Effects of American Fruit Extracts on Antioxidant-Related Genes in Cultured Cells & on Modulation of Metabolomic Profile in Poultry

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Table of Contents

Abstract

With the rising threat of antibiotic resistant infections as a risk to human health, multiple countries, including Canada, are making efforts to reduce the use of antibiotics in livestock. Cranberry and blueberry extracts have shown potential as antibiotic alternatives in broiler production due to their polyphenolic compounds which act as antioxidants and immune system modulators. To investigate potential methods of action of the extracts during gram positive infections, in-vitro assays were conducted using chicken hepatic cell line LMH. Cell viability was significantly increased after 24 hours in the presence of lipoteichoic acid (LTA) at the concentrations of 0.1, 1, 10, 50, 100μg/mL or a combination of LTA and peptidoglycan in a 1:1 ratio (0.1μg/mL or 1μg/mL). Quercetin is found in both cranberries and blueberries and is considered one of their main flavonoid components. Thus, the effect of cranberry and blueberry extracts was investigated with quercetin. The effects of quercetin at concentrations of 1.5 or 30.2 μg/mL in the absence or presence of LTA (0.1 μg/mL) did not affect cell viability, suggesting that quercetin was able to negate the proliferative effect LTA had on the LMH cell line. Expression of five genes related to cellular antioxidant status (NRF2, Gclc, GPX2, Txn, and HO-1) was not affected by the presence of LTA and fruit extracts. In the second part of this thesis, cecal samples from an in vivo study were used for metabolomic analysis using nuclear magnetic resonance (NMR). The broiler chickens came from ten treatment groups: bacitracin methylene disalicylate (BMD), wild cranberry pomace (CB) (0.5% and 1%), wild blueberry pomace (BB) (0.5% and 1%) alone or in combination with a mixture of digestive enzymes (7 pens/treatment, 45 chicks/pen) over a 5-week experimental period. Cecal samples were collected at day 21 (D21) and 35 (D35). The chosen metabolites were acetate, butyrate, propionate, glutamate, valerate, ethanol, and glucose (combined glucose & glucose-6-phosphate due to signal overlaps). Before

removal of outliers no significant differences were observed in any of the metabolites among treatments. Removal of outliers did not change statistical results for most comparisons. However, the following metabolites showed statistically different results among treatment groups: acetate (D21), ethanol (D21), and glucose (D35) but none of the three showed significant differences from the control (antibiotic) group. In addition, a time effect was observed for several metabolites. Acetate, butyrate, propionate, and valerate were all significantly higher at D35 than at D21. In contrast, ethanol levels were higher at D21 than those at D35. Overall, the addition of fruit pomaces and/or an enzyme mixture showed no significant effects on the chicken gut metabolites from the standard antibiotic treatment. Our results suggest that cranberry and blueberry extracts could be used for broiler production.

Résumé

Avec la menace croissante d'infections résistantes aux antibiotiques comme un risque pour la santé humaine, nombreux pays, incluant le Canada, font un effort de réduire l'usage des antibiotiques chez le bétail et la volaille. Les extraits de canneberge et bluet ont manifesté le potentiel comme des alternatives à l'usage des antibiotiques dans les poules, à cause de leur composés polyphénoliques qui s'agissant comme les antioxydants et modulateurs du système immunitaire. Pour enquêter les méthodes d'action potentielle des extraits durant les infections causées par les bactéries gram-positives, les essais in-vitro ont été fait avec lignée de cellules hépatiques « LMH. » La viabilité cellulaire s'est accrue de façon significative après 24 heures dans la présence de l'acide lipotéichoïque (LTA) aux concentrations de 0.1, 1, 10, 50, 100µg/ml ou avec une combinaison du LTA et peptidoglycan dans un 1:1 ratio (0.1µg/ml ou 1µg/ml). La quercétine se trouve dans les deux canneberges et bluets et c'est considéré l'une des composants flavonoïdes principals. Donc, l'effet des extraits a été examiné en utilisant la quercétine. La présence de la quercétine aux concentrations du 1.5 ou 30.2 µg/mL dans la présence ou absence du 0.1µg/mL LTA n'avait pas effet sur la viabilité cellulaire, suggérant que la quercétine a pu annuler l'effet prolifératif que LTA avoir sur la lignée de cellules hépatiques « LMH. » L'expression de cinq gènes liés au statut antioxydant (NRF2, Gclc, GPX2, Txn et HO-1) n'a pas été affectée par la présence du LTA et les extraits de fruit. Pour la deuxième partie de cette thèse, des échantillons du caecum d'une étude in-vivo ont été utilisé pour l'analyse métabolique utilisant la résonance magnétique nucléaire (RMN). Les poules de chair font partir de dix groupes: du bacitracine méthylène disalicylate (BMD), le marc de canneberge sauvage (CB) (0.5 % et 1 %), le marc de bluet sauvage (BB) (0.5 % et 1 %) seul ou en combination d'un mélange des enzymes digestifs (7 enclos/traitement, 45 poussins/enclos) sur la durée de cinq semaines.

Les échantillons du caecum ont été collectées au jour 21 (D21) et 35 (D35). Les métabolites choisent ont été l'acétate, le butyrate, le propionate, le glutamate, le valérate, l'éthanol et le glucose (un combinaison de glucose et glucose-6-phosphate à cause des chevauchements de signal). Avant l'élimination des valeurs aberrantes il n'y avait pas de différence significatives dans n'importe quels métabolites dans les traitements. L'élimination des valeurs aberrantes n'avait pas changé les résultats statistiques pour la majorité des comparaisons. Cependant, les métabolites suivants ont montré des différences significativement différents : acétate (D21), éthanol (D21), et glucose (D35); mais aucun des trois ont montré des différences significativement du groupe d'intervention. De plus, un effet du temps a été observer dans plusieurs métabolites. L'acétate, le butyrate, le propionate, et le valerate ont été sensiblement plus élevés durant D35 que D21. En revanche, le niveau d'éthanol a été plus haut durant D21 que D35. Globalement, l'addition des marcs de fruit et/ou un mélange des enzymes digestifs n'ont pas montré des effets significatifs sur les métabolites des poules de chair comparés au traitement standard avec l'antibiotique. Nos résultats suggèrent que les extraits de la canneberge et bluet peuvent être utiliser pour la production des poules.

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Foremost, I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Xin Zhao, for providing me the opportunity to study with his lab, all his time spent helping me work on my writing, and input and ideas on my experiments. Also, I would like to thank my lab-mates for their assistance and insight during my studies. Particularly I want to thank Mengmeng Yu for helping me to learn about cell culture and cell culture experiments, and Muhmmed Bilal for helping me with just about everything else in the lab. I would also like to thank Dr. Wei Si and Dr. Andree Gravel for their assistance and guidance for the metabolomics portion of my thesis. Finally, I would like to thank my friends and family for their endless patience and for keeping me motivated and sane through these years. I would not have been able to complete my education without their support.

Preface and Contribution of Authors

This thesis is the final work of my Master study in Animal Science at McGill University. It serves as documentation of my research during time of study, which has been from January 2019 to December 2021. It presents the results of my research into the effects of phenolic-rich cranberry and blueberry extracts on chicken hepatic cells, and the effects of the addition of these extracts on the cecal metabolic profiles of broiler chickens.

My contribution to this work is as follows: and I wrote the entire manuscript under the guidance of my supervisor, Dr. Xin Zhao. I designed experiments, executed them, and performed statistical analysis for the in-vitro portion. I prepared raw fecal samples for NMR processing, which was run by Dr. Andree Gravel at the Drug Discovery Platform, Research Institute of the McGill University Health Center, after which I prepared and analyzed the spectral data.

List of Figures

4.5.2

Figure 6: Effect of LTA and three concentrations of blueberry or cranberry pomace on expression of NRF2 in LMH cells. Results are shown as the result of three experiments with the means and SEM calculated.

Figure 7: Effect of LTA and three concentrations of blueberry or cranberry pomace on expression of Gclc in LMH cells. Results are shown as the result of three experiments with the means and SEM calculated.

Figure 8: Effect of LTA and three concentrations of blueberry or cranberry pomace on expression of Txn in LMH cells. Results are shown as the result of three experiments with the means and SEM calculated.

Figure 9: Effect of LTA and three concentrations of blueberry or cranberry pomace on expression of HO-1 in LMH cells. Results are shown as the result of three experiments with the means and SEM calculated.

5.5.1

Figure 10: Sample showing acetate at approximately 1.9ppm with integral value of 13.1821, and TSP at 0.0ppm with integral value of 1.0000.

Table 4: Summary table of relative concentrations of acetate during the two sampling points, before removal of outliers.

Figure 11: Relative concentrations of acetate at day 21. The chart shows data visualized from Table 4. Relative levels of acetate were determined by comparison to an internal standard of a known concentration. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Figure 12: Relative concentrations of acetate at day 35. The chart shows data visualized from Table 4. Relative levels of acetate were determined by comparison to an internal standard of a

known concentration.

Table 5: Relative concentration of acetate after removal of outliers.

Figure 13: Relative concentrations of acetate at day 21, after removal of 8 outlier points. The chart shows data visualized from Table 5. Relative levels of acetate were determined by comparison to an internal standard of a known concentration. Groups that share letters indicate no significant differences. After removal of the initial outliers, two new mild outliers were formed in the CB-1.0 group. Mild outliers are marked by a °.

5.5.2

Table 6: Cluster centers of metabolites of interest identified by Chenomx. Bold values indicate chosen clusters of focus during fitting process.

Figure 14: The fitting pattern of butyrate (b) and valerate (a).

Figure 15: Fitting of butyrate (b), valerate (a), propionate (c).

Figure 16: Fitted acetate (d) with concentration 202.7540mM, contrasted with butyrate (b) concentration 44.1769mM; concentrations are determined by the total area under the curve of all peaks a compound consists of.

Figure 17: This doublet is shared by glucose-6-phosate and glucose; but not by the other named glucose compounds in the table. The blue line represents glucose while the green line represents glucose-6-phosphate. The red line is the sum of the two doublets. Both compounds have been matched to under their maximum, by looking in the other regions with signal and making sure they do not go above the spectrum line in their sum fit. The spectrum line is the produced signal: because in this case it is so much higher than the sum line, this means that signal is being produced by other compounds. Thus the fitted sum line can only serve as an educated estimate; in reality it may be lower or higher.

Figure 18: Estimated marginal means of acetate for two time points.

Table 7: Levels of acetate during two time points, before removal of outliers.

Figure 19: Concentrations of acetate, in mM, at day 21. The chart shows data visualized from Table 7. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Figure 20: Concentrations of acetate, in mM, at day 35. The chart shows data visualized from Table 7.

Table 8: Acetate concentrations after the removal of outliers.

Figure 21: Concentrations of acetate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 8. Groups that share letters indicate no significant differences. After removal of the initial outliers, one new outlier was formed in the CB-1.0 group. Mild outliers are marked by a °.

Figure 22: Estimated marginal means of butyrate for two time points.

Table 9: Concentrations of butyrate for two time points.

Figure 23: Concentrations of butyrate, in mM, at day 21. This chart shows data visualized from Table 9. Mild outliers are marked by a °.

Figure 24: Concentrations of butyrate, in mM, at day 35. This chart shows data visualized from Table 9. Extreme outliers are marked by a *.

Table 10: Butyrate concentrations for two time points after the removal of outliers.

Figure 25: Concentrations of butyrate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 10. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Figure 26: Concentrations of butyrate, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 10.

Figure 27: Estimated marginal means of ethanol for two time points.

Table 11: Concentrations of ethanol during two time points.

Figure 28: Concentrations of ethanol, in mM, at day 21. This chart shows data visualized from Table 11. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Figure 29: Concentrations of ethanol, in mM, at day 35. This chart shows data visualized from Table 11. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Table 12: Concentrations of ethanol for two time points, after removal of outliers.

Figure 30: Concentrations of ethanol, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 12. Groups that share letters indicate no significant differences.

Figure 31: Concentrations of ethanol, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 12.

Figure 32: Estimated marginal means of glutamate for two time points.

