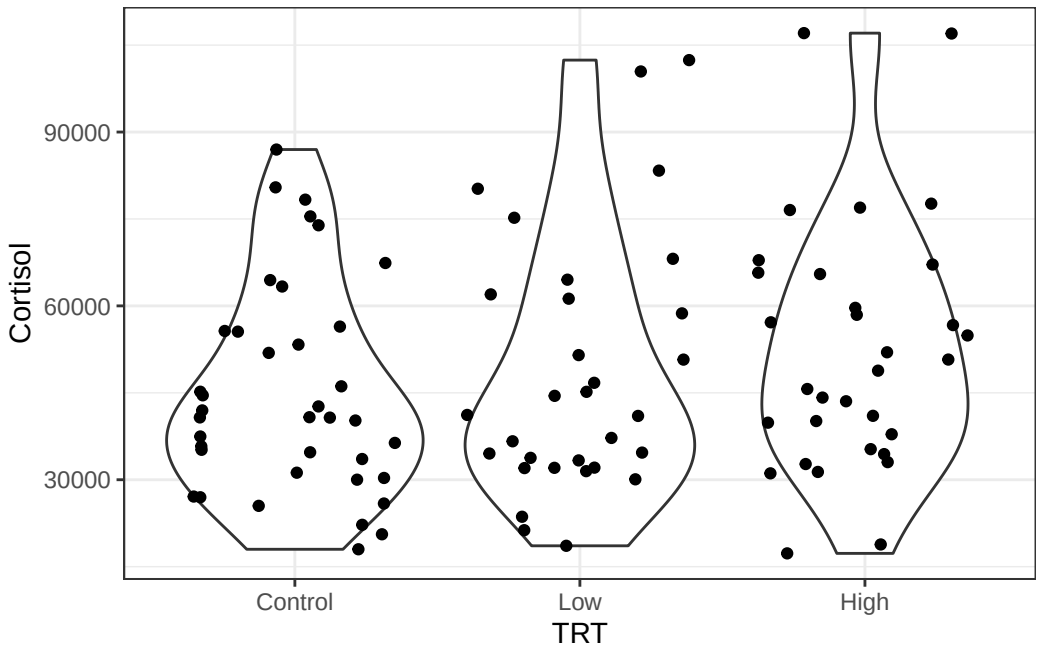
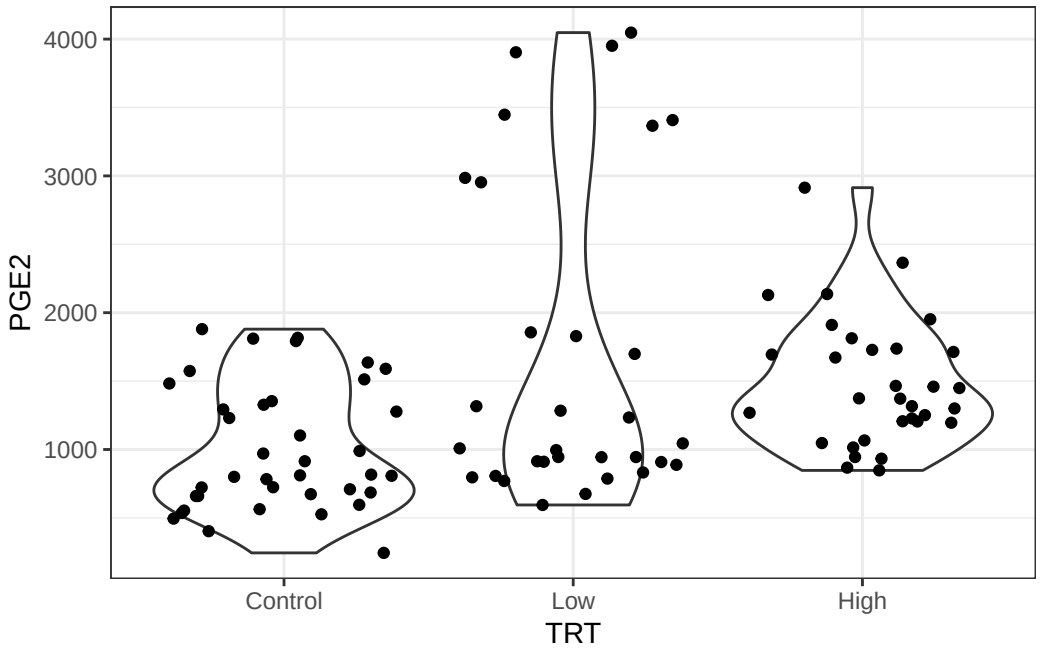
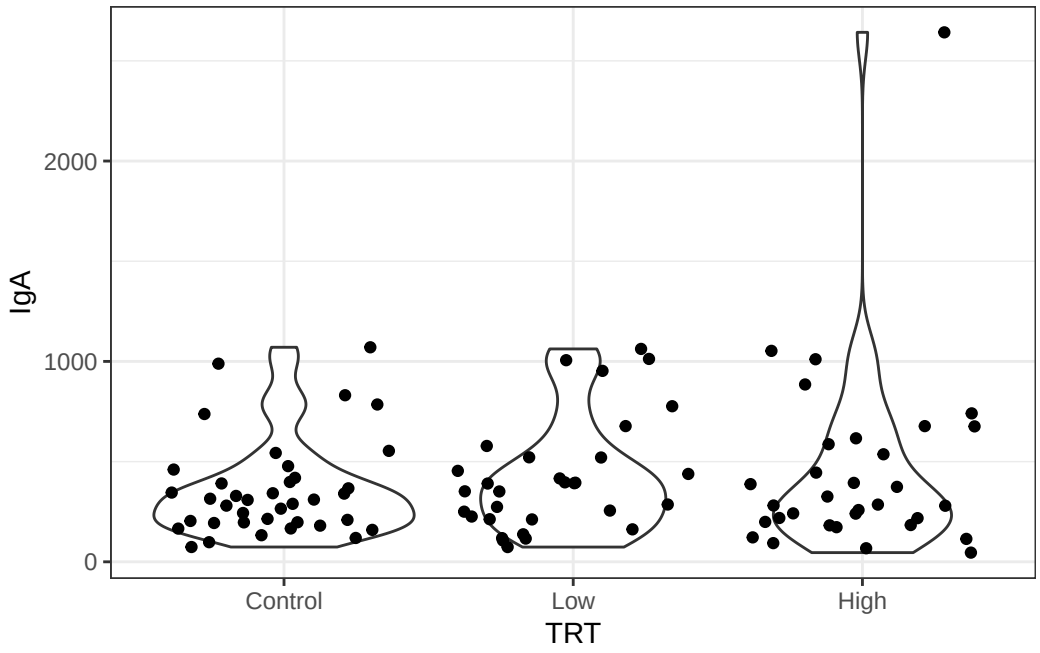
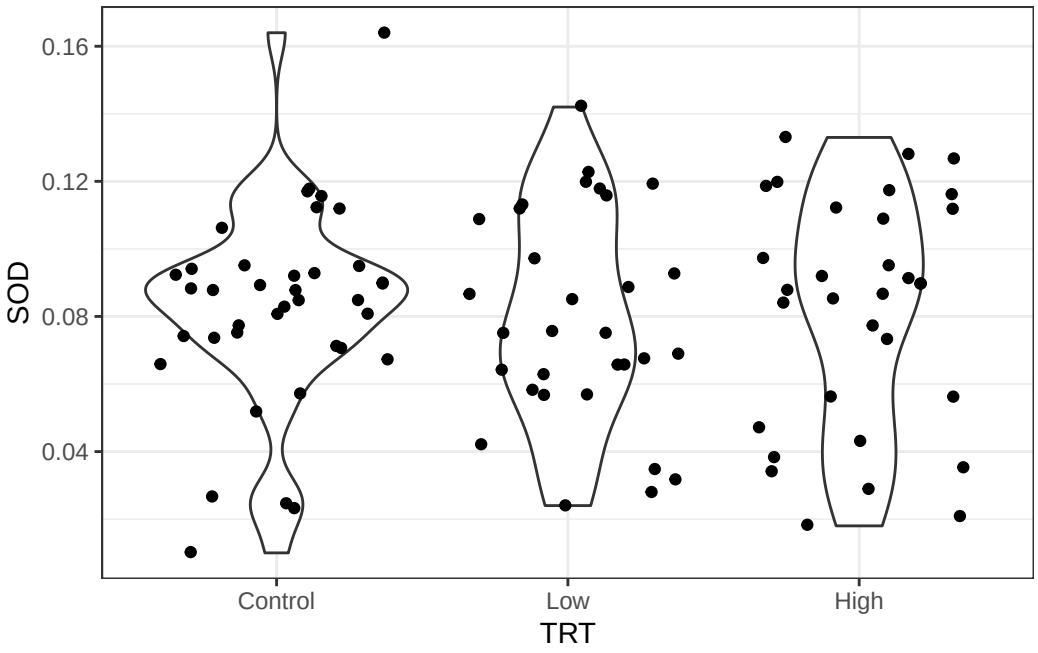
Stat 541 Experimental Design Project 2

Ben DeVries, Eric Folsom, Ryan Kardoes, Alex Trauner




4000
3000
2000
1000
PGE2
Control
Low
High
TRT


2000
1000
0
IgA
Control
Low
High
TRT



Part 1: Experimental Design Plan (9-step checklist)

1. Define the objectives:
    1. The objective of this experiment was to compare how three levels of postbiotic supplementation may attenuate stress, inflammation, and indicators of leaky gut in young horses undergoing an exercise challenge.
2. Identify response and sources of variation.
    1. Treatment factors and levels: One treatment factor (postbiotic supplementation) with 3 levels: 0 mg/kg BW, 46 mg/kg BW, and 92 mg/kg BW.
    2. Response: Multiple response factors measured, including serum cortisol, PGE2, SOD, and fecal IgA. Repeated measures over time surrounding the SET (submaximal exercise test): PRE (before exercise), h0 (immediately post), h1, h6 post-exercise.
    3. Sources of variation: Individual horse, consumption of full amount of supplement, baseline stress/inflammation levels.
    4. Blocking factor: Sex
3. Choose a rule for assigning the experimental units to trt (design).
    1. We would choose a randomized complete block design.

