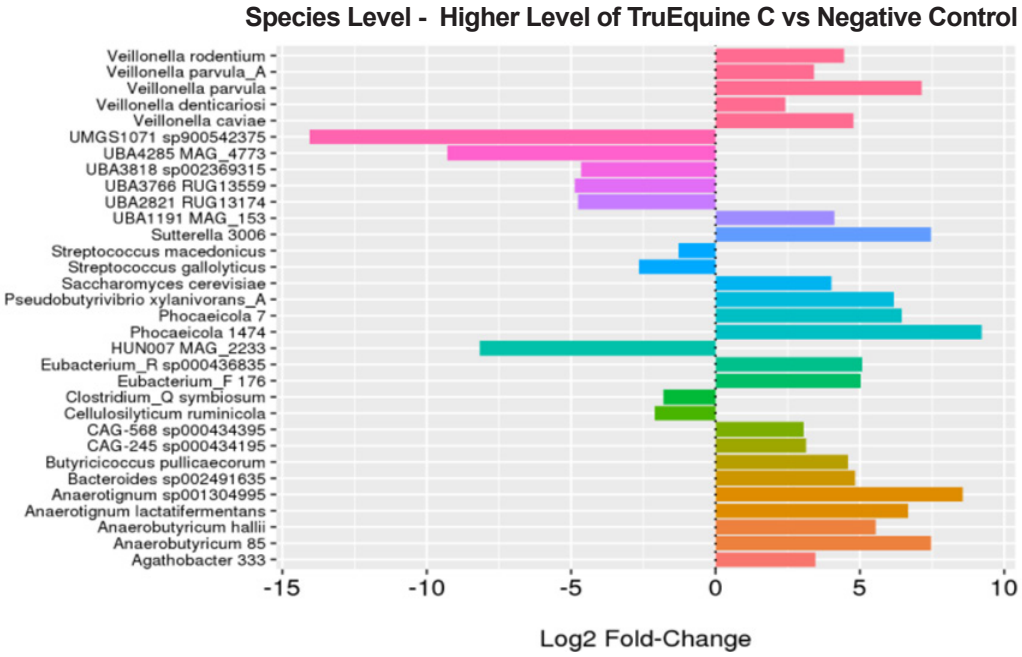
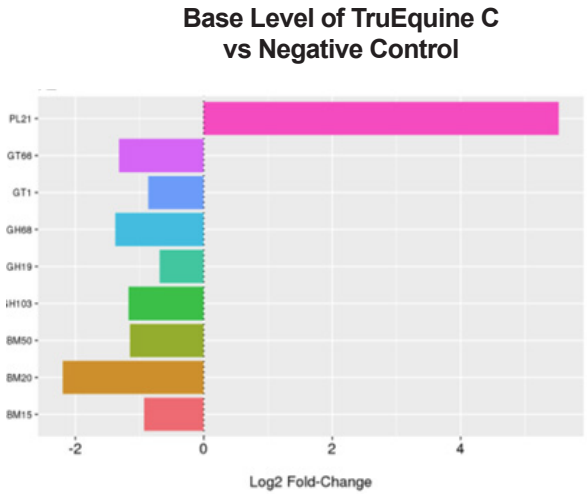


Figure 7. Carbonhydrase Enzyme Potential Function
Left Panel: Log_2 fold change for CAZys with differential abundance. A positive Log_2 fold change means higher presence while a negative Log_2 fold change means lower presence



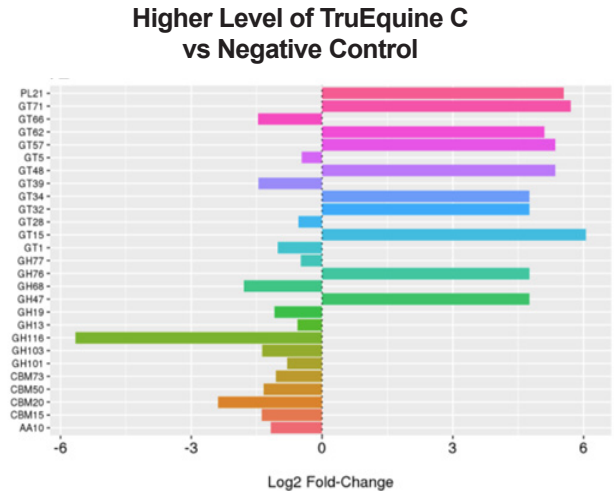
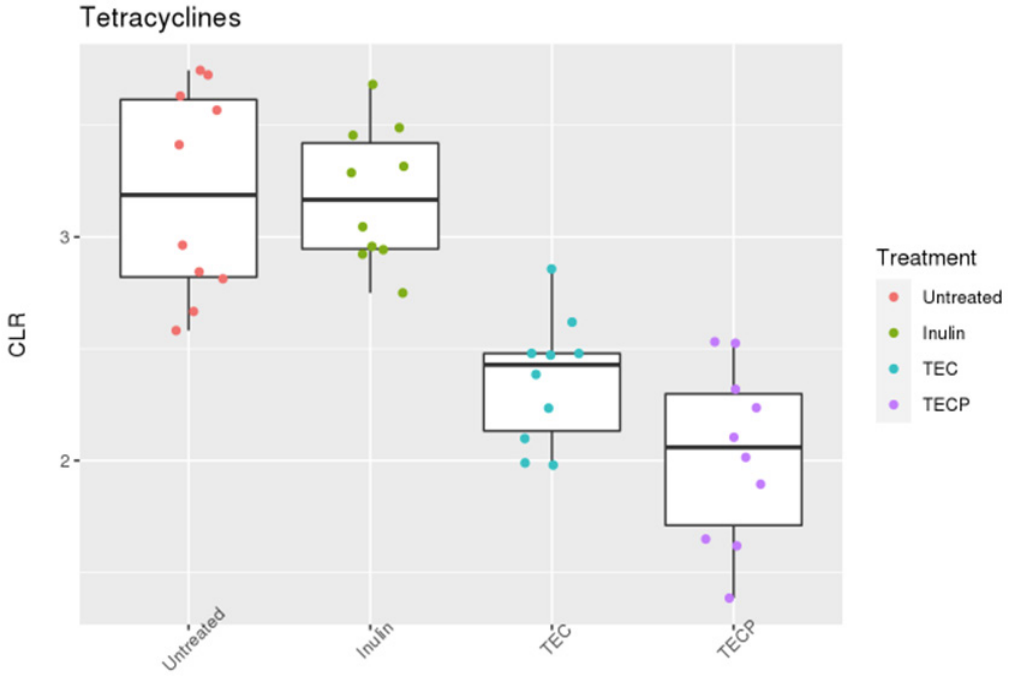


Figure 8. Antibiotic Resistance Genes
Both levels of TruEquine™ C had a beneficial effect decreasing the presence of tetracycline resistance genes whereas inulin did not have an effect on any resistome genes when compared to the negative control.



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