Table 13: Concentrations of glutamate for two time points.

Figure 33: Concentrations of glutamate, in mM, at day 21. This chart shows data visualized from Table 13. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Figure 34: Concentrations of glutamate, in mM, at day 35. This chart shows data visualized from Table 13. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Table 14: Concentrations of glutamate after removal of outliers.

Figure 35: Concentrations of glutamate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 14. After removal of initial outliers, one new outlier was formed in the CB-0.5 group. Mild outliers are marked by a °.

Figure 36: Concentrations of glutamate, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 14.

Figure 37: Estimated marginal means of propionate for two time points.

Table 15: Concentrations of propionate for two time points.

Figure 38: Concentrations of propionate, in mM, at day 21. This chart shows data visualized from Table 15. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Figure 39: Concentrations of propionate, in mM, at day 35. This chart shows data visualized from Table 15. Mild outliers are marked by a °.

Table 16: Concentrations of propionate for two time points, after removal of outliers.

Figure 40: Concentrations of propionate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 16. After removal of initial outliers, three new outliers formed: one in group CB-1.0, and two in group BB-0.5-Enz. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Figure 41: Concentrations of propionate, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 16. After removal of initial outliers, two new outliers formed in the CB-1.0-Enz group. Mild outliers are marked by a °.

Figure 42: Estimated marginal means of valerate for two time points.

Table 17: Concentration of valerate for two time points.

Figure 43: Concentrations of valerate, in mM, at day 21. This chart shows data visualized from Table 17. Mild outliers are marked by a °.

Figure 44: Concentrations of valerate, in mM, at day 35. This chart shows data visualized from Table 17. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Table 18: Concentration of valerate at two time points, with outliers removed.

Figure 45: Concentrations of valerate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 18. After removal of initial outliers, two new ones formed; one in group CB-1.0, and one in group BB-0.5. Extreme outliers are marked by a *.

Figure 46: Concentrations of valerate, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 18.

Figure 47: Estimated marginal means of glucose for two time points.

Table 19: Concentrations of glucose at two time points.

Figure 48: Concentrations of glucose, in mM, at day 21. This chart shows data visualized from Table 19. Mild outliers are marked by a °.

Figure 49: Concentrations of glucose, in mM, at day 35. This chart shows data visualized from Table 19. Mild outliers are marked by a °, while extreme outliers are marked by a *.

Table 20: Concentrations of glucose at two time points, after removal of outliers.

Figure 50: Concentrations of glucose, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 20. After removal of initial outliers, two new outliers formed in the Ab group. Extreme outliers are marked by a *.

Figure 51: Concentrations of glucose, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 20. Groups which share a letter have no significant differences.

Table 21: Summary showing the time effect differences observed in certain metabolites as birds mature.

List of Abbreviations

LTA– Lipoteichoic Acid

NE – Necrotic Enteritis

NMR – Nuclear Magnetic Resonance

LPS – Lipopolysaccharide

WTA – Wall Teichoic Acid

Nrf-2 – Nuclear Factor Erythroid 2-Related Factor 2

HO-1 – Heme Oxygenase 1

GPx – Glutathione Peroxidase

MOA – Mode of Action

ROS – Reactive Oxygen Species

RNS – Reactive Nitrogen Species

RSS – Reactive Sulfer Species

NO – Nitric Oxide

iNOS – Inducible Nitric Oxide Synthase

HSHF – High Sugar and High Fat

SOD – Superoxide Dismutase

UTI – Urinary Tract Infections

CP – Cranberry Pomace

SCFA – Short Chain Fatty Acid

GAE – Gallic Acid Equivalent

BP – Blueberry Pomace

NDM – Non-Dialyzable Material

LDH – Lactate Dehydrogenase

Gclc – Glutamate Cysteine Ligase Catalytic Subunit

Txn – Thioredoxin

MS – Mass Spectrometry

PCA – Principal Component Analysis

BMD – bacitracin methylene disalicylate (BMD),

CRP – Wild Cranberry Pomace

BLP – Wild Blueberry Pomace

TSP – Sodium 3-Trimethylsilyl (2,2,3,3-d4) propionate

1. Introduction

In response to the growing threat of antibiotic resistant bacterial strains on human health, many countries, including Canada, have begun to enforce stricter regulations in an attempt to reduce antibiotic use in livestock. Prophylactic use of class I and class II antibiotics has been entirely banned in Canada, a practice which many livestock farmers relied on to maintain flock health. Without this tool, many farmers fear loss of productivity or more disease-ridden flocks. To this day, it is frequently cited that global economic loss due to necrotic enteritis (NE) in broilers is over $2 billion annually (Van der Sluis, 2000). However, this estimate is now over 20 years old and the number could be higher. Over two decades the global production of chicken meat has more than doubled, from approximately 37 million tonnes in 1998 to almost 100 million tonnes in 2016, with demand steadily increasing each year (Roenigk, 1999; Mottet & Tempio, 2017). Evenly scaled, this translates to a global loss of around $6 billion per year, however with many countries making efforts to reduce antibiotic use in livestock, the true cost of NE may be even higher. It has also been estimated that subclinical NE, afflicting an estimated 20% of flocks in the United States, causes loss of about $878.13-1480.52USD per flock (Skinner et al., 2010). When the European Union banned the use of prophylactic antibiotics in livestock, there was a sharp increase in cases of NE in poultry (Van Immerseel et al., 2009), thus it should be expected that as Canada moves towards reducing antibiotic use in poultry agriculture a similar pattern will follow. Both farmers and researchers are now exploring alternative methods to ensure animal health with minimal use of antibiotics. These methods include increasing biosecurity, quality of animal care, and novel products such as probiotics, prebiotics, or essential plant oils, in an attempt to minimize disease occurrence. In order to protect animal and human health as well as productivity and profitability of farms it is vital to create products which can

serve as antibiotic replacements. NE is a disease with high prevalence on broiler farms, even with the present use of antibiotics. Thus, the transition to rearing practices free of prophylactic antibiotic use presents a modern challenge for researchers and farmers.

The causative agent of necrotic enteritis is the bacterium *Clostridium perfringens*, a normal occupant of the chicken digestive tract. *C. perfringens* is a gram-positive, spore-forming anaerobe which has proven difficult to study, as the conditions required for disease induction are not fully understood. *C. perfringens* can be characterized into toxino-types A-G according to their toxin production (García et al., 2019). These types are characterized by levels of production of common toxins, and a strain can be categorized into the type which is the best fit. Of these toxins, alpha-toxin and NetB toxin are considered significant, but not vital, to disease-induction. These toxins are produced by type A *C. perfringens* strains and are responsible for the majority of necrotic enteritis in poultry. However, strains genetically positive for both toxins have been found in healthy broilers, suggesting that the presence of pathogenic bacteria alone is not enough to induce disease (Mwangi et al., 2019). The complex nature of *C. perfringens* and its pathogenesis alongside its prevalence on farms has led many farmers to rely on prophylactic antibiotic use to prevent losses.

Traditionally, antibiotics have been used on a regular basis to prevent necrotic enteritis. Nowadays, with considerable pressure to decrease the use of antibiotics, these medications are no longer a sustainable strategy to control NE and alternative measures are being studied. Among them, there have been several studies conducted on using fruit (including cranberries and blueberries) pomace for poultry production. Studies have found fruit pomace to have reduced early mortality in chickens, and in in-vitro studies their concentrated extracts have demonstrated inhibitory or destructive properties towards pathogenic bacteria (Islam et al., 2016; Lacombe et

al., 2010; Leusink et al., 2010). These benefits have been attributed to polyphenols, a family of metabolites produced by plants. There exist thousands of polyphenol molecules, many with multiple forms and structures. Cranberries have consistently high levels of polyphenols, particularly anthocyanins, which are large molecules best known for producing dark pigment in fruit with strong antioxidant properties (Govers et al., 2017). As natural products begin to make their way into mainstream markets, especially as they begin to replace antibiotics, there is need to be able to ensure the quality of these products.

Recent studies have shown that the addition of fruit pomace to the diets of broiler chickens may offer benefits to the health of the animals. However, the exact mechanisms by which they function are not yet fully understood. As fruit pomace is a by-product of the juice industry, this offers a potential cost-efficient replacement for preventative antibiotic use on farms. The most popular hypothesis is that the beneficial effects of the extracts are due to their high antioxidant content, helping to reduce cellular oxidative stress and thus perhaps helping to protect animals from stress caused by various factors present in the barn environment, such as bacteria, parasites, or air quality. By using in vitro cell culture and in vivo measurement of metabolites after pomace supplementation, we attempt to discern more specifically how fruit extracts act on a line of chicken hepatic cells.

1.1 Overview of the Research

In order to continue to expand our knowledge regarding the unique effects cranberry and blueberry extracts have on poultry, we performed in-vitro assays by using the gram-positive cell wall component LTA as an infection agent and extracts or the polyphenol quercetin as treatments first in culture chicken hepatic cells. Next, we determined eight significant metabolites in cecal contents through Nuclear Magnetic Resonance (NMR) assays. These cecal samples were from a

feeding trial supplemented with one of two concentrations of either fruit extract, in the presence or absence of a mixture of supplemental digestive enzymes theorized to increase bioavailability of the berry compounds in the poultry digestive system.

2. Literature Review

2.1 Antibiotic Use in Livestock

Shortly after their initial discovery and use in humans, antibiotics were introduced to livestock systems. Since then, livestock have become the largest consumer of antibiotics worldwide; in the United States it has been estimated that 80% of all antibiotics are used by livestock (Van Boeckel et al., 2015). Antibiotics are used to treat disease when it occurs, prophylactically prevent common infections, or be used as growth promoters. In Canada, antibiotics have been classified into four groups based on their importance in human medicine, with Class I drugs being the most critical for human medicine and not used for livestock treatment. Class II and III drugs are of high and medium importance and currently being phased out of unregulated use in livestock. Class IV drugs are those which are not used in humans. The European Union began active phasing out of unnecessary antibiotic use in livestock in 2006. In Canada, restriction of medically important antibiotics to prescription only came into effect at the end of 2018. The use of antibiotics for growth promotion has been banned entirely. As far as chickens are concerned, it is questionable if an old estimate of a 5-6% increase in body weight is even relevant in a modern barn with superior genetics, nutrition, and husbandry (Diarra & Malouin, 2014). When used as growth promoters, antibiotics were often used at very low doses. This constant exposure to sub-lethal levels of antibiotics may create an environment which promotes creation and spread of antibiotic resistance genes.

The threat of antibiotic resistant bacteria to human and animal health is not a new one. However, in many countries, especially in developing countries, there has been little to no regulation of how antibiotics are used in agriculture. The effects of antibiotic use on the resistome- the collection of all antibiotic resistance genes in an environment- remain poorly

understood. Many antibiotics have originated from bacterial or fungal sources, originally employed by microorganisms as natural defence mechanisms. As natural consequence of evolution, the ability to confer resistance to these compounds followed, as microorganisms struggled to survive. Diverse antibiotic resistomes have been found in natural areas all over the world, and long pre-date any human use (Brown & Wright, 2016). It remains difficult to discern whether, for example, a soil resistance profile has elevated resistance levels due to anthropogenic sources or due to the naturally present resistome. Connections have been established between amounts of antibiotic consumption in a region and related antibiotic resistance gene levels found in livestock animals (Van Boeckel et al., 2014). On poultry farms, antibiotic resistance genes have been found to persist for quite some time even after withdrawal of antibiotics. A study of Canadian broiler farms found that removing antibiotic use for 15 months was not able to lower overall levels of antibiotic resistance genes in broiler ceca. However, 6 years of responsible antibiotic use- that is only using antibiotics to treat sick birds- was able to reduce levels of antibiotic resistance in bird ceca (Turcotte et al., 2020). These results are promising in that long-term responsible action can reduce resistance problems in poultry, while still allowing occasional treatment of sick birds. This suggests that controlling antibiotic use on farms will successfully reduce amounts of antibiotic resistance genes eventually transferring to outside soil, water, or human environments.