4. Specify measurements to be made, experimental procedure, and the anticipated difficulties.
    1. Measurements:
        1. Serum cortisol, PGE2, SOD (measured by commercially available ELISA kit).
        2. Fecal IgA (measured by commercially available ELISA kit).
    2. Procedure: 30 quarter horse yearlings were be stratified by age, body weight, and sex and assigned to one of three treatment groups: a control, not receiving any postbiotic, a low dose group receiving 46 mg/kg of the postbiotic, and a high dose group receiving 92 mg/kg postbiotic product, which was top-dressed on concentrate fed two times daily. Horses also had ad lib (free-choice) access to water and hay. Horses underwent progressive exercise training for 30 minutes a day, 5 days a week to simulate industry-standard sales preparation protocols. At the end of the study, a submaximal exercise test, or SET, was used to simulate a prolonged intense exercise bout. Horses were balanced by treatment and assigned to one of 4 SET groups which started exercise on 4 consecutive days, with one group per day. Horses arrived at day - 30 to adapt to the new location. At day - 14, baseline samples were collected and at day - 7, basal diet adaptation began. Day 0 marks initiation of experimental treatments via postbiotic supplementation. Sample collection occured at days -14, 0, 11, 45, and pre- and post-SET. Post-SET samples were taken at 0 h post, 1 hour, 6 hours, and 24 hours post exercise. For the purposes of this project, we will focus on samples surrounding the SET. To evaluate stress and inflammation, serum samples will be analyzed by ELISA for cortisol, prostaglandin E2 (PGE2), and superoxide dismutase (SOD). Fecal samples will be analyzed by ELISA for fecal IgA. Altogether, these markers allow for evaluation of inflammatory responses to stress events and gut dysfunction. Samples will be numerically coded and processed randomly to prevent any bias from researchers.
    3. Anticipated difficulties:
        1. Working with animals - Sometimes dangerous to obtain samples, leading to missing observations for some animals. Injuries/lameness can prevent animals from participating in exercise. Animals may not always eat the entirety of their diet, leading to differences in supplement dosing - monitored by collecting and weighing refusals. Individual differences in levels of stress and inflammation in any given animal.
        2. Lab work - Difficulty in troubleshooting and optimizing kits, sample preservation and degradation, human error in lab techniques, environmental conditions in lab.
        3. Equipment - For this particular study, there were issues with the hot walker (how we exercise the horses) that prevented the SET from being as intense as it should have been to illicit a true stress response.
5. Pilot experiment:
    1. For the purposes of this project, cortisol values will be used as a “Pilot Study” to

determine sample size through a power analysis. A true difference of 20,000 pg/mL serum cortisol will be used (found to be significantly different in a similar study in horses). The standard deviation used is from the current cortisol samples as previously analyzed.

Power test for approximate delta of serum cortisol found to be significantly different in a similar study, SD from current cortisol samples. Sample Size: 3

6. Specify the model: $Y_{ijkht} = \mu + \alpha_i + \beta_j + \gamma_k + T_{h(i)} + \epsilon_{ijkht}$

   Where:

   Y_{ijkht} is the response (cortisol, PGE2, SOD, or fecal IgA)

   μ is a constant

   α_i is main effect of treatment (CON, LOW, HIGH postbiotic supplementation)

   β_j is main effect of time

   γ_k is the block effect (sex)

   $T_{h(i)}$ is random effect of horse within treatment

   ϵ_{ijkht} is the error term

7. Outline the analysis:

   1. Results will be analyzed using ANOVA for mixed effects models with repeated measures of time.

8. Calculate number of observations and time/budget.

   1. 30 horses were used for this study (n = 10 per trt).
   2. The study was completed over a span of 69 days in Fall of 2022.

9. Review and revise. Following the pilot study power analysis, the sample size for this study (n = 10/trt) should be more than sufficient to detect a difference if there truly is one.

Part 2: Report

Introduction

A healthy intestinal barrier helps aid in nutrient absorption and immune defense to pathogens in the gut. When this barrier becomes compromised by things such as illness or stress, pathogens can enter the bloodstream via gaps in cell junctions. This condition is most commonly known as "leaky gut". This leaky gut can challenge the immune system and lead to weight loss, decreased performance, and other health complications for horses. Dietary supplementation with postbiotics may be used to support proper gut function and overall health, as well as mitigating stress responses. Postbiotics are beneficial byproducts that are created as a result of probiotic activity. Supplementing directly with postbiotics can support normal intestinal barrier integrity and decrease stress responses. Little is known about the response of young horses to supplementation with postbiotics when faced with exercise stress, therefore the objective of this study was to investigate the use of dietary Saccharomyces cerevisiae fermentation product in mitigating the impacts of training and exercise on intestinal integrity and stress in the young performance horse.

Experimental units and randomization

Thirty Quarter Horse yearlings (374 $\pm$ 25 kg BW; 562 $\pm$ 16 d of age; 15 fillies and 15 geldings) were used for this study. Treatment allocations (n = 10 per treatment) were fixed, while experimental units were assigned random numbers and stratified within treatment groups until optimally balanced groups were created. Horses were stratified by BW, age, and sex and randomly assigned to one of three dietary treatments (n = 10/treatment): CON (0), LOW (46), or HIGH (92 mg/kg BW postbiotic). Blood and fecal samples will be obtained at predetermined time points surrounding the SET and analyzed by ELISA for biological markers indicating stress and inflammation in response to an exercise challenge.

Pilot study and sample size calculations

From a power analysis based on cortisol values with a true difference of 20,000 pg/mL and a standard deviation of 5419 pg/mL, 3 horses per treatment will be required for a power level of 0.90 and an alpha of 0.05. The sample size of n = 10/trt in the present study is sufficient.

Data overview

For our analysis of the effects of postbiotic treatments on prostaglandin E2 (PGE2) levels in quarter horse yearlings, we will control for time and sex while accounting for repeated measures on the horses. Figure 1 displays a panel of plots used to visualize the response against the

predictors, and explore the potential for interactions between our covariates. The violin plots in (A) suggest the treatment may have some effect as the observed PGE2 tends to be higher for horse with a larger postbiotic supplement. The violin plots in (B) appear nearly identical, providing little to no evidence for a sex effect. In plot (C), we explore the potential for changing PGE2 over time. There is no clear discernible trend, but there appears to be slightly less variation in PGE2 prior to applying treatments. This may suggest a potential treatment effect, as PGE2 measurements were more similar, prior to applying treatments. In plot (D), we see PGE2 measurements for geldings were slightly higher on average then fillies in the control and low probiotic treatments; but the reverse is true for the high treatment group. This hints at a potential interaction, but it's quite possible that patterns may be due to random chance. Both plots (E) and (F) show slightly different patterns in PGE2 measurements over time by treatment and sex respectively. These variations are slight and provide little to no evidence of interactions.

Data analysis and results

After fitting our model for prostaglandin E2, we checked modelling assumptions via visual diagnostics. We see a clear violation of the assumption of homoscedasticity in the plot (A) of Figure 2 where the spread of residuals tends to increase with predicted values. The Q-Q plot in (B) indicates a moderately strong violation of our assumption of normally distributed residuals, showing heavy tails. This indicates intervals from the model will be overly wide and power will be below the nominal level. Next we checked our assumptions of linearity with the effects plot in Figure 3. The distributions of partial residuals are all roughly centered at the marginal means, suggesting linearity is met. The partial residuals for Low in (A) show a bimodal distribution. We did not see any interaction that could explain this in our exploratory data analysis, suggesting a potentially unobserved omitted variable. Finally, we checked the assumption of normally distributed random effects with a Q-Q plot displayed in Figure 4. The plot displays a slightly light left tail, and heavy right, indicating a mild violation of normality. Intervals are likely to be slightly wider and power slightly lower than desired.

Even though our model violates multiple assumptions, we proceeded to assess the effect of postbiotic treatments on prostaglandin E2 via a type II ANOVA. Under the null hypothesis, the mean effect of low, high, and no probiotics are the same ($\alpha_1 = \alpha_2 = \alpha_3$); once sex, time, and horse are accounted for. Our alternative hypothesis states at least one of these means differ. The test provides very weak evidence of a treatment effect ($\chi^2_2 = 3.855$, p-value=0.146). Using the Bonferroni correction, we then constructed contrasts for the treatment levels. The Low probiotic treatment had a PEG2 667 pg/mL lower than the control (95% confidence interval for control-low: [-1563, 229]). The high probiotic treatment group had a PGE2 that was 497 pg/mL higher than the control on average (95% confidence interval for control-high [-1405, 411]). Finally, the contrast for low minus high was estimated to be 170 pg/mL (95% confidence interval: [-775, 1115]). These results do not indicate a clear difference in PGE2 among horses based on postbiotic treatment, but a relationship is cerrtainly possible. Addressing modelling

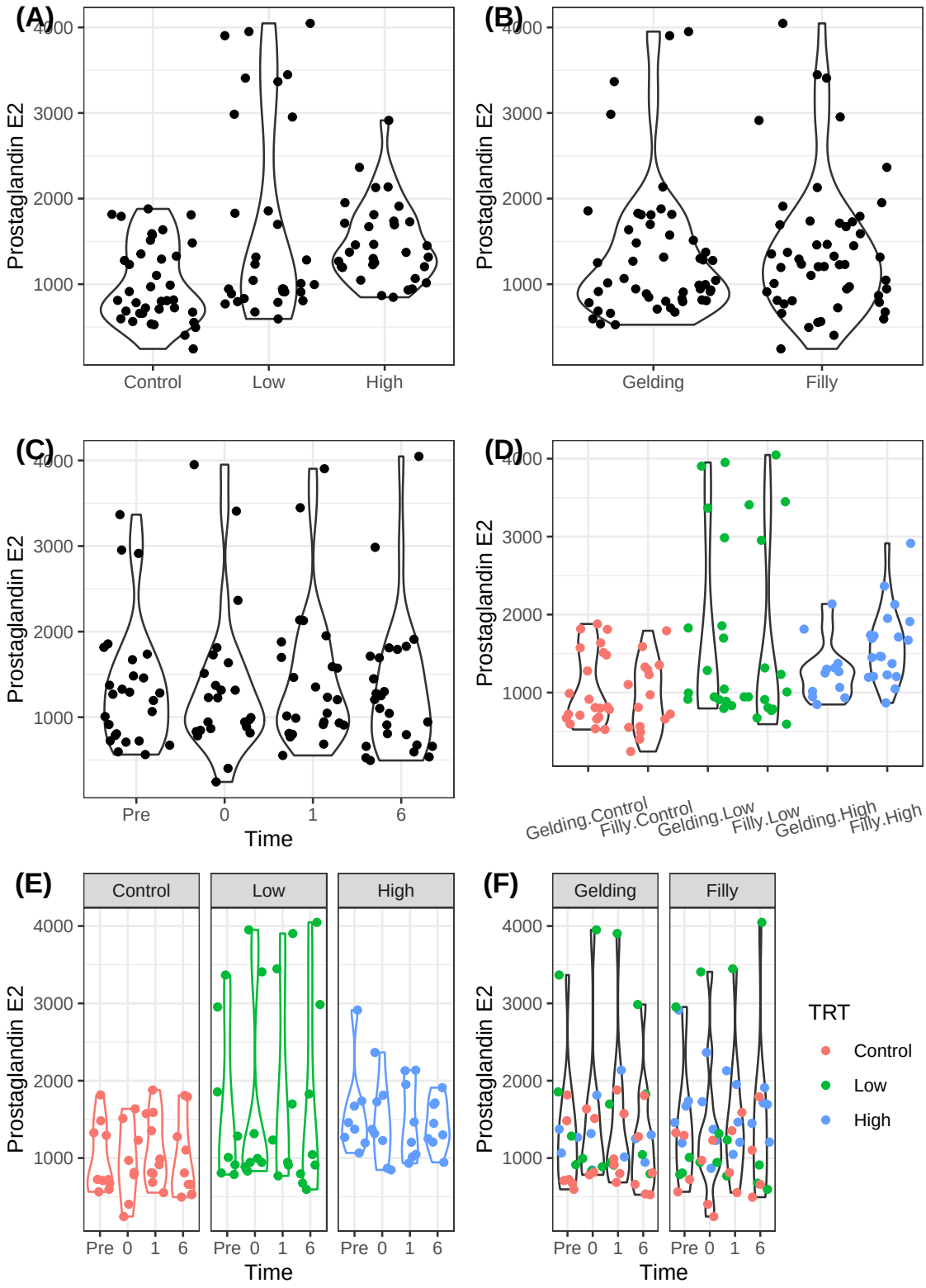
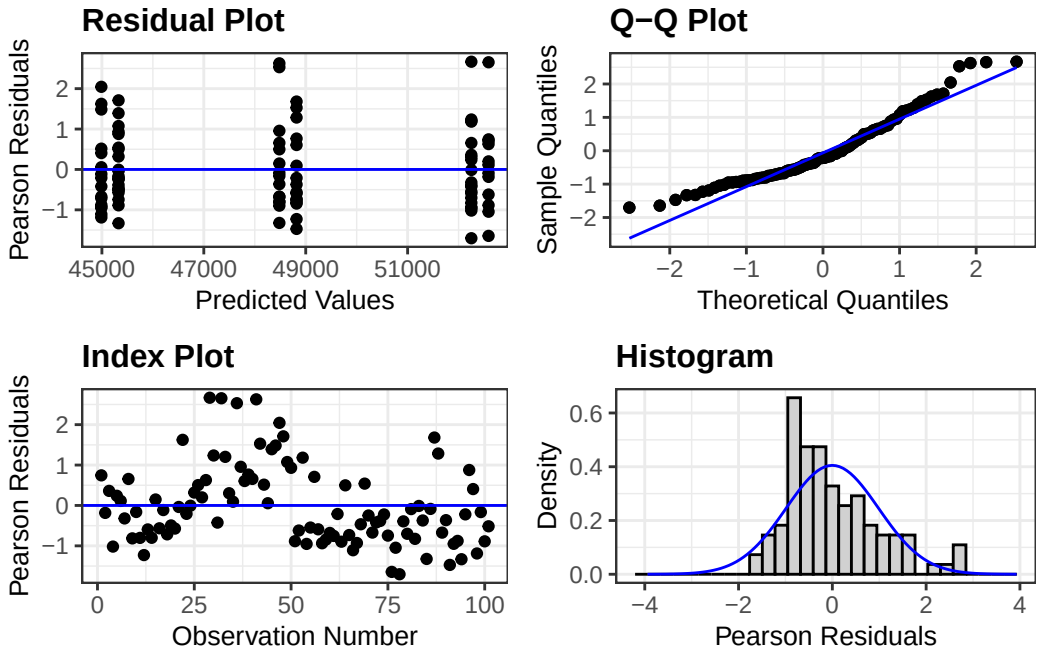

(A)
(B)
(C)
(D)
(E)
(F)
Prostaglandin E2
4000
3000
2000
1000
Control
Low
High
Gelding
Filly
Pre
0
1
6
Time
Gelding.Control
Filly.Control
Gelding.Low
Filly.Low
Gelding.High
Filly.High
TRT
Control
Low
High

Figure 1

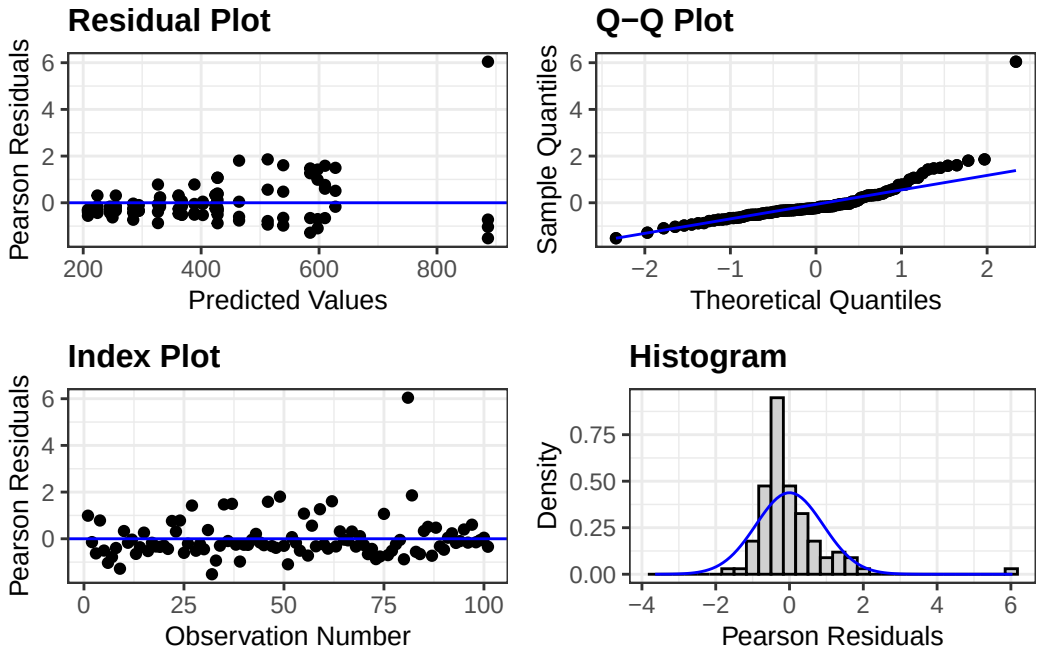
assumptions and increased sample size may provide stronger evidence for a postbiotic effect. A log transform may address these violations.

```
Linear mixed model fit by REML ['lmerMod']
Formula: Cortisol ~ TRT + SEX + TRT:(1 | ID)
   Data: HorseDat

REML criterion at convergence: 2215.4

Scaled residuals:
    Min      1Q  Median      3Q     Max
-1.7028 -0.7326 -0.2115  0.6049  2.6666

Random effects:
 Groups   Name        Variance  Std.Dev.
 ID       (Intercept)         0     0
 Residual             421541501 20531
Number of obs: 101, groups:  ID, 26

Fixed effects:
            Estimate Std. Error t value
(Intercept)  45326.8     3760.7  12.053
TRTLow        3493.6     4975.9   0.702
TRTHigh       7261.9     4998.2   1.453
SEXFilly      -337.8     4147.6  -0.081

Correlation of Fixed Effects:
         (Intr) TRTLow TRTHgh
TRTLow   -0.569
TRTHigh  -0.512  0.455
SEXFilly -0.464 -0.052 -0.169
optimizer (nloptwrap) convergence code: 0 (OK)
boundary (singular) fit: see help('isSingular')
```