2.1.1 Clostridium perfringens and Necrotic Enteritis

Necrotic enteritis is a disease caused by pathogenic *C. perfringens* which can lead to high mortality in young broiler chickens. The disease is characterized by necrotic patches throughout the gastrointestinal tract of the birds. The visible effects of necrotic enteritis have been well documented. The epithelial cells slough off in large sheets in the lumen and blood vessels

congest in the lamina propria and submucosa. Studies on the earlier stages of the disease have found that villus damage begins in the basement membrane and lateral domain of the enterocytes before spreading upwards to the epithelial region (Van Immerseel et al., 2009).

Acute illness caused by NE often occurs rapidly, with few symptoms beforehand, and can lead to losses as high as 50% of a flock (Timbermont et al., 2011). While many *C. perfringens* strains exist as commensal bacteria within the healthy microbiome, there are multiple pathogenic strains which are categorized into toxino-types A-G, with type A long thought to be the main cause of disease in poultry. The strain types A-G are sorted by their production of the long-known toxins α, β, ε, ι, which are released into the surrounding environment by the bacteria (Keyburn et al., 2010). More recently a new toxin, NetB, has also been found in many, but not all, pathogenic strains of *C. perfringens* (Keyburn et al., 2008). Since its discovery, NetB has become a main focus of NE research, and it has been found the toxin producing genes are located on a bacterial plasmid (Zhou et al., 2017). However, the presence of a pathogenic strain does not guarantee that disease will occur. NE has been traditionally difficult to research as even challenge-type studies would often not produce disease. Currently, it is believed that the coccidia-causing parasite *Eimeria*, which damage the epithelial lining, create spaces for *C. perfringens* to replicate rapidly or stimulate production of large amounts of toxins (Van Immerseel et al., 2009). Nowadays, it is common to use both *C. perfringens* and *Eimeria* for inducing experimental NE in order to better re-create the conditions which are thought lead to the occurrence of this disease (Yang et al., 2019).

While the acute form of the disease itself is of significant concern, a subclinical form of NE may persist in as many as 20% of US broiler flocks (Skinner et al., 2010). Instead of sudden death, the subclinical form can persist for the life of the bird, causing damage to the intestinal

mucosa, which leads to reduced weight gain and poorer feed conversion. Through intestinal barrier damage, bacteria may cross the intestinal barrier and colonize the liver of the birds, causing cholangiohepatitis, with liver lesions frequently observed at slaughter with no other noticeable signs in the live birds (Timbermont et al., 2011). Through decreased feed conversion and higher levels of carcass condemnation due to organ lesions, it is the subclinical form that is estimated to cause the highest economic loss in the poultry industry (Van Immerseel et al., 2009). Prophylactic antibiotics offered protection against this form of disease in the past. With their usage being phased out in Canada, the cost of subacute NE is likely to greatly increase. It has already been found that short term withdrawal of antibiotics on Canadian poultry farms causes an increase in *C. perfringens* levels as well as a decrease of bacterial families that are considered important for energy metabolism (Turcotte et al., 2020). This is in agreement with what was observed as in other countries which reduced antibiotic use, thus it is quite likely that in the coming years necrotic enteritis will pose an increasing threat to poultry producers.

The production of bacterial toxins is considered one of the major causes of disease in poultry and other species, and the strain types A-G are sorted by their production of the long-known toxins α, β, ε, ι, which are released into the surrounding environment by the bacteria (García et al., 2019, Keyburn et al., 2010). These are pore-forming toxins, which cause cell death through leakage by puncturing holes in the cell wall (Tilley & Saibil, 2006). Type A strains produce α-toxin and are the most common type of *C. perfringens,* existing in the digestive tracts of various animals as well as in soil environments (Keyburn et al., 2010). In humans, the type A strains can cause gas gangrene and are also a common cause of food poisoning (Keyburn et al., 2010). In 2008 researchers identified a new toxin of *C. perfringens* which replaced α-toxin as the theorized main causative toxin of NE. This toxin, named NetB, has been isolated from multiple

NE-causing strains of *C. perfringens* (Keyburn et al., 2008). Though originally theorized to be a critical factor for pathogenicity, recent surveys have found non-pathogenic NetB-producing strains of *C. perfringens* (Mwangi et al., 2018). Thus far, no exact cause of pathogenicity has been found in *C. perfringens*, though it is likely that there is some variety in pathogenicity factors which lead a strain to cause disease.

2.1.2 Gram-Positive Bacteria & Infection

Bacteria can be divided into two functional groups based on staining differences: gram-positive or gram-negative. A major difference between the two classes of bacteria is the cell wall. While the gram-negative cell wall is only a few nanometers thick, the gram-positive cell wall can commonly be between 3-100nm thick. Both gram-positive and gram-negative bacteria contain an ingredient known as peptidoglycan, alongside other components. In gram-negative bacteria, peptidoglycan only represents 5-10% of the total cell wall. The buck of cell wall is the outer membrane. The outer membrane is composed of a lipid bilayer, and it differs from the cell membrane by the presence of large molecules known as lipopolysaccharide (LPS), which are anchored into the outer membrane and project from the cell into the environment (Dorr et al., 2019). LPS are well known for their immunostimulatory functions. In gram-positive bacteria, peptidoglycan can represent up to 90% of the cell wall and the secondary components are teichoic acids, which are embedded within the peptidoglycan layers. Teichoic acids that are anchored to the lipid membrane are referred to as lipoteichoic acids (LTAs), whereas teichoic acids that are covalently bound to peptidoglycan are referred to as wall teichoic acids (WTAs). LTA offers protection from antimicrobial peptides and cationic antibiotics. In *Staphylococcus aureus* infected macrophages, the NLRP6 inflammasome is activated through interactions with cytosolic LTA, leading to caspase-1 activation and cytokine activation. Kupffer cells are liver-

resident macrophages which are able to capture bacteria such as *S. aureus* by binding to LTA (van Dalen et al., 2020). WTA can bind to receptors and surfaces during infection, as well as serves as a phage receptor. The WTAs of *S. aureus* are known to bind to receptors on innate antigen-presenting cells, scavenger receptors presented on a range of cell types, as well as soluble serum receptors such as mannose-binding lectin (van Dalen et al., 2020).

LTA can bind non-specifically to host cells to aid in colonization; specifically, it is known to bind to CD14, TLR-2, and TLR-4 (Ginsburg, 2002). Injection of 100ug of LTA into mice was able to induce fever and disrupt sleep patterns, suggesting that LTA is able to at least partially replicate symptoms of gram-positive bacterial infection in vivo (Szentirmai et al., 2021). Sepsis is another method by which gram positive bacteria can cause severe damage to organisms. When large amounts of bacteria undergo lysis (which may be caused by bacteriolytic drugs), the bacterial cell wall components LTA and peptidoglycan act in synergy to illicit immunostimulatory responses. In sepsis studies, these two components are used simultaneously, as much lower amounts of LTA are required to stimulate a strong response when peptidoglycan is present. Combined, these findings show that LTA is a potent enough component of gram-positive bacteria to warrant continued research.

2.2 Polyphenols

Polyphenols are compounds characterized by multiples of phenyl rings with at least one hydroxyl substituent (Singla et al., 2019). They are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens (Manach et al., 2004). Polyphenols can be divided into one of two groups based on structure: flavonoids or nonflavonoids. The flavonoid group is further divided into multiple sub-groups, including

anthocyanins, flavan-3-ols, flavones, flavanones, and flavonols. Nonflavonoids are further sorted into groups including phenolic acids, stilbenes, and lignans.

Polyphenols exist in varying concentrations and forms, depending not only on plant species, but on environmental factors such as abiotic stresses and location in the plant tissue. Most polyphenols concentrate on surface areas such as fruit skin in order to better protect the plant. Different cultivars of the same crop species can have significantly varied polyphenol profiles. Red grapes, for example, have long been considered a good source of flavonols and anthocyanins. However, a study by Mattivi et al. (2006) found great variance in these chemicals depending on cultivar. For instance, the cultivar Casetta had the highest flavonol content at 80.37mg/kg, while the cultivar Grignolino had only 3.81mg/kg, showing a large range in flavonol content depending on red grape cultivar. This means in the development of future health products, continuous testing will be needed to ensure that products contain effective levels of compounds to benefit recipients. Analysis of fruit products used will be required to measure levels of beneficial compounds and compare with previous studies on the species of interest. There will need to be streamlining of testing procedures to ensure quality and desired effects.

2.2.1 Polyphenols: Their Action in the Digestive System

It has been commonly believed that the main benefits of polyphenols come from their strong antioxidant properties. The biochemical scavenger theory suggests that polyphenols act by neutralizing free radicals they come into contact with, reducing oxidative stress in surrounding cells (Cory et al., 2018). Polyphenols have been found to upregulate antioxidant related genes, such as nuclear factor erythroid 2-related factor 2 (Nrf-2), heme oxygenase 1 (HO-1), and glutathione peroxidase (GPx) (Bernardi et al., 2019). This combination of acting as antioxidants

and upregulating genes that improve host antioxidant status may be the reason that polyphenols are so potent even when they are generally only consumed in small quantities.

Intestinal microbiota play a large role in digestion, metabolism, and immunology of monogastric animals. Therefore, many attempts to improve animal production have been focused on the gut microbiota. Probiotics are used to introduce or raise populations of bacteria perceived to be beneficial to the animal. Often these are simple products containing only one or a few species of bacteria. Prebiotics are non-digestible (to the animal) plant products which are thought to offer substrate to raise populations of native beneficial bacteria. Polyphenols are xenobiotic compounds which can fall under the classification of prebiotics due to their positive effects on beneficial gut bacteria (Anhê et al., 2015).

In mammals, it has been estimated that as little as 10-15% of polyphenols are taken up in the small intestine, with the vast majority of them reaching the colon unchanged (Manach et al., 2005). Despite this, they are able to exert strong effects on the body, leading to their functionality being coined as a low bioavailability/high bioactivity paradox. Polyphenols which reach the liver generally undergo extensive enzymatic processes, such as deglycosylation or hydroxylation, which alter their chemical structures (Luca et al., 2020). These altered metabolites may be more or less potent than their parent chemicals, thus it is important to understand how individual polyphenols are metabolized to fully comprehend how they may function in different regions of the body. The remaining ingested polyphenols reach the colon and ceca, especially in birds, where they can interact with the microbiota. Due to this, a large number of studies have looked at the benefits of polyphenols in the intestinal environment, while less research has been done on the impacts of polyphenols taken up into the blood and interacting with other organs. Bacteria have been shown to interact with polyphenols in a variety of ways. For instance,

chlorogenic acid, a polyphenol found in plant products such as coffee, is metabolized by gut bacteria into compounds including caffeic acid, 3-phenylpropionic acid, and benzoic acid. It has been found that combining chlorogenic acid alongside its three microbial products was able to significantly decrease the rate of proliferation of cancerous intestinal cells by regulating the apoptosis pathway, suggesting potential anti-cancer actions can be augmented when polyphenols are able to be metabolized by gut bacteria (Ekbatan et al., 2018). They are known to act as bacterial substrates; for instance, quercetin has been found to act as a substrate for the human gut bacteria *Eubacterium ramulus, Clostridium orbiscindens,* and *Eubacterium oxidoreducens* (Luca et al., 2020). In addition to this, polyphenols have also been found to have negative effects on harmful bacteria; for instance, in vivo supplementation of polyphenols in humans was found to significantly reduce populations of pathogenic *Clostridium* bacteria (Ma & Chen, 2020). Whether this is due to bactericidal effects or through increasing competition of other species is not clear. In-vitro studies have found that polyphenol-rich cranberry extracts had strong inhibitory effects on pathogenic *Listeria monocytogenes* and *S. aureus* (Diarra et al., 2013; Diarra et al., 2020). Polyphenols in the intestinal environment can also interact directly with enterocytes, regulating gene expression and interacting with pattern-receptor proteins. In summary, polyphenols from cranberry and blueberry play complex roles in multiple regions of the body, interacting with both host cells and bacterial cells.

2.2.2 Direct Actions of Polyphenols against Pathogenic Bacteria

Even though there has been ongoing research since the late 1980's, a definite mode of action (MOA) is still not clear for flavonoids. Part of this is due to inconsistencies in research methods and conclusions, but another part of this is due to the sheer volume of flavonoid molecules known, and different forms each molecule identified having different levels of

efficacy. Currently it is still unknown whether all flavonoids have one MOA with slight variation between molecules, or multiple MOAs depending on the specific molecule.

The initial MOAs of flavonoids on bacterial cells theorized have been as follows: cytoplasmic membrane damage caused by perforation and/or a reduction in membrane fluidity; inhibition of nucleic acid synthesis caused by topoisomerase inhibition; and inhibition of energy metabolism caused by NADH-cytochrome c reductase inhibition (Cushnie & Lamb, 2011). More specifically, the classes flavonol, flavan-3-ol, and flavolan are thought to damage cytoplasmic membrane; the classes flavan-3-ols and isoflavones are suggested to inhibit nucleic acid synthesis through topoisomerase and/or dihydrofolate reductase inhibition; the classes flavonol, flavan-3-ol, and flavone inhibit energy metabolism through ATP synthase inhibition. In reality, flavonoids may have an aggregatory effect on bacterial cells, based on the combination of previously mentioned mechanisms.

Another documented function of flavonoids is their reduction of pathogenicity. One specific member of the flavan-3-ol family found in green tea, (-)-epicatechin gallate, has been studied in regard to its effects on the gram-positive bacteria *Streptococcus mutans*, which causes tooth decay. It was found that a level of (-)-epicatechin gallate below minimum inhibitory concentration was able to significantly downregulate the expression of four genes associated with the pathogenicity of the bacterium (Xu et al., 2011). Flavonoids have also been found to inhibit sortase, an enzyme used by many gram-positive bacteria to catalyse the assembly of surface proteins such as adhesins; these proteins have been shown to be important for establishment of infection through biofilm formation and attachment to host cells (Wallock-Richards et al., 2015).

Many bacterial species, including *Clostridium perfringens*, release a variety of toxins that cause pathogenesis and can persist in the host long after the bacteria have been killed. One study found that polymerised catechin negates the alpha-toxin of S. aureus both in-vitro and in-vivo (Shah et al., 2008). The NetB toxin produced by *C. perfringens* has been theorized to be similar in form and function to S. aureus alpha-toxins and thus it is possible that flavonoids also may be able to play a role in neutralizing this key toxin in *C. perfringens* pathogenesis (Keyburn et al., 2008). This same study also found secretion of the enzyme coagulase was also inhibited, suggesting that this reduction of activity was due to action of the flavonoids.

2.2.3 Cranberries and Their Phenolic Properties

Cranberries have been studied in the past for benefits arising from their phenolic properties. Several screenings have identified several polyphenols found in cranberries. One study detected, in µg/g FW (Fresh Weight), the phenolic acid p-coumaric acid (20.28+-1.35), as well as trans-resveratrol (19.29+-1.53), and the flavonoid quercetin (5.15+-0.40) **(**Ehala et al., 2005). A review of several studies reported the following polyphenols in concentrations of µg/gFW: anthocyanins: Cyanidin (464.3), Delphinidin (77), Malvidin (40), Peonidin (492), Pelargonidin (3); the flavan-3-ols: (-)-Epicatechin (44), (+)-Catechin (40); the flavonols: quercetin (148), myricetin (66); and the stilbenoids: Resveratrol (1.35), Trans-resveratrol (19.29) (Govers et al., 2017). The varying levels of quercetin between the two publications should be noted as evidence of variability between samples.

Studies looking at flavonols and their benefits have showed that in vitro isolations of certain compounds did not yield any results or gave results which were contrary to what was later found in vivo. This demonstrates the importance of looking at the benefits of fruit pomace through a holistic, whole-food approach, as there is likely synergy between multiple chemical

compounds acting together which cannot be recreated using a single compound (Govers et al., 2017). However, this indicates promise in the future use of these by-products as health stimulants with little need for manipulation or filtering. Despite this, there is need to develop a better understanding of the roles of chemical compounds in the cranberry if we are to use it most effectively.

While reactive oxygen species (ROS) tend to be the main focus of antioxidant studies, there exist other damaging compounds such as reactive nitrogen species (RNS) and reactive sulfur species (RSS). Though their roles may be less compared to ROS, their potential to cause oxidative damage to the body should not be overlooked. A novel study found that cranberry extract at a concentration as low as 1mg/mL to human kidney cells (HEK293) challenged with the RSS hydrogen sulfide (H_2S) was able to significantly lower the levels of H_2S by oxidation to polysulfides (Olson et al., 2021). This study also found that the polyphenol quercetin was able to oxidize H_2S, and when combined with the enzyme superoxide dismutase (SOD), oxidation was increased with an additive effect, which may provide a clue into how polyphenols work alongside the body's natural pathways.

Specific phytochemical families from fruits have to be studied to see which family is responsible for the majority of antibacterial action. Studies done by Lacombe et al. (2013) examined the effects of 5 categories of cranberry constituents on *E. coli* and *L. monocytogenes* bacteria. All phytochemical families examined were found to have high antimicrobial effects on the bacteria. Cranberries are naturally acidic, with cranberry juice having a pH of 2.5. As phytochemicals are produced to service the plant, it is likely that they may be most efficient at the native pH of their producer. However, through the digestive process the phytochemicals are exposed to different pH levels which may lower their efficiency, with the large intestine pH of

broilers at 6.4, the caecum at 6.62 (Mabelebele et al., 2014). Thus, the efficiency of cranberry compounds was also investigated at neutral pH to confirm their activity remained in different conditions. It was found that the polyphenol fractions including anthocyanins maintained their antibacterial properties against *E. coli* and *L. monocytogenes* in neutral pH (Lacombe et al., 2013).

If multiple phytochemicals in cranberry show antimicrobial activity, then a holistic approach should be considered. Much like multi-drug therapies used to treat resistant disease, the complex chemical mixture in the cranberry pomace may be more potent. Multiple compounds attacking through the same or different mode of action at the same time will make it more difficult for a strain of bacteria to develop resistance. The cranberry flavonoid quercetin has been found to reduce inflammatory response during LPS challenge. In murine macrophages, polyphenols including quercetin were found to lower NO production by inhibiting production of the inducible nitric oxide synthase (iNOS) protein through two transcription pathways, NF-κB and STAT-1 (Hämäläinen et al., 2007). Whole cranberry extracts have been used in a variety of live human and animal studies where they have shown antioxidant, immune boosting, and protective effects. They have found to be quite potent in protecting from negative effects of high sugar and high fat (HSHF) diets, indicating that cranberries may be beneficial to humans suffering from diseases such as diabetes. These types of diets can lead individuals to develop non-alcoholic fatty liver disease, characterized by scarring and fat accumulation in liver tissue. When supplemented with cranberry extracts, rats on HSHF diets were protected from excessive weight gain and showed improved liver health index scores (Faheem et al., 2020). The cranberry extract additionally reduced insulin resistance levels, levels of several inflammatory markers, and upregulated levels of the antioxidant regulator gene Nrf-2 which was lowered by the HSHF.

A similar study which focused more on the effects supplementing cranberry extract to HSHF-fed mice found a similar protective effect from weight gain, fatty liver disease, as well as less insulin resistance and reduced intestinal inflammation (Anhê et al., 2015). This study identified a marked increase in abundance of the bacterial genus *Akkermansia*, a group of bacteria which have been strongly linked to offering protective benefits against obesity caused diseases. The high fat diet resulted in increased circulating LPS, a source of inflammation. The high fat diet supplemented with cranberry extract showed a reduction in intestinal inflammation due to lower levels of COX-2 and TNF-α expression alongside normalization of NF-κB/IκB ratio compared with mice not supplemented with cranberry extract. In addition to this, the cranberry extract prevented a drop in SOD2 activity, an enzyme responsible for clearing reactive oxygen species, thus protecting antioxidant function in mice (Anhê et al., 2015). This suggests that cranberry extract plays a strong role both in regulating inflammatory and antioxidant status in challenged intestinal systems. Cranberries have been long thought to offer protection against urinary tract infections (UTIs) in humans. It has been theorized that this is due to the unique A-type linkage structure of cranberry proanthocyanins. The majority of proanthocyanin-heavy foods have differently structured B-type linkages; this small structural difference is thought to have higher antiadhesion activity against both antibiotic-sensitive and resistant UTI-causing *E. coli* strains (Duda-Chodak et al., 2015). It can be concluded that cranberry extracts have a wide range of benefits throughout the digestive system of mammals, as well as benefits for the natural microbiome.

Several studies have also been conducted on chickens fed cranberry extracts. In terms of production performance, cranberry extract supplementation has had negligible effects on factors such as weight gain and feed conversion in broilers (Leusink et al., 2010; Islam et al., 2016). One

study did find that an ethanolic extract of cranberry was able to improve feed efficiency (Das et al., 2020). A 2% cranberry pomace (CP) diet was found to improve serum levels of the antibody IgY, but had no effect on IgM, in 21-day old broilers whereas a diet with 1% CP or cranberry ethanolic extracts did not affect any serum Ig levels (Das et al., 2021). By 28 days levels in all treatment groups were similar. A secondary study found that additional of a cranberry fraction resulted in higher serum IgM levels in 35-day old broilers (Islam et al., 2016). The bursa of Fabricius is an avian immune organ responsible for the development of B cells which control Ig production. Cranberry pomace was found to upregulate expression of anti-inflammatory genes IL-6, IL-10, and IL-1R1 in the bursa while reducing the expression of inflammation genes IL-4 and interferon gamma in the liver (Das et al., 2021). These health benefits may help protect birds from disease.

Cranberry supplementation may have effects on the microbiome community; in the past cranberry fruit extract was found to result in lower cecal levels of *Enterococcus* spp. (Leusink et al., 2010); however, it was not found to lower cecal levels of *Campylobacter jejuni* in challenged birds (Woo-Ming et al., 2016) or *E. coli* in non-challenged birds (Leusink et al., 2010). A 1% or 2% CP diet was able to lower prevalence of necrotic enteritis, while a 2% CP diet was able to lower *E. coli* levels to match antibiotic-treated chickens (Das et al., 2020). Supplementation of cranberry extracts versus cranberry ethanolic extracts have shown to offer different benefits, which still needs to be further examined to determine the most desirable benefits for poultry production.

2.2.4 Blueberries and Their Phenolic Properties

Similar to cranberries, antioxidant-rich blueberries have been the subject of many studies interested in their potential health benefits. *E. coli* infection was found to reduce transepithelial

electrical resistance in an in-vitro model of the human intestinal lining using Caco-2 cells. The addition of the anthocyanin-enriched fraction, but not proanthocyanin-enriched fraction of blueberries, was able to attenuate this effect, suggesting that the anthocyanin component could have bactericidal activity or inhibit the adhesion of *E. coli* to intestinal cells (Polewski et al., 2019). Similar to cranberries, blueberry extract added at a concentration as low as 1mg/mL to human kidney cells (HEK293) challenged with the RSS hydrogen sulfide (H_2S) was able to significantly lower the levels of H_2S by oxidation to polysulfides (Olson et al., 2021). Rats fed a high fat diet experienced physiological changes in the gut through a decrease in ileal villus height and expression of a gene involved in mucin production, Muc2. These negative effects were reversed through supplementation with 10% blueberry powder (Lee et al., 2018). Additionally, an increase in the class Gammaproteobacteria was noticed, which was theorized to have also led to increased levels of acetate and propionate. These short chain fatty acids (SCFA) are able to stimulate genetic pathways related to insulin sensitivity (Tolhurst et al., 2012). This suggests that blueberry may help mitigate conditions such as diabetes, which may be caused by high fat or high sugar diets, through modulation of the microbiome as well as host gene expression.