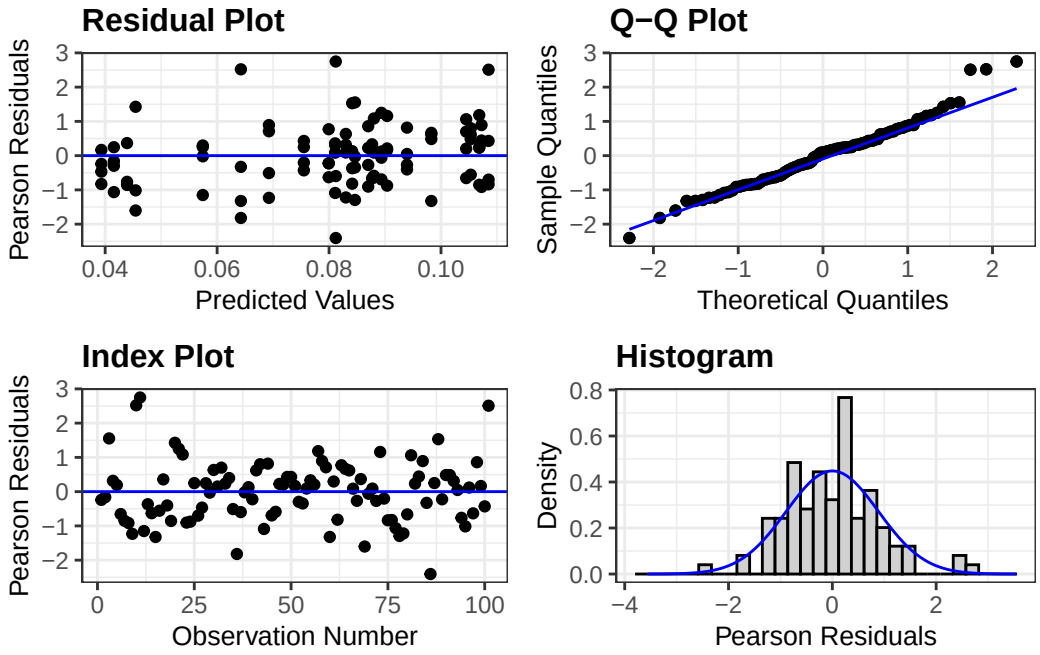

```
Linear mixed model fit by REML ['lmerMod']
Formula: IgA ~ TRT + SEX + TRT:(1 | ID)
   Data: HorseDat

REML criterion at convergence: 1411.9

Scaled residuals:
    Min      1Q  Median      3Q     Max
-1.5189 -0.4484 -0.2277  0.3102  6.0428

Random effects:
 Groups   Name        Variance Std.Dev.
 ID       (Intercept) 38934    197.3
 Residual             84425    290.6
Number of obs: 101, groups:  ID, 26

Fixed effects:
            Estimate Std. Error t value
(Intercept)   399.03      88.01   4.534
TRTLow         78.68     117.50   0.670
TRTHigh       115.82     118.70   0.976
SEXFilly      -96.06      98.29  -0.977
```

```
Correlation of Fixed Effects:
            (Intr) TRTLow TRTHgh
TRTLow      -0.561
TRTHigh     -0.505  0.452
SEXFilly    -0.456 -0.071 -0.179
```




```
Linear mixed model fit by REML ['lmerMod']
Formula: SOD ~ TRT + SEX + TRT:(1 | ID)
   Data: HorseDat

REML criterion at convergence: -413.8

Scaled residuals:
     Min       1Q   Median       3Q      Max
-2.40448 -0.63336  0.08979  0.44126  2.74791

Random effects:
 Groups   Name        Variance  Std.Dev.
 ID       (Intercept) 0.0005384 0.02320
 Residual             0.0004895 0.02213
Number of obs: 101, groups:  ID, 26

Fixed effects:
```

```
             Estimate Std. Error t value
(Intercept)  0.074400   0.009176   8.108
TRTLow      -0.001811   0.012286  -0.147
TRTHigh     -0.001789   0.012433  -0.144
SEXFilly     0.014772   0.010289   1.436

Correlation of Fixed Effects:
         (Intr) TRTLow TRTHgh
TRTLow   -0.558
TRTHigh  -0.503  0.452
SEXFilly -0.454 -0.077 -0.182
```




```
Linear mixed model fit by REML ['lmerMod']
Formula: PGE2 ~ TRT + SEX + TIME + TRT:(1 | ID)
   Data: HorseDat

REML criterion at convergence: 1427

Scaled residuals:
     Min       1Q   Median       3Q      Max
-1.89508 -0.49953 -0.00625  0.32188  2.73689

Random effects:
```

```
 Groups   Name        Variance Std.Dev.
 ID       (Intercept) 539325   734.4
 Residual              83379   288.8
Number of obs: 101, groups:  ID, 26

Fixed effects:
            Estimate Std. Error t value
(Intercept)   985.20     265.60   3.709
TRTLow        667.18     356.60   1.871
TRTHigh       497.07     361.48   1.375
SEXFilly        7.00     298.96   0.023
TIME.L        -11.91      56.86  -0.209
TIME.Q        -17.49      57.75  -0.303
TIME.C        -97.67      58.70  -1.664

Correlation of Fixed Effects:
         (Intr) TRTLow TRTHgh SEXFll TIME.L TIME.Q
TRTLow   -0.556
TRTHigh  -0.501  0.451
SEXFilly -0.451 -0.082 -0.185
TIME.L   -0.005  0.004  0.002  0.003
TIME.Q   -0.008  0.002  0.005  0.002  0.006
TIME.C    0.014 -0.011 -0.006 -0.009 -0.024 -0.018


Analysis of Deviance Table (Type II Wald chisquare tests)

Response: PGE2
      Chisq Df Pr(>Chisq)
TRT  3.8548  2     0.1455
SEX  0.0005  1     0.9813
TIME 2.9404  3     0.4009


 contrast       estimate  SE df t.ratio p.value
 Control - Low      -667 357 22  -1.871  0.1708
 Control - High     -497 361 22  -1.375  0.3707
 Low - High          170 376 22   0.452  0.8940

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 3 estimates


 contrast       estimate  SE df lower.CL upper.CL
```

```
 Control - Low      -667 357 22    -1563      229
 Control - High     -497 361 22    -1405      411
 Low - High          170 376 22     -775     1115

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
Confidence level used: 0.95
Conf-level adjustment: tukey method for comparing a family of 3 estimates
```

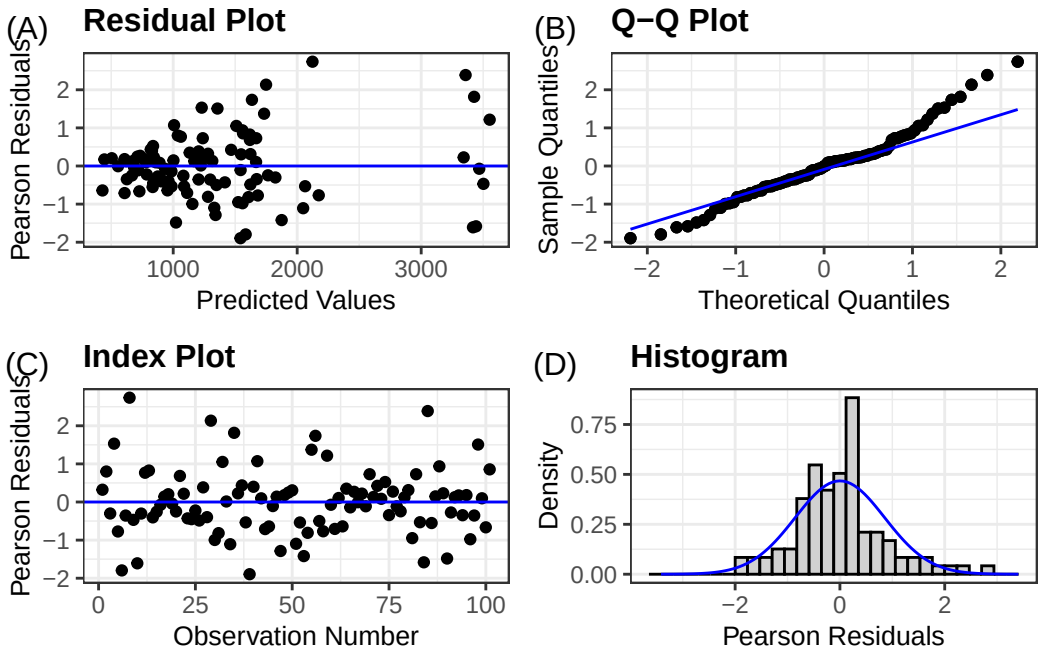


Figure 2: Panel of residual diagnostics for prostaglandin E2 model

```
Linear mixed model fit by REML ['lmerMod']
Formula: log(PGE2) ~ TRT + SEX + TIME + TRT:(1 | ID)
   Data: HorseDat

REML criterion at convergence: 63.3

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.6259 -0.5493  0.1305  0.5639  1.7126

Random effects:
 Groups   Name        Variance Std.Dev.
```

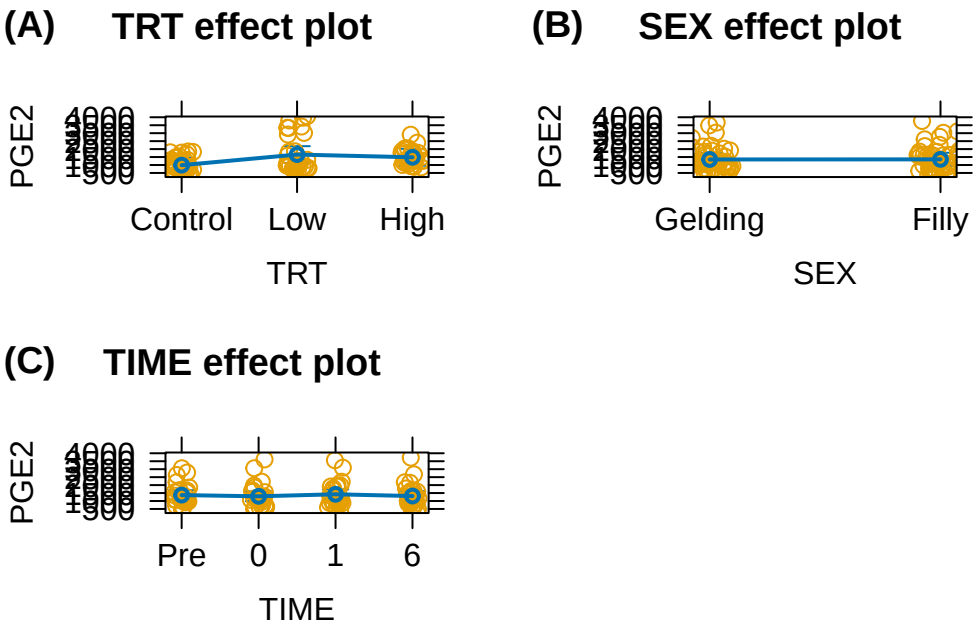


Figure 3: Panel of effects plot for prostaglandin E2 model

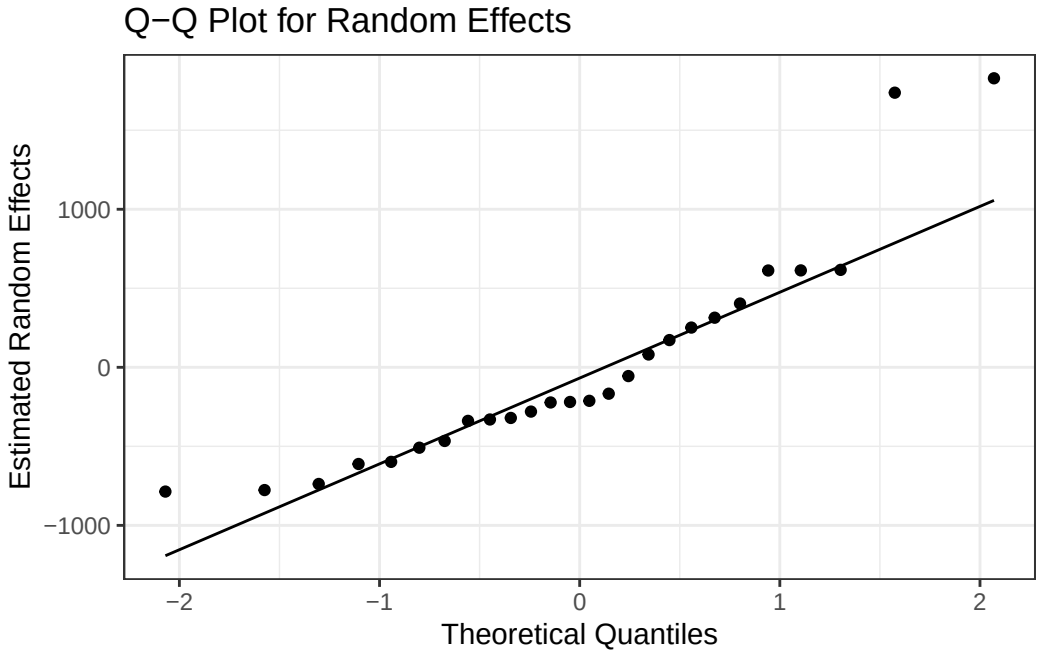


Figure 4: Quantile-Quantile plot of random intercepts for horse nested within treatment

```
 ID       (Intercept) 0.21397  0.4626
 Residual             0.04465  0.2113
Number of obs: 101, groups:  ID, 26

Fixed effects:
             Estimate Std. Error t value
(Intercept)  6.799448   0.168476  40.359
TRTLow       0.429077   0.226139   1.897
TRTHigh      0.477243   0.229197   2.082
SEXFilly    -0.028029   0.189568  -0.148
TIME.L      -0.017095   0.041613  -0.411
TIME.Q      -0.001518   0.042259  -0.036
TIME.C      -0.110626   0.042956  -2.575

Correlation of Fixed Effects:
         (Intr) TRTLow TRTHgh SEXFll TIME.L TIME.Q
TRTLow   -0.556
TRTHigh  -0.502  0.451
SEXFilly -0.452 -0.082 -0.185
TIME.L   -0.006  0.004  0.002  0.004
TIME.Q   -0.009  0.002  0.006  0.002  0.006
TIME.C    0.016 -0.013 -0.007 -0.010 -0.024 -0.018


Analysis of Deviance Table (Type II Wald chisquare tests)

Response: log(PGE2)
      Chisq Df Pr(>Chisq)
TRT  5.4885  2    0.06430 .
SEX  0.0219  1    0.88245
TIME 6.8619  3    0.07643 .
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


 contrast       estimate    SE df t.ratio p.value
 Control - Low   -0.4291 0.226 22  -1.897  0.1631
 Control - High  -0.4772 0.229 22  -2.082  0.1168
 Low - High      -0.0482 0.239 22  -0.202  0.9778

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
Results are given on the log (not the response) scale.
P value adjustment: tukey method for comparing a family of 3 estimates
```

```
 contrast       estimate    SE df lower.CL upper.CL
 Control - Low   -0.4291 0.226 22   -0.997   0.1390
 Control - High  -0.4772 0.229 22   -1.053   0.0986
 Low - High      -0.0482 0.239 22   -0.648   0.5512

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
Results are given on the log (not the response) scale.
Confidence level used: 0.95
Conf-level adjustment: tukey method for comparing a family of 3 estimates
```

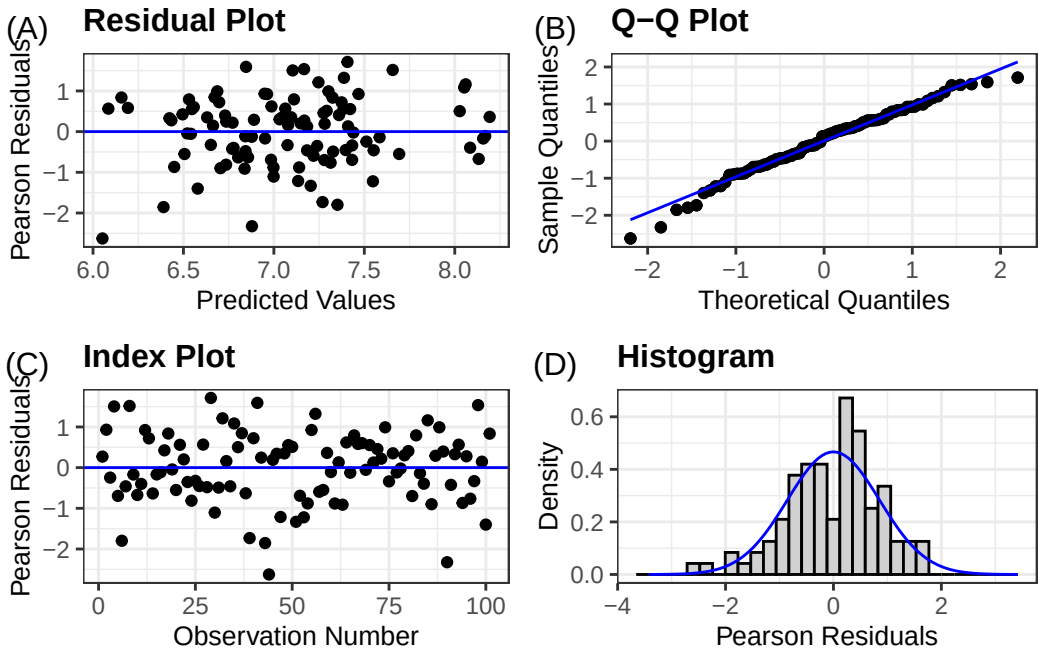


Figure 5: Panel of residual diagnostics for prostaglandin E2 model (Log Transformed)

```
Linear mixed model fit by REML ['lmerMod']
Formula: PGE2 ~ TRT + SEX + TIME + (1 | ID)
   Data: HorseDat

REML criterion at convergence: 1427

Scaled residuals:
     Min       1Q   Median       3Q      Max
-1.89508 -0.49953 -0.00625  0.32188  2.73689
```

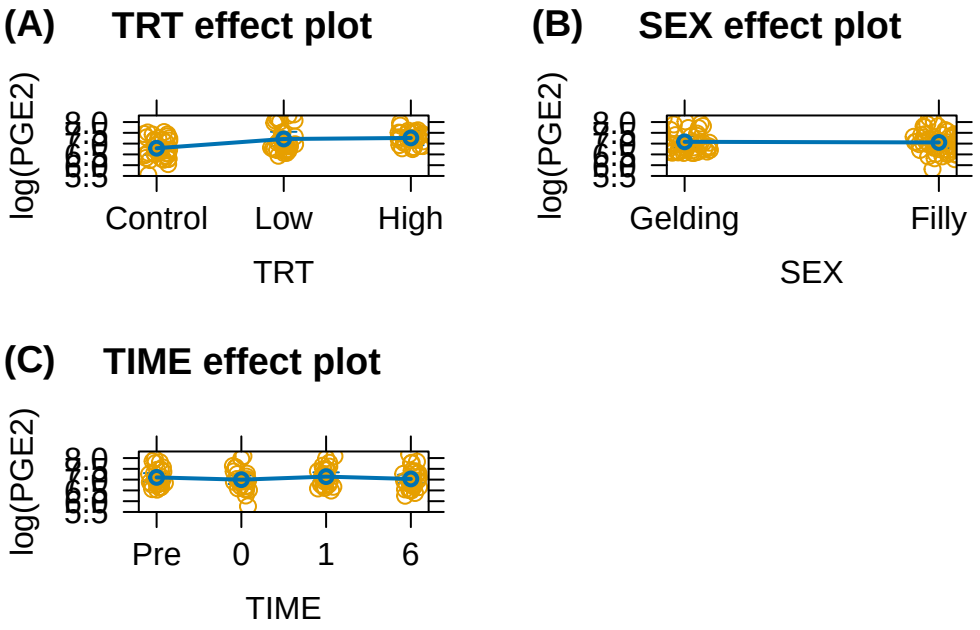


Figure 6: Panel of effects plot for prostaglandin E2 model (Log Transformed)

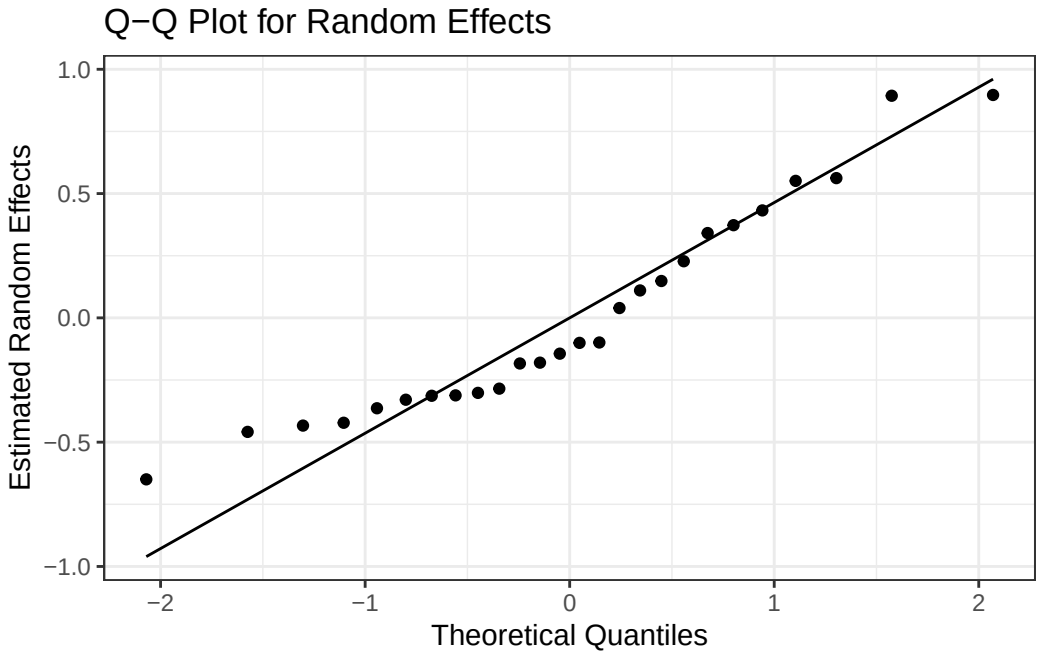


Figure 7: Quantile-Quantile plot of random intercepts for horse nested within treatment (Log Transformed)

```
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TIME.Q        -17.49      57.75  -0.303
TIME.C        -97.67      58.70  -1.664

Correlation of Fixed Effects:
         (Intr) TRTLow TRTHgh SEXFll TIME.L TIME.Q
TRTLow   -0.556
TRTHigh  -0.501  0.451
SEXFilly -0.451 -0.082 -0.185
TIME.L   -0.005  0.004  0.002  0.003
TIME.Q   -0.008  0.002  0.005  0.002  0.006
TIME.C    0.014 -0.011 -0.006 -0.009 -0.024 -0.018


Analysis of Deviance Table (Type II Wald chisquare tests)

Response: PGE2
      Chisq Df Pr(>Chisq)
TRT  3.8548  2     0.1455
SEX  0.0005  1     0.9813
TIME 2.9404  3     0.4009


 contrast       estimate  SE df t.ratio p.value
 Control - Low      -667 357 22  -1.871  0.1708
 Control - High     -497 361 22  -1.375  0.3707
 Low - High          170 376 22   0.452  0.8940

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 3 estimates
```

```
 contrast       estimate  SE df lower.CL upper.CL
 Control - Low      -667 357 22    -1563      229
 Control - High     -497 361 22    -1405      411
 Low - High          170 376 22     -775     1115

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
Confidence level used: 0.95
Conf-level adjustment: tukey method for comparing a family of 3 estimates
```