In poultry, a few studies have looked at health effects from blueberry addition to feed. A 2% blueberry extract supplement was found to improve feed conversion and final weight of broiler chickens (Ölmez et al., 2021). A second study only found that blueberry pomace ethanolic extracts increased body weight of birds during the starting and growing phases, but did not improve feed conversion (Das et al., 2020). A 1:1 combination of blackberry and blueberry extract, measured by phenolic strength using Gallic Acid Equivalent (GAE), was found to successfully reduce colonization of *C. jejeuni* in challenged chicks (Salaheen et al., 2017). In

pastured slow growing broilers raised for 64 days, supplementation of blueberry extract enriched populations of *Lactobacillus*, *Bacteroides*, and *Bifidobacterium*, lowering abundance of *E. coli*, *Clostridium Clostridiaceae*, *Heliobacter*, and *Enterococcus* by the end of the growing period (Islam et al., 2019). A 1% blueberry pomace (BP) supplement was found to significantly lower prevalence of necrotic enteritis in broilers (Das et al., 2020). Ethanolic extracts of blueberry pomace increased cloacal populations of Acidobacteria and Lactobacillaceae, two beneficial groups of bacteria (Das et al., 2020). Similar to cranberry supplementation, addition of a blueberry fraction to poultry diets offers potential benefits that warrant further investigation.

2.3 The Avian Metabolome

The avian gut metabolome is composed of small molecules produced by host cells and microbial cells, and exogenous compounds consumed by birds. As with the microbiome, the metabolome of birds develops and changes over the maturation of the birds. Large amounts of data have been collected on the development of the commensal microbiome of broilers, as well as how it may be affected by stressors such as infection (Rehman et al., 2007). Metabolomic data can offer a better understanding of how to improve animal productivity. Metabolites constantly interact as signalling molecules and with host enzymes where undergo modifications such as phosphorylation (Lee et al., 2022). Host-derived metabolites include mucins and bile acids, the latter of which are hydrolysed by multiple bacterial genera (Rehman et al., 2007). Bile acids play vital roles in modulation of the metabolic and immune-inflammatory responses impacted by bacterial activity in the gut. While bile acids are known to be essential to host health, there is still a lack of understanding as to how microbial metabolites of bile acids may impact poultry health and production. Studies which are able to correlate changes in bacterial groups with changes in metabolite levels, gene expression, and protein expression could offer improved understanding

of why products such as feed additives often show contradictory results between different studies.

Glucose and glutamate are two diet-derived metabolites essential for cellular functions. Glucose is essential for glycolysis, where it is broken down into pyruvate for further metabolic processing, with the generation of ATP along the way. Glutamate is metabolized from glutamine and is a vital component of many metabolic pathways including, but not limited to, gluconeogenesis in the kidneys to produce glucose, conversion to the neurotransmitter γ-aminobutyric acid, as well as ornithine which can lead to urea synthesis in the liver or arginine synthesis as a precursor nitric oxide as a signaller of apoptosis and leukocytes (Newsholme et al., 2003). Ethanol presents an example of a less desirable metabolite in the gut, and in humans is associated with dysbiosis (Elshaghabee et al., 2016). Ethanol produced by the microbes in the gut is known as endogenous ethanol. Many species of bacteria are able to use multiple compounds as substrates, which may be fermented into different end products. For instance, while Bifidobacteria are known as producers of acetate and lactate, in some circumstances they also can produce ethanol (Elshaghabee et al., 2016). The microbiome shifting to increase more endogenous ethanol production could be considered a sign of a dysfunctional microbiome as ethanol offers no benefit to the host. In the case of poultry, this could lead to reduced weight gain and poorer feed conversion.

Perhaps the best known and understood metabolites from bacterial fermentation are SCFA; these include acetate, butyrate, propionate, and valerate. These compounds contribute to a significant amount of total energy to a host, between 5-15% in humans and as high as 30% in pigs (Rinttilä & Apajalahti, 2013). Production of SCFA is regulated by numerous factors including diet, fermentation rate, transit time in the gut, and microbiome composition (Rehman

et al., 2007). The ratios of these compounds are often of interest, as increases or decreases in one or another can have significant effects on the host. Butyrate is known to be beneficial to enterocytes and can promote healthy epithelial development, leading to improved host immunity (Rinttilä & Apajalahti, 2013). Propionate is able to promote gluconeogenesis in hepatocytes (Armstrong et al., 2016). Lactic acid is often produced in high quantities and contributes to lowering the pH of the intestinal environment; however, it is not normally found in high quantities in the cecal region (Rehman et al., 2007; Rinttilä & Apajalahti, 2013). Overall, the SCFA produced by healthy commensal bacteria are considered an essential component of host health, helping to maintain proper pH through the gastrointestinal tract and through offering bacteriostatic effects on potential pathogens.

2.3.1 Microbial Influence on Metabolome

While the metabolome is influenced by many internal and external factors, the gastrointestinal metabolome could be argued to be most heavily influenced by the resident microbiome, due to the sheer volume and variety of bacterial cells constantly consuming and producing metabolites. Under normal conditions, the chicken microbiome undergoes large changes between birth and maturity which are also reflected in the metabolites produced. It has been observed that during early life, the microbiome in broilers is less diverse and highly dynamic, becoming more stable and mature around 42 days of life (Bilal et al., 2021). As broilers are commonly processed between 4-6 weeks, most, if not all, of their lifespan is spent with an immature microbiome. During the early stages of life, the gastrointestinal system is more oxygenated and thus contains a higher level of facultative anaerobes which can tolerate some amount of oxygen. Over time, oxygen levels decrease, allowing for the obligate anaerobes which make up the mature microbiome to dominate the gut. In poultry this role is filled by the phylum

Proteobacteria, which make up a larger fraction of the early microbiome but are replaced by obligate anaerobes as birds age (Richards et al., 2019). The replacing phyla are largely composed of Bacteroidetes and Firmicutes. In one study it was seen that firmicutes concentration increased in broilers gradually at day 14 of life and continued to increase at day 42 of life, however it was noted that there are conflicting studies finding variances in the percentage makeup of these two phyla in birds (Richards et al., 2019). While encompassing many species with different substrates and products, in the cecum, Firmicutes are generally greater producers of butyrate, while Bacteroides are greater producers of propionate (Richards et al., 2019). The levels of butyrate-producing Firmicutes may be one important connection in ensuring healthy epithelial development in young birds. Connecting microbiome analysis to metabolomic analysis may offer new understanding into the roles various bacterial groups play in developing birds and create a better understanding of an ideal microbiome for various utilities of poultry.

Antibiotic caused dysbiosis in chickens saw a connection with the increase in Proteobacteria correlating with several metabolomic lipogenesis indicators; the decrease in Bacteroidetes and Firmicutes was also correlated with increase in lipogenesis indicators (Zhang et al., 2021). Proteobacteria are less sensitive to oxygen and have been linked as markers of epithelial dysfunction causing an increase in oxygen levels in the human gastrointestinal system (Litvak et al., 2017). Bacteria involved in carbohydrate and lipid metabolism were enriched post antibiotic treatment; while bacteria involved in amino acid metabolism and nucleotide metabolism were reduced in population. These relations show a strong suggestion that antibiotic-induced dysbiosis can lead to undesirable weight gain in birds. Bacterial infections of the gastrointestinal tract can also cause imbalances in the microbiome and thus the metabolome. *Campylobacter jejuni* infection was found to reduce levels of propionate, butyrate, isovalerate,

892 and isobutyrate in growing broilers; these metabolites may serve as preferable energy sources for
893 the pathogen (Awad et al., 2016). Gene regulation in the cecal tonsils of chickens was found to
894 be affected by subclinical *Salmonella* sp. infection in the form of upregulation of arginine and
895 proline metabolism related genes (Mon et al., 2020). These results were taken to be a potential
896 precursor to preparation for nitric oxide synthesis as these amino acids are both essential to the
897 pathway. Thus, it is likely that pathogenic bacteria not only effect the metabolome in multiple
898 ways through competition with the host microbiome, but also illicit responses in the host cells,
899 altering metabolite production in order to defend from infection. Understanding what composes
900 an ideal host metabolomic profile alongside how host cells and the microbiome respond to
901 various challenges may offer insights into methods to promote host health.

3 Overall Research Objectives

The objective of this research was to build upon our knowledge of how cranberry and blueberry fruit extracts can be incorporated into use in poultry feed. We attempted to create an in-vitro model of infection using immortalized chicken hepatic cells and cell wall components from gram-positive bacteria. We also wanted to see if the extracts would have influence on genes which were of previous interest in a live animal trial using q-PCR. Next, we quantified metabolites of interest from the cecal contents of broilers fed diets containing the fruit extracts. Any changes in metabolite levels, such as short chain fatty acids, could impact animal health and performance. Overall, the goal was to gather information about whether the extracts can be used without negatively impacting animal health, and to try and gain insight into beneficial effects previously observed when chickens were fed the extracts.

4 Experiment #1: In-Vitro Analysis

4.1 Introduction

While in-vivo experiments offer a complete picture of animal production, in-vitro experiments offer the ability to conduct more trials in a shorter length of time, with reduced interference from uncontrolled variables. Despite its widespread prevalence and decades of research, necrotic enteritis remains difficult to induce in an experimental setting. It is believed that there are multiple complex factors which are needed to predispose birds to disease. Due to these reasons, there is rationale to work on developing an in-vitro infection model for necrotic enteritis in poultry. Cell culture experiments allow the observation of changes in genetic and proliferative changes of animal tissues in response to stimuli. Bacteria or bacterial products can be applied to cultured cells in order to measure and detect changes in viability as well as genetic

924 expression that may be precursors to disease caused by those bacteria. Additionally chemical
925 compounds of interest may also be applied to cultured cells to measure potential negative or
926 beneficial effects through gene expression or viability. Such experiments can be performed using
927 primary or immortalized cells. Primary cell lines are harvested directly from animals and are
928 only able to survive for a limited number of mitosis cycles; while immortalized cell lines can be
929 maintained indefinitely and are often commercially available and offer the opportunity for
930 uniform comparisons between research groups. Toxins produced by *C. perfringens* cause
931 damage to the small intestine, liver lesions, and mortality (Løvland and Kaldhusdal; 1999). There
932 were no available established cell lines of poultry intestinal cells. So, we elected to study the
933 effect of NE on liver cells, using LMH line of chicken hepatic cells. The liver possesses a vital
934 role in the digestive system as a metabolic powerhouse and filter for toxins which have passed
935 through the intestinal epithelium and entered the portal vein blood. As such, it is the first organ
936 pathogenic bacteria encounter if they break through the intestinal epithelium. Likewise, it is also
937 the second organ that consumed polyphenolic extracts would come into contact with. Thus, it is
938 important to understand how polyphenol-rich fruit extracts may interact with liver tissue.

939 In the following experiments, we attempted to use bacterial cell wall components to try to
940 induce a necrotic effect on hepatic poultry cells. LMH (hepatic cells) have been previously used
941 as a model for *C. perfringens* infection in poultry (Parreira et al., 2017; Zhou et al., 2017). We
942 used LTA, with or without the additional component peptidoglycan, to attempt to induce an
943 inflammatory or necrotic response in the cells. Next, we tested the effect of a single polyphenol
944 quercetin on the cells. Additionally, we applied fruit extracts to the cells in varying
945 concentrations in order to measure changes in five genes examined previously in a live animal
946 trial with cranberry and blueberry feeding.

4.2 Objective

The first objective of this research was to determine if cell wall components from gram-positive bacteria (LTA & peptidoglycan) could be used as a model for infection in LMH cells. The second objective was to determine if polyphenol-rich fruit extracts (cranberry & blueberry) or the polyphenol quercetin could attenuate oxidative stress caused by LTA and/or peptidoglycan by reducing cell mortality or altering expression of antioxidant related genes.

4.3 Hypothesis

We hypothesized that the bacterial cell wall components would affect viability of LMH cells, and that cells treated with fruit extracts or quercetin would show reduced mortality through increased antioxidant actions when challenged with LTA and/or peptidoglycan.