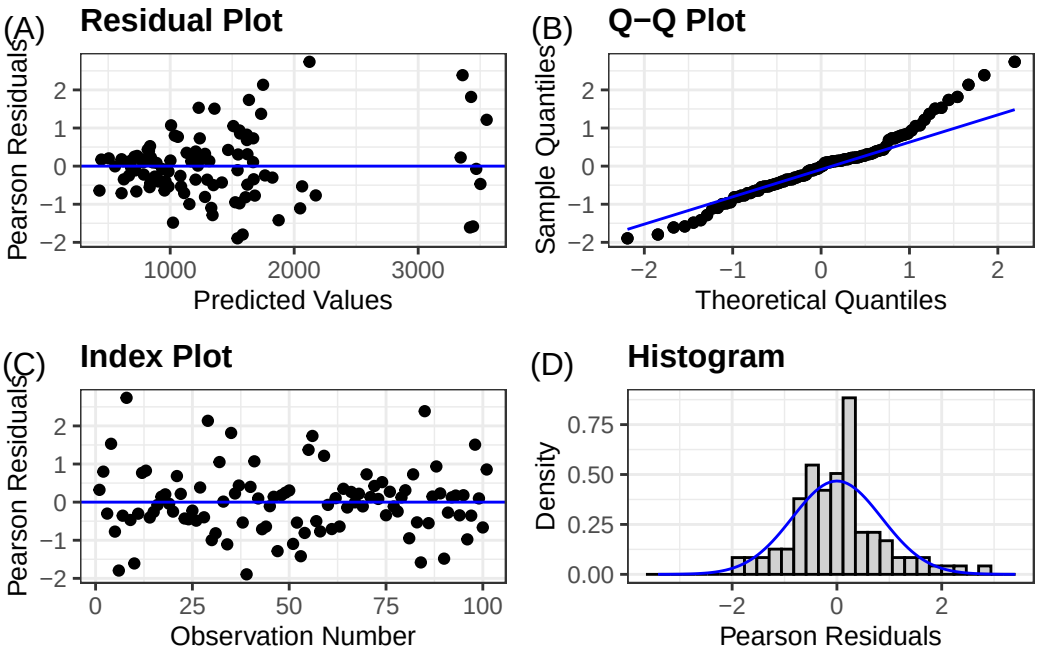

Figure 8: Panel of residual diagnostics for prostaglandin E2 model (ID not nested but random effect)

```
Linear mixed model fit by REML ['lmerMod']
Formula: log(PGE2) ~ TRT + SEX + TIME + (1 | ID)
   Data: HorseDat

REML criterion at convergence: 63.3

Scaled residuals:
    Min      1Q  Median      3Q     Max
-2.6259 -0.5493  0.1305  0.5639  1.7126
```

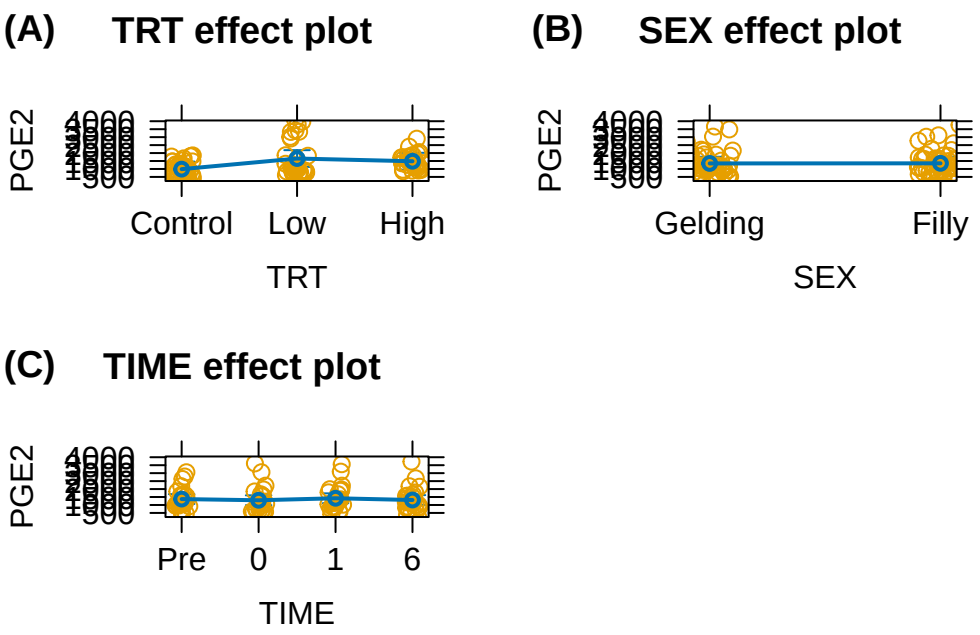

Figure 9: Panel of effects plot for prostaglandin E2 model (ID not nested but random effect)

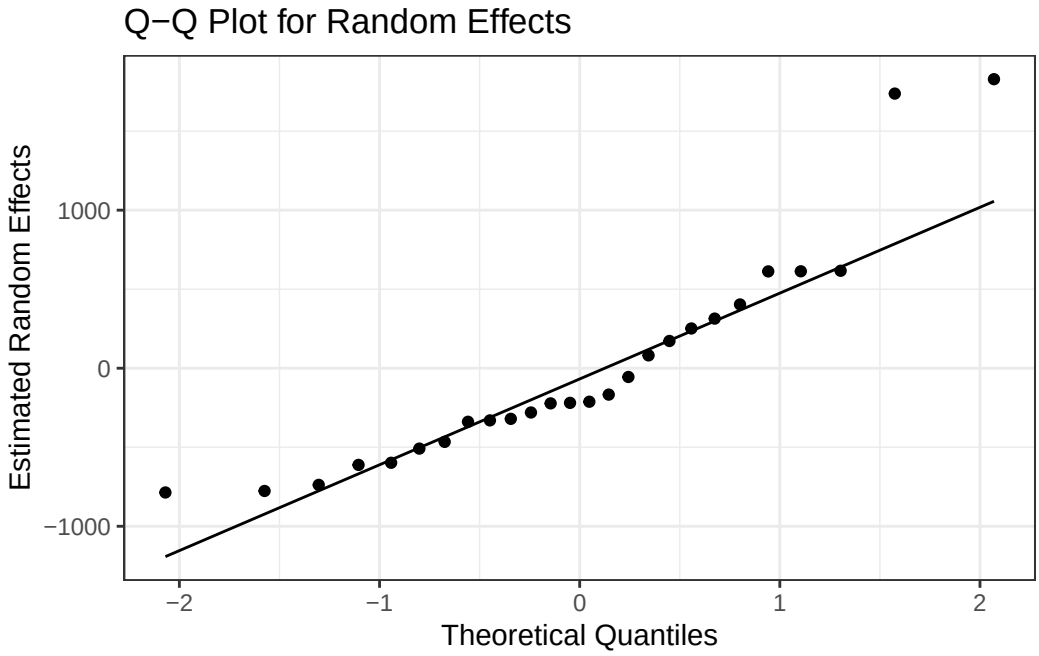

Figure 10: Quantile-Quantile plot of random intercepts for horse nested within treatment (ID not nested but random effect)

```
Random effects:
 Groups   Name        Variance Std.Dev.
 ID       (Intercept) 0.21397  0.4626
 Residual             0.04465  0.2113
Number of obs: 101, groups:  ID, 26

Fixed effects:
             Estimate Std. Error t value
(Intercept)  6.799448   0.168476  40.359
TRTLow       0.429077   0.226139   1.897
TRTHigh      0.477243   0.229197   2.082
SEXFilly    -0.028029   0.189568  -0.148
TIME.L      -0.017095   0.041613  -0.411
TIME.Q      -0.001518   0.042259  -0.036
TIME.C      -0.110626   0.042956  -2.575

Correlation of Fixed Effects:
         (Intr) TRTLow TRTHgh SEXFll TIME.L TIME.Q
TRTLow   -0.556
TRTHigh  -0.502  0.451
SEXFilly -0.452 -0.082 -0.185
TIME.L   -0.006  0.004  0.002  0.004
TIME.Q   -0.009  0.002  0.006  0.002  0.006
TIME.C    0.016 -0.013 -0.007 -0.010 -0.024 -0.018


Analysis of Deviance Table (Type II Wald chisquare tests)

Response: log(PGE2)
      Chisq Df Pr(>Chisq)
TRT  5.4885  2    0.06430 .
SEX  0.0219  1    0.88245
TIME 6.8619  3    0.07643 .
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


 contrast       estimate    SE df t.ratio p.value
 Control - Low   -0.4291 0.226 22  -1.897  0.1631
 Control - High  -0.4772 0.229 22  -2.082  0.1168
 Low - High      -0.0482 0.239 22  -0.202  0.9778

Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
```

```
Results are given on the log (not the response) scale.
P value adjustment: tukey method for comparing a family of 3 estimates
```

```
 contrast       estimate    SE df lower.CL upper.CL
 Control - Low   -0.4291 0.226 22   -0.997   0.1390
 Control - High  -0.4772 0.229 22   -1.053   0.0986
 Low - High      -0.0482 0.239 22   -0.648   0.5512
```

```
Results are averaged over the levels of: SEX, TIME
Degrees-of-freedom method: kenward-roger
Results are given on the log (not the response) scale.
Confidence level used: 0.95
Conf-level adjustment: tukey method for comparing a family of 3 estimates
```