4.4 Methodology

4.4.1 Cell Culture

LMH (ATCC® CRL-2117™) cells were maintained in standard growth conditions of 90% Waymouth's medium and 10% fetal bovine serum. Cells were incubated in a humidified environment of 5% CO_2 at 37C (Kawaguchi *et al*., 1987). The medium usually contains antibiotics penicillin (50µg/mL) and streptomycin (50units/mL) (Thermo Fisher Cat#15140148, Canada) unless indicated otherwise. Cell counting was done manually with a hemacytometer. All experiments except RNA analysis were performed in 96 well plates. Cells were seeded at a density of 1×10^4 cells/mL with 100uL of media to cover them. Cells were allowed to adhere to the plate for 12 hours. After 12 hours, media were aspirated and replaced with 150 uL of antibiotic-free, FBS-free media (to reduce interference from plasma components) before the experiments. Six well plates were used in order to obtain sufficient cells for RNA analysis. Cells were seeded at a density of 5×10^5 cells/mL with 1000uL of media to cover them. Cells were

allowed to adhere and grow for 24 hours, after which media were aspirated and replaced with 2000uL of fresh antibiotic-free treatment media before the experiments. After 24 hours, absorbance was measured using a microplate reader.

4.4.2 Preparation of Chemicals

a. Preparation of LTA

Powdered LTA was diluted in molecular grade water as recommended by the supplier (Sigma Aldrich Cat#L2515, Canada). Diluted LTA was allocated in 100uL components to avoid multiple freeze/thaw cycles. The concentration of diluted LTA was 1,000,000ng/100uL. LTA was then diluted to required concentrations in cell media. For preliminary studies, several concentrations of LTA were tested to create a dose response curve. The chosen concentrations were: 0.1ugl/mL, 1ug/mL, 10ug/mL, 50ug/mL, and 100ug/mL. Once it was determined that the concentration of 0.1ug/mL was enough to induce a significant effect on the cells, this dose was used for later experiment with quercetin and for RNA analysis.

b. Preparation of Quercetin

Quercetin is the flavonol found in the largest quantity in both cranberry (Islam et al., 2016) and blueberry (Sezer et al., 2019). Concentrations of quercetin (Sigma Aldrich Cat#337951, Canada) were selected from prior literature using human cell culture (van der Woude et al., 2003; Garcia-Mediavilla et al., 2006). Serial dilution using 80% ethanol alcohol was performed to achieve desired concentrations. The initial stock solution was syringe filtered to achieve sterility, and the dilutions were performed in a sterile biosafety hood. The extra stock solution was stored at -20C. At the chosen treatment doses, ethanol would not make up more than 1% of the well volume to minimize the effect of ethanol on the cells.

c. Preparation of Fruit Extracts

Organic cranberry and blueberry 80% ethanol soluble extracts were previously prepared by Kelly Ross (Summerland, BC). The tested concentrations of blueberry and cranberry extract were chosen as 2.5mg/mL and 10mg/mL. This was based on a related study (Islam et al., 2016) used more concentrated non-dialyzable materials (NDM) of cranberry in an in-vitro study with chicken heterophil cells. The NDM extracts had significantly higher phenolic content than the ethanol soluble extractives used, but the concentrations tested are based off this paper adjusted for the differences in strength.

d. Qualities of Fruit Extracts

The qualities of the extracts were determined in a previous study (Ross et al., 2017).

Phenolic Levels	Organic cranberry 80% ethanol soluble extractives	Organic blueberry 80% ethanol soluble extractives
Total phenolics (mg gallic eq./g)	36.25 +- 1.39	55.67 +- 1.58
Tartaric esters (mg caffeic acid eq./g)	10.29 +- 0.41	15.03 +- 0.41
Flavonols (mg quercetin eq./g)	11.74 +- 0.48	18.34 +- 0.51
Anthocyanins (mg cyanidin-3-glucoside eq./g)	11.14 +- 0.39	38.53 +- 1.09

Table 1: Levels of various phenolic families in the cranberry and blueberry extracts.

Antioxidant Activity	Organic cranberry 80% ethanol soluble extractives	Organic blueberry 80% ethanol soluble extractives
ABTS (umol Trolox eq./g)	306.77 +- 10.67	468.79 +- 11.64
FRAP (umol Trolox eq./g)	243.61 +- 5.11	372.22 +- 9.01

Table 2: Measured antioxidant activity of the cranberry and blueberry extracts determined by two methods: [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] Assay (ABTS) and Ferric Reducing Antioxidant Power Assay (FRAP).

4.4.3 Viability Assays

In order to measure cell viability more accurately, two cell viability assays were performed. The LDH release assay (ThermoFisher Cat#C20302, Canada) measures levels of lactate dehydrogenase, which is released from cells once the cellular membrane's permeability increases due to cell death. On the other hand, Cell Counting Kit-8 (Sigma Aldrich Cat#96992) measures the NADH dehydrogenase activity of live cells. The LDH assay measures cell death, while the CCK-8 confirms cell viability levels.

The following formulae were used to determine cell viability (as suggested by each kit):

LDH Assay: $Cell\ Viability = 100 - \left(\frac{LTA\ Sample\ Absorbance - Spontaneous\ Activity\ Absorbance}{Maximum\ Absorbance - Spontaneous\ Activity\ Absorbance}\right) x100$

CCK-8 Assay: $Cell\ Viability = \left(\frac{LTA\ Sample\ Absorbance - Media\ Blank\ Absorbance}{LTA - free\ Control\ Absorbance - Media\ Blank\ Absorbance}\right) x100$

4.4.4 RNA Analysis

RNA was harvested from cells treated with fruit extracts or with LTA. Two concentrations of each fruit extract (2.5mg/mL & 10mg/mL), were used and each experiment was conducted in triplicate. Total RNA was extracted from cells using Trizol (Applied Biosystems, U.S.). The RNA was re-suspended in RNase-free water, and the concentration and purity were measured using a spectrophotometer.

RNA (2μg) was used with a high-capacity RNA-cDNA kit (ThermoFisher Cat#4387406). For RT-qPCR analysis, PowerUp SYBR Green Master Mix (5uL) (ThermoFisher Cat#A25742) was combined with 1uL of cDNA, 1uL of forward primer, 1uL of reverse primer, and 2uL H_20. Primers were purchased from Thermo Fisher Scientific and are listed in Table 3. A gradient PCR step was run to optimize annealing temperature for the chosen genes, with the temperature chosen at 60C. The antioxidant-related genes detected included nuclear factor E2-related factor 2 (***Nrf2***), glutamate cysteine ligase catalytic subunit (***Gclc***), Glutathione peroxidase 2 (***Gpx2***),

thioredoxin (***Txn***) and heme oxygenase-1(***HO-1***). B-actin was used as the reference gene. Relative gene expression levels were calculated using the 2−ΔΔCt method.

Gene Name	Primer Sequence (5'-3')
Nrf2	Nrf2-F **CTG CTA GTG GAT GGC GAG AC** Nrf2-R **CTC CGA GTT CTC CCC GAA AG**
Gclc	Gclc-F **GGA CGC TAT GGG GTT TGG AA** Gclc-R **AGG CCA TCA CAA TGG GAC AG**
Gpx2	GPX2-F **ACC ACG GTG AGG GAT TAC A** GPX2-R **TTC AGG TAG GCG AAG ACG G**
Txn	Txn-F **GTG CAT GCC AAC ATT CCA GT** Txn-R **CTC CAT GGC GGG AGA TTA GAC**
HO-1	HO-1-F **AGC TTC GCA CAA GGA GTG TT** HO-1-R **GGA GAG GTG GTC AGC ATG TC**
B-actin	β-actin-F **ATC TTT CTT GGG TAT GGA GTC** β-actin-R **GCC AGG GTA CAT TGT GG**

Table 3: Primers used in RNA Analysis

4.4.5 Statistical Analysis:

Statistical results were determined using IBM SPSS 27. For cell proliferation assays, a one-way ANOVA followed by Tukey's post hoc test was performed to determine significance. qPCR statistical analysis was performed as outlined in Taylor et al., 2019. A one-way ANOVA test with Tukey's post hoc test was performed to determine significance.

4.5 Results

4.5.1 Cell Viability

In order to determine whether LTA could affect LMH viability, the LDH release assay was first performed. The results from the LDH release assay indicate that LTA did not induce cell death (Figure 1). In order to confirm the result from the LDH assay, we repeated the trial with a secondary cell viability kit, the CCK-8 which measures NADH dehydrogenase activity of

live cells. From this experiment, we saw that the cell viability actually increased to over 100%, meaning that proliferation was increased when LTA was applied (Figure 2).

a. Effect of LTA on LMH Cell Viability

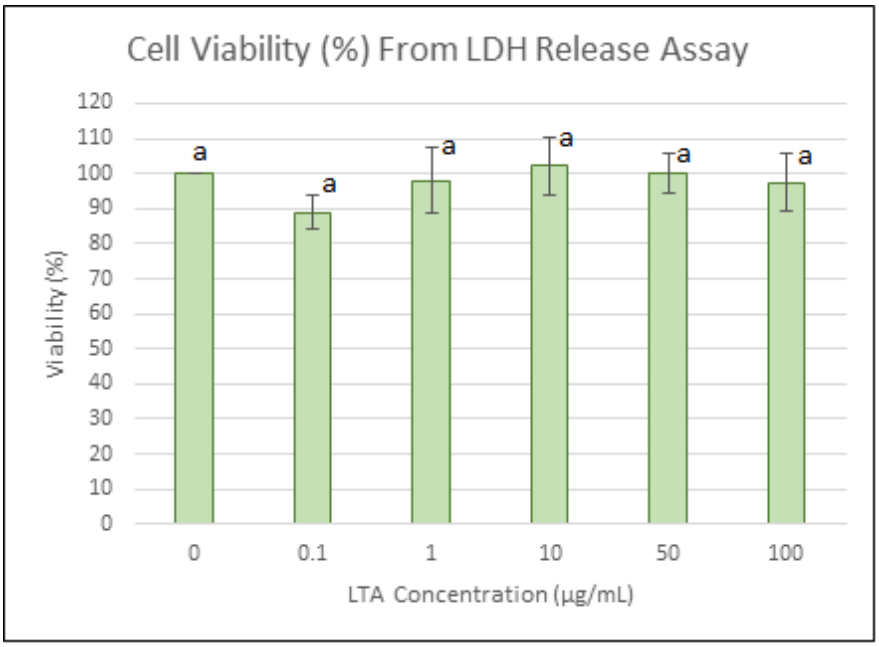


Figure 1: Effects of LTA dosage on LMH Cell death. Results are shown as the result of three experiments with the means and SEM. Shared letters indicate no significant difference.

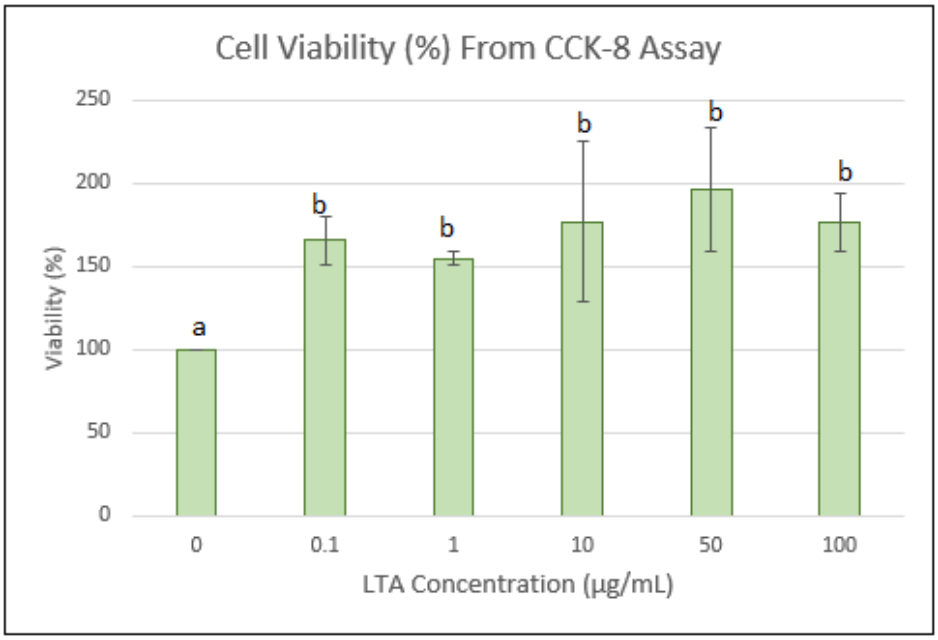


Figure 2: Effects of LTA dosage on LMH Cell viability. Results are shown as the result of three experiments with the means and SEM. Shared letters indicate no significant difference.

b. Effect of LTA and Peptidoglycan on LMH Cell Proliferation

We were surprised to see the stimulative effects of LTA on LMH cells. In order to see whether combination of LTA and peptidoglycan on proliferation, we repeated the trial testing 0.1 and 1ug/mL of LTA, alongside which we added two treatments with a 1:1 ratio of peptidoglycan. Literature review suggested that in some cell lines, an equal ratio of the two cell wall components would induce cell death even if the single components did not (Wu et al., 2020). However, we found that with this cell line, results showed that even with peptidoglycan added, LTA did not decrease LMH cell proliferation (Figure 3).