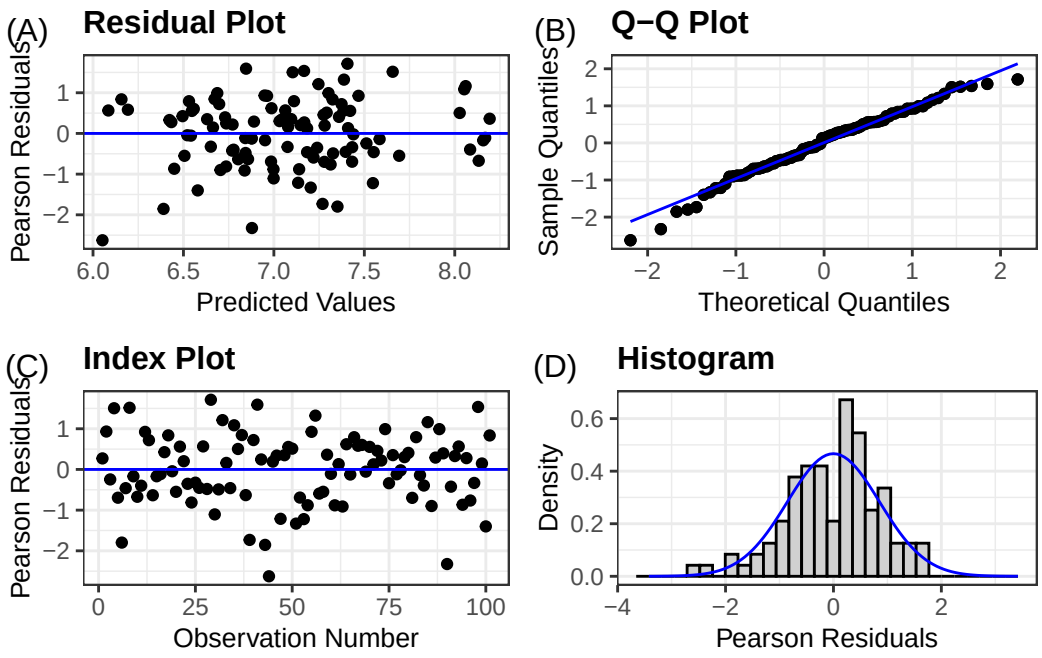

Figure 11: Panel of residual diagnostics for prostaglandin E2 model (Log Transformed) (ID not nested but random effect)

```
Call:
lm(formula = PGE2 ~ TRT + SEX + TIME, data = HorseDat)

Residuals:
```

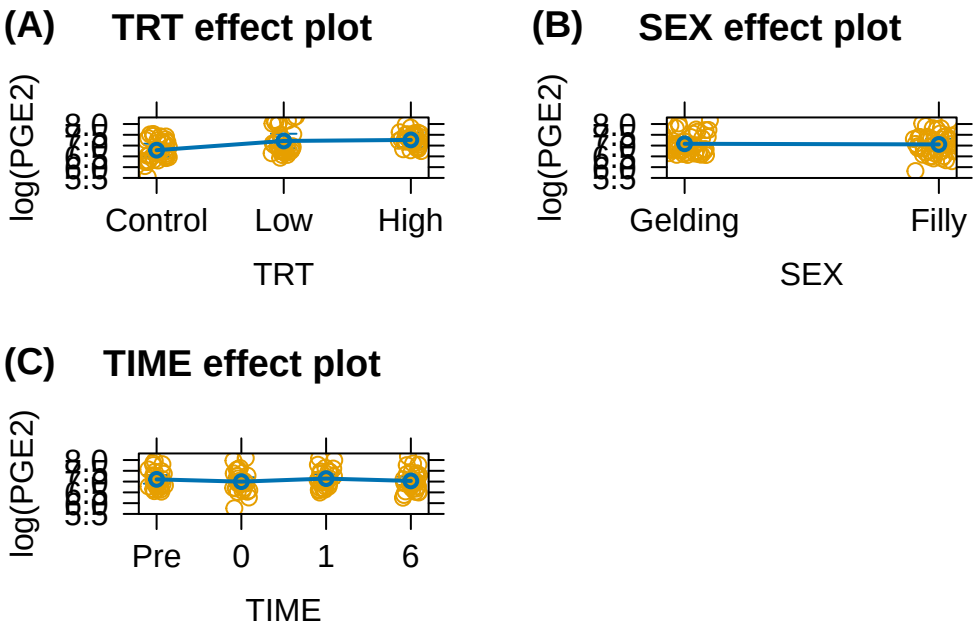

Figure 12: Panel of effects plot for prostaglandin E2 model (Log Transformed) (ID not nested but random effect)

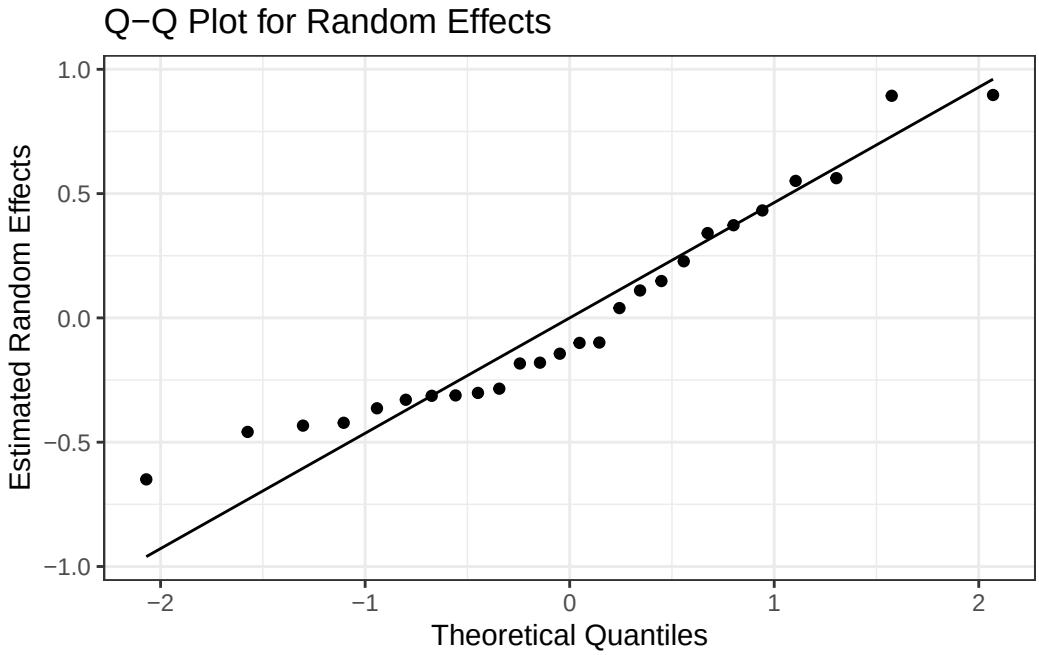

Figure 13: Quantile-Quantile plot of random intercepts for horse nested within treatment (Log Transformed) (ID not nested but random effect)

```
    Min      1Q  Median      3Q     Max
-1040.1  -532.9  -175.7   307.8  2412.9

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  1001.04     139.58   7.172 1.67e-10 ***
TRTLow        674.75     184.48   3.658  0.00042 ***
TRTHigh       478.01     185.18   2.581  0.01139 *
SEXFilly       12.16     153.71   0.079  0.93713
TIME.L        -10.91     149.60  -0.073  0.94199
TIME.Q        -47.26     151.43  -0.312  0.75568
TIME.C       -100.66     153.48  -0.656  0.51353
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 760.3 on 94 degrees of freedom
Multiple R-squared:  0.1377,    Adjusted R-squared:  0.08263
F-statistic: 2.501 on 6 and 94 DF,  p-value: 0.02734


Anova Table (Type II tests)

Response: PGE2
            Sum Sq Df F value   Pr(>F)
TRT        8344273  2  7.2175 0.001213 **
SEX           3616  1  0.0063 0.937127
TIME        312581  3  0.1802 0.909561
Residuals 54337145 94
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


 contrast       estimate  SE df t.ratio p.value
 Control - Low      -675 184 94  -3.658  0.0012
 Control - High     -478 185 94  -2.581  0.0303
 Low - High          197 193 94   1.020  0.5661

Results are averaged over the levels of: SEX, TIME
P value adjustment: tukey method for comparing a family of 3 estimates


 contrast       estimate  SE df lower.CL upper.CL
 Control - Low      -675 184 94    -1114     -235
 Control - High     -478 185 94     -919      -37
 Low - High          197 193 94     -263      656
```

```
Results are averaged over the levels of: SEX, TIME
Confidence level used: 0.95
Conf-level adjustment: tukey method for comparing a family of 3 estimates
```