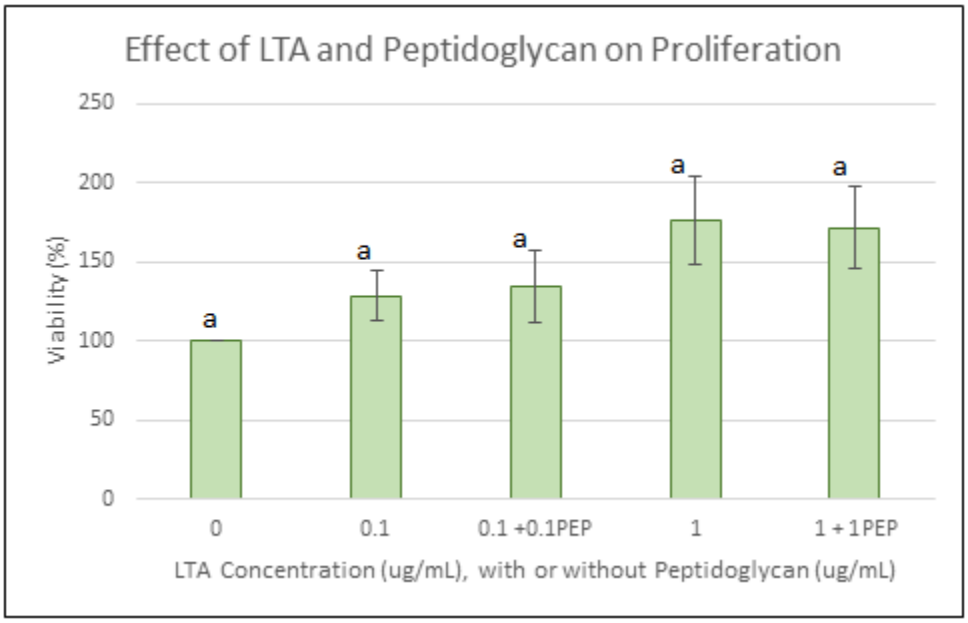


Figure 3: Effect of LTA, with or without equal proportion of peptidoglycan, on cell viability. Results were measured using the CCK-8 kit and are shown as the result of three experiments with the means and SEM. Shared letters indicate no significant differences.

c. Effect of Quercetin on LMH Cell Proliferation

We tested if quercetin would have any effects on cell proliferation. We elected to use a single purified polyphenol as even with dilution in alcohol, the fruit extracts produced a strong colour that interfered with the colorimetric assays. As shown in Figure 4, the four concentrations

tested did not significantly reduce cell viability. However, a trend suggests concentrations greater than 15.1µg/mL may start to reduce viability.

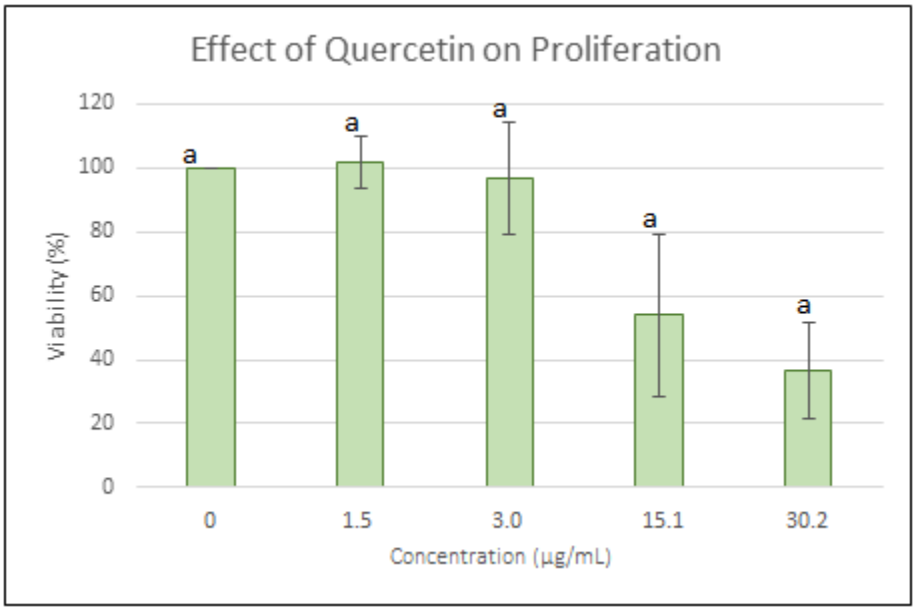


Figure 4: Effect of quercetin on cell proliferation. Results were measured using the CCK-8 kit and are shown as the result of three experiments with the means and SEM. Shared letters indicate no significant differences.

d. Effects of Quercetin and LTA on LMH Cell Proliferation

Finally, we used one dosage of LTA (the lowest dose as all previously tested had similar effects) combined with concentrations of quercetin. The combined doses showed no significant differences from the untreated group in cell viability (Figure 5).

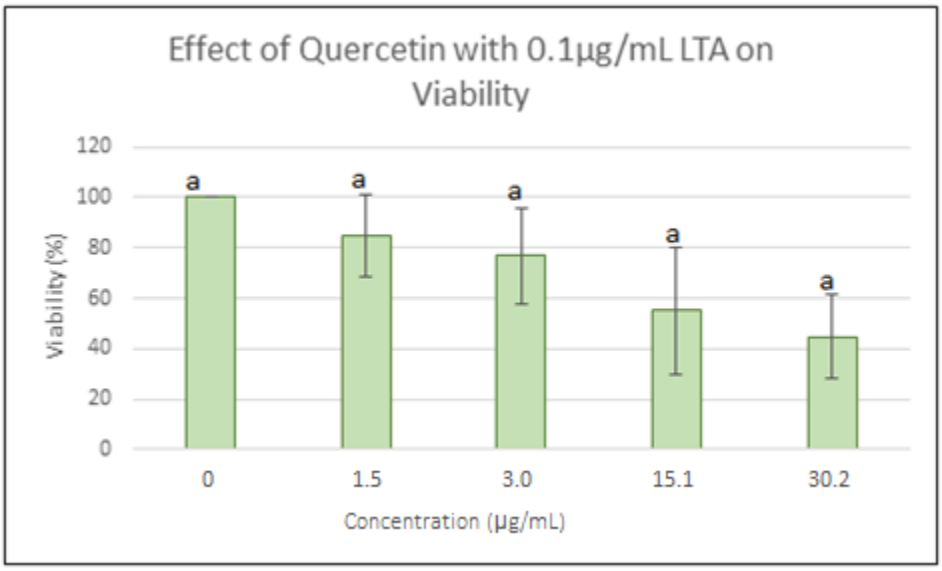


Figure 5: Effect of 0.1µg/mL LTA and four concentrations of quercetin on cell proliferation. Results were measured using the CCK-8 kit and are shown as the result of three experiments with the means and SEM. Shared letters indicate no significant differences.

4.5.2 rt-qPCR

Next, we used real-time q-PCR to determine if LTA or fruit extracts had any effects on expression of five genes related to antioxidant expression: nuclear factor E2-related factor (Nrf2), glutamate cysteine ligase catalytic subunit (Gclc), Glutathione peroxidase 2 (Gpx2), thioredoxin (Txn), and heme-oxygenase-1 (HO-1). Beta-actin was used as a reference gene. The gene Gpx2 was not detected in any treatment groups, suggesting a primer error for this cell line, as our reference gene was confirmed through muscle tissue, or the possibility that this gene is not expressed in this cell line. The treatment group Blueberry 10mg/mL was dropped, as it very rarely produced a Ct value. Of the remaining groups, no significant differences were detected in NRF2 (Figure 6), Gclc (Figure 7), Txn (Figure 8), or HO-1 (Figure 9).

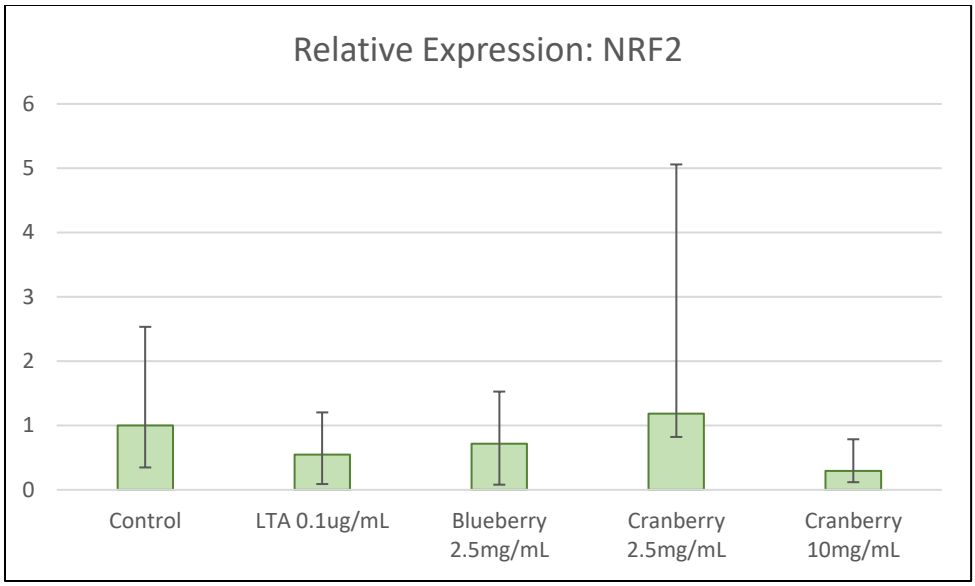


Figure 6: Effect of LTA and three concentrations of blueberry or cranberry pomace on expression of NRF2 in LMH cells. Results are shown as the result of three experiments with the means and SEM calculated.

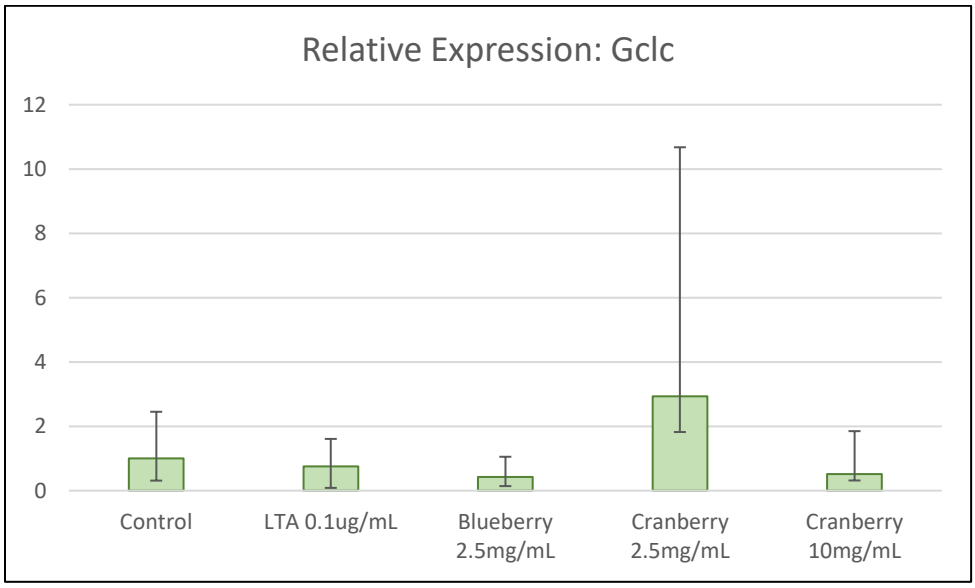


Figure 7: Effect of LTA and three concentrations of blueberry or cranberry pomace on expression of Gclc in LMH cells. Results are shown as the result of three experiments with the means and SEM calculated.