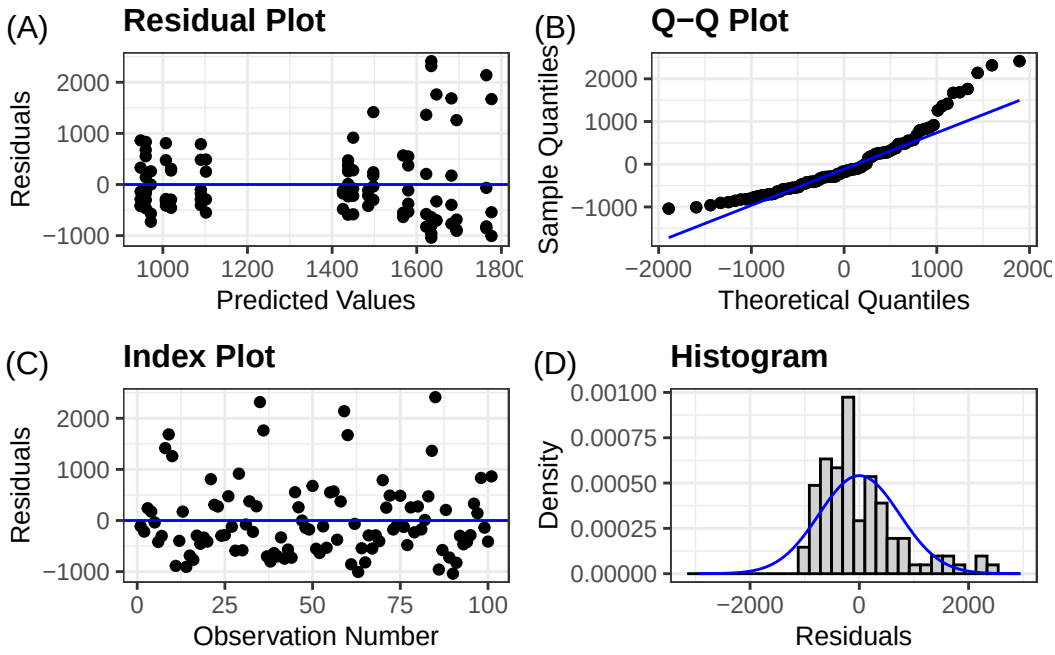

Figure 14: Panel of residual diagnostics for prostaglandin E2 model (No ID)

```
Call:
lm(formula = log(PGE2) ~ TRT + SEX + TIME, data = HorseDat)

Residuals:
     Min       1Q   Median       3Q      Max
-1.23710 -0.36614 -0.07823  0.33739  1.13965

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  6.81795    0.08990  75.839  < 2e-16 ***
TRTLow       0.42646    0.11882   3.589 0.000529 ***
TRTHigh      0.45985    0.11927   3.856 0.000211 ***
SEXFilly    -0.02981    0.09900  -0.301 0.763975
TIME.L      -0.01909    0.09635  -0.198 0.843386
TIME.Q      -0.02443    0.09753  -0.250 0.802777
```

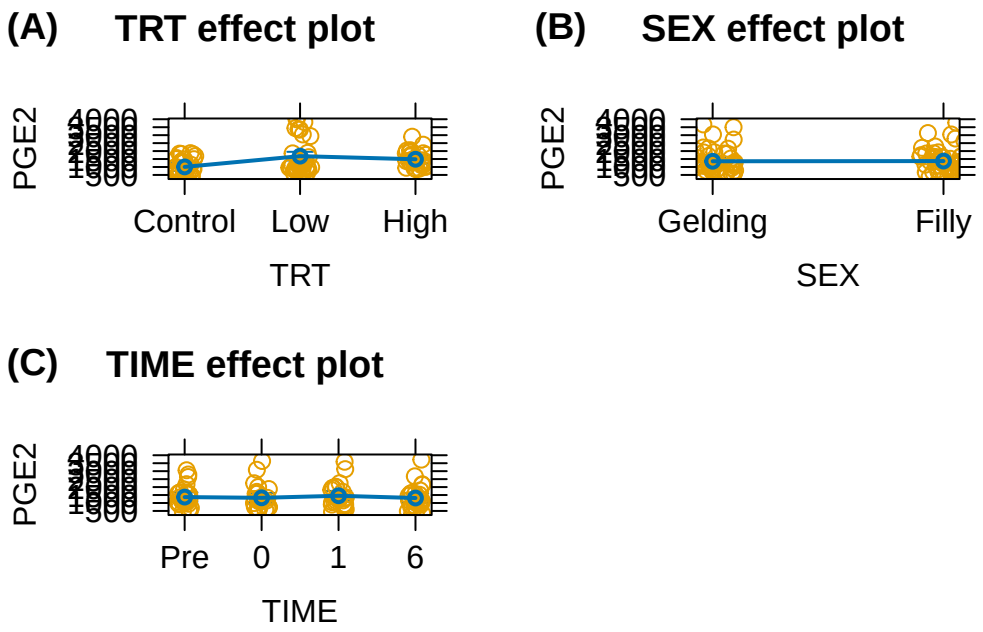

Figure 15: Panel of effects plot for prostaglandin E2 model (No ID)

```
TIME.C      -0.10464    0.09885  -1.059 0.292497
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.4897 on 94 degrees of freedom
Multiple R-squared:  0.1765,    Adjusted R-squared:  0.124
F-statistic: 3.359 on 6 and 94 DF,  p-value: 0.004822


Anova Table (Type II tests)

Response: log(PGE2)
          Sum Sq Df F value    Pr(>F)
TRT       4.5812  2  9.5523 0.0001673 ***
SEX       0.0217  1  0.0907 0.7639748
TIME      0.2970  3  0.4129 0.7441289
Residuals 22.5408 94
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


 contrast       estimate    SE df t.ratio p.value
 Control - Low   -0.4265 0.119 94  -3.589  0.0015
 Control - High  -0.4599 0.119 94  -3.856  0.0006
```

```
 Low - High      -0.0334 0.124 94  -0.269  0.9610

Results are averaged over the levels of: SEX, TIME
Results are given on the log (not the response) scale.
P value adjustment: tukey method for comparing a family of 3 estimates

 contrast       estimate    SE df lower.CL upper.CL
 Control - Low   -0.4265 0.119 94   -0.709   -0.144
 Control - High  -0.4599 0.119 94   -0.744   -0.176
 Low - High      -0.0334 0.124 94   -0.329    0.262

Results are averaged over the levels of: SEX, TIME
Results are given on the log (not the response) scale.
Confidence level used: 0.95
Conf-level adjustment: tukey method for comparing a family of 3 estimates
```

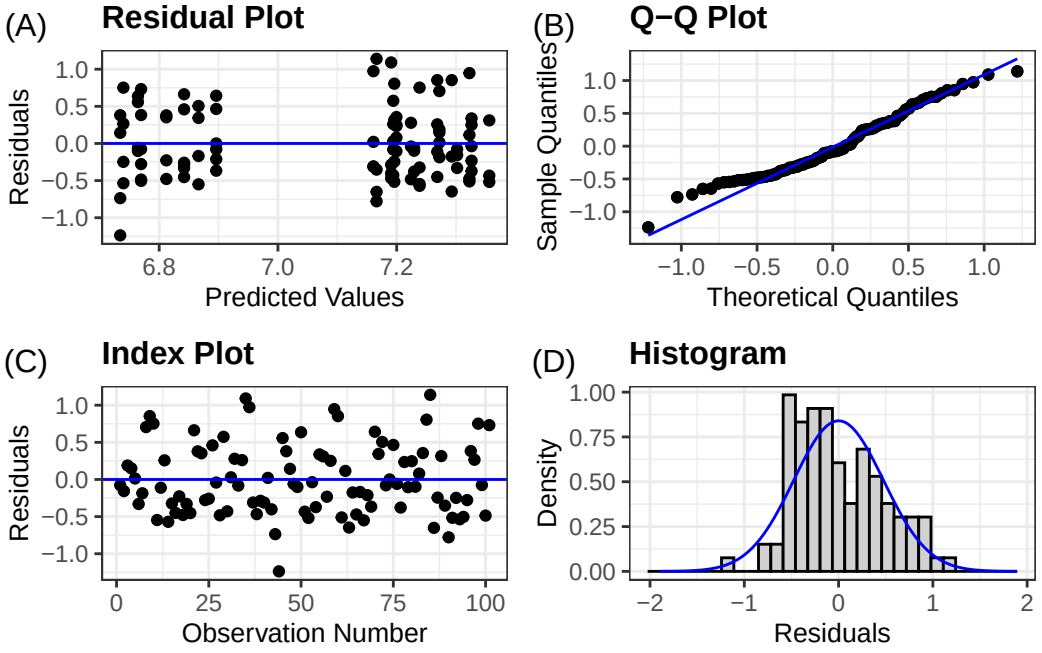

Figure 16: Panel of residual diagnostics for prostaglandin E2 model (Log Transformed) (No ID)

Discussion

Results suggest that there is very weak evidence for a true difference in PGE2 levels (a marker of inflammation) due to postbiotic supplementation (dietary treatment) in yearling

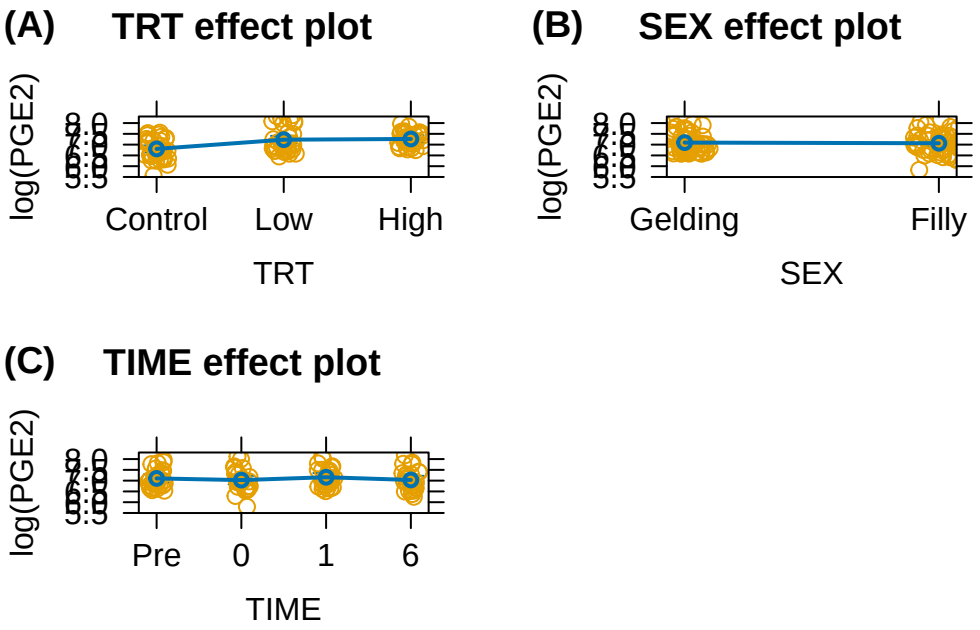

Figure 17: Panel of effects plot for prostaglandin E2 model (Log Transformed) (No ID)

quarter horses faced with an exercise challenge.

In terms of experimental design and analysis, a number of changes may have improved this study. During this study, equipment difficulties caused the hot walker to be unusable at certain speeds. Due to this, the SET was not as intense as originally planned in order to avoid injury to the horses due to uneven/suboptimal footing. As a result, the SET was likely not a true "exercise stressor" and did not induce the desired level of stress to be able to parse out differences in stress responses by dietary treatments.

As PGE2 differed by treatment group at the PRE timepoint, it may be appropriate to use baseline PGE2 values (PRE values) as a covariate to normalize starting inflammation levels between groups, as individual animal levels of stress/inflammation may influence the analysis of these markers.