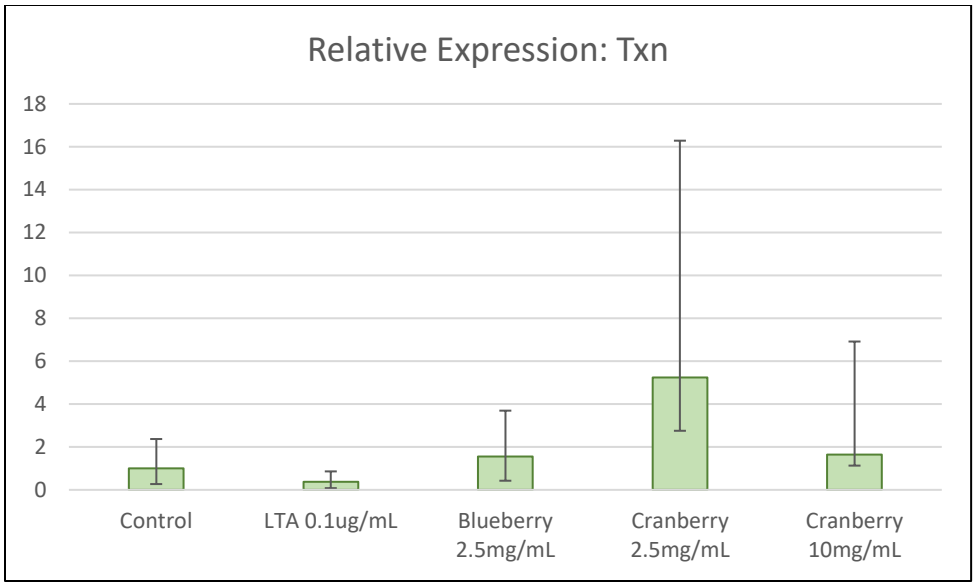


Figure 8: Effect of LTA and three concentrations of blueberry or cranberry pomace on expression of Txn in LMH cells. Results are shown as the result of three experiments with the means and SEM calculated.

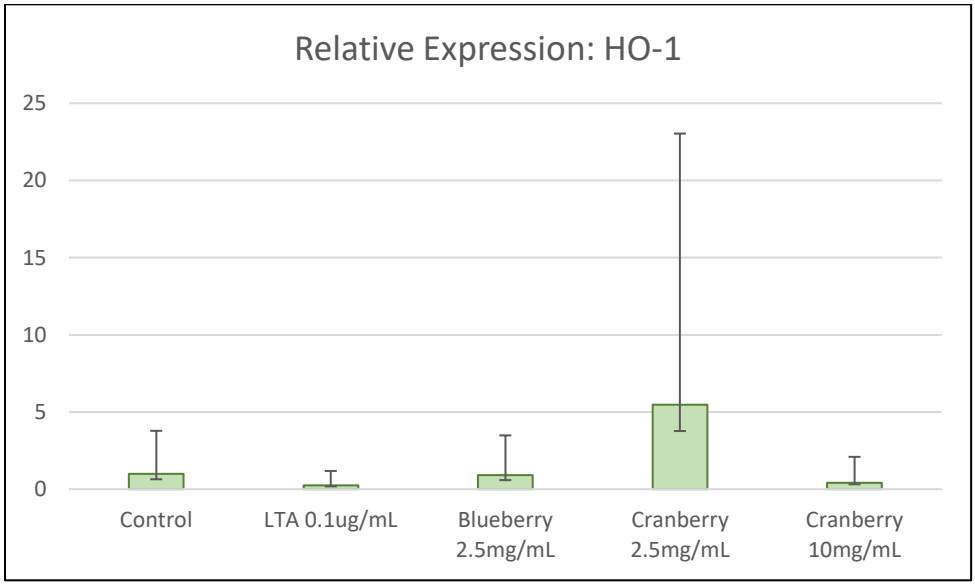


Figure 9: Effect of LTA and three concentrations of blueberry or cranberry pomace on expression of HO-1 in LMH cells. Results are shown as the result of three experiments with the means and SEM calculated.

4.6 Discussion

For the first part of this thesis, our goal was to start to work on developing an in-vitro model for necrotic enteritis infection using an immortalized chicken hepatic cell line. Liver cells are a valid subject for this type of research. Being part of the digestive system, the liver is frequently involved in processing digested compounds, and *C. perfringens* infected poultry can show signs of liver infiltration through the formation of pustules. Bacterial cell wall components can play a role in initial infection when they make contact with host cells. LTA is a cell wall component unique to gram positive bacteria and may play a role in host infection (García et al., 2019). Cells were first stimulated with LTA, a cell wall component of gram-positive bacteria such as *C. perfringens*. Results were measured through an LDH release assay, followed by the Cell Counting Kit-8 (CCK-8) assay. These are colorimetric assays which produce colour changing reactions measured using a microplate at 490nm and 450nm, respectively. Our experiments to learn the effect LTA has on LMH cells found that even at concentrations as low as 0.1ug/mL it produced a proliferative effect, increasing cell populations by approximately 150% from the control group. These results are similar to results from Hattar et al. (2017), who observed LTA stimulated proliferation of human lung cells A549. They attribute the LTA induced proliferation to IL-8 liberated from A549 cells activating adjacent A549 cells in an auto- or paracrine way. Whether the similar mechanisms are responsible for our observed results needs to be further investigated.

When LTA alone failed to produce a lethal effect on the cells we were looking for, we attempted to combine LTA with an equal proportion of peptidoglycan. Peptidoglycan, while found in both gram-positive and gram-negative bacteria as a cell wall component, exists in a much larger quantity in gram-positive bacteria. Literature review indicated that in some

instances, LTA and peptidoglycan can have a synergistic effect which greatly increases cell death (Wu et al., 2020). The two components were combined in equal ratios to see if this effect would be present, however even with peptidoglycan added the LMH cells still showed a large increase in viability from the CCK-8 test, indicating a proliferative effect.

The colour of the blueberry and cranberry extracts interfered with the colour-based viability assays, so instead we chose to see if the flavonoid quercetin would affect the cells. Quercetin is the most common polyphenol in cranberries, and one of the most common in blueberries (Govers et al., 2017; Sezer et al., 2019). We tested four concentrations, 1.5, 3.0, 15.1, and 30.2 μg/mL, and found none of these concentrations had a significant impact on cell viability or proliferation (Figure 4). However, while not statistically significant, there was a trend suggesting that the 15.1 and 30.2 μg/mL concentrations may start to have a negative impact on the cells.

Finally, we combined the lowest dose of LTA which produced a significant response with the four concentrations of quercetin. In order to examine potential effects of phenolic-rich fruit pomaces on the cells, viability tests were conducted using concentrations of quercetin, a flavonoid found in both cranberries and blueberries and known to have beneficial effects as an antioxidant. This was done as the highly pigmented fruit pomaces caused disruptions in the colour-based assays. The addition of quercetin prevented the strong proliferative effect that LTA had on the LMH cells. Further work into the exact mechanism which caused this effect is needed, however a previous paper examining the effects of quercetin on multiple cell lines found that it caused changes in regulation of apoptotic and the wnt signalling pathways (Srivastava & Srivastava, 2019).

We also wanted to examine if LTA or the fruit extracts would cause any changes in gene expression of genes of interest in previous related studies. In-vitro, it was noted that the addition of the cranberry extract had improved antioxidant status by increasing mRNA levels of Nrf, Gpx2, and HO-1 in muscle tissue (Xu et al., 2020). For RNA analysis, cells were treated with LTA, or two concentrations of blueberry and cranberry pomace. The antioxidant-related genes detected included nuclear factor E2-related factor 2 (Nrf2), glutamate cysteine ligase catalytic subunit (Gclc), Glutathione peroxidase 2 (Gpx2), thioredoxin (Txn) and heme oxygenase-1 (HO-1). B-actin was used as the reference gene. Results were calculated using delta-delta Ct analysis.

Expression of five genes related to cellular antioxidant status was tested: NRF2, Gclc, GPX2, Txn, and HO-1. The gene GPX-2 was not detected in any sample. As this gene is known to be expressed in live chicken liver tissue, it is possible that this cell line has lost the ability to express this gene (Liu et al., 2014). Literature review failed to find any mentions of the gene GPX-2 in the LMH cell line specifically. RNA purity was measured using a spectrophotometer, RNA from treatments of fruit extract often had poor nucleic purity, indicated by a 260/280 ratio under a value of 1.8. The 10mg/mL blueberry treatment very rarely produced any signal after PCR, and plant polyphenols are known to interfere with PCR experiments. This treatment group had to be dropped from the results due to high phenolic content being suspected of interfering with the PCR reactions. In addition, the 10mg/mL cranberry group produced few readings as well, so results for this group had to be calculated using fewer repetitions. The use of fruit extracts caused significant impacts on the purity of RNA leading to unclear results, further testing may require additional rinsing of cells to remove as much of the extract as possible before qPCR.

4.7 Conclusion

We found that LTA, with or without peptidoglycan, did not inhibit the viability of LMH cells, and the addition of quercetin was able to negate the proliferative effect. Further research is required to determine the exact mechanisms of action at play, and further trials using more specific toxins, such as *Clostridium perfringens* toxins, may offer a better in-vitro model of infection. In qPCR tests, the GPX-2 gene was not detected in this cell line, which may be due to lack of expression in the cell line. Further experiments are needed to confirm the effects of fruit extracts on cultured liver cells.

5 Experiment #2: In-Vivo Analysis

5.1 Introduction

Metabolomics is the study of the metabolites produced by an organism. It encompasses a vast number of small molecules (<1500Da) of all types, including both those produced by a host organism or those which enter the host system through exogenous means. An organism's metabolome consists of small molecules produced by host cellular functions, including nucleic fragments, proteins, fatty acids, and by-products of cellular processes. In an animal, the intestinal metabolome will also include compounds from the diet and microbiome, as well as host metabolized compounds from these sources.

There are multiple methods to elucidate the nature of small molecules in a sample; however, the most frequently used are mass spectrometry (MS) and nuclear magnetic resonance (NMR). Of the two, MS is able to detect smaller concentrations of compounds in a mixture than NMR. Despite increased sensitivity of MS, there are benefits to NMR that make it the preferable choice for many studies. NMR spectra have greater reproducibility than MS spectra, and MS spectra often contain unknown compounds which are not yet identifiable. NMR experiments can be one dimensional (1D), producing a linear signal, or two dimensional (2D), producing a dual axis signal. The two main types of NMR experiments are proton based to determine numbers of hydrogen atoms (1H), and carbon based (13C), focusing on carbon atoms. The most common type of experiment is 1D H NMR.

The NMR protocols for processing and analyzing simple biofluids such as blood or urine have been optimized and standardized, allowing for relatively simple and accurate analysis. The use of NMR with more heterogenous substances such as feces or soil remains a less refined

process for which standardized protocols are still being developed (Cui et al., 2020). Samples can either be frozen wet or freeze-dried to remove water content. Wet freezing of samples is thought to better preserve concentrations of metabolites; however, the freeze-drying process removes the natural water weight variation in fecal samples. A solid fecal sample needs to be processed to a liquid slurry to perform NMR; thus, fecal NMR is limited by the chosen solvent. Solvents such as water, DMSO, or ethanol affect which types of metabolites will be detected; most often water-soluble compounds are examined. Finally, different instruments or protocols may measure at different frequencies, which may produce discrepancies between experiments.

A further complication of fecal NMR is that fecal samples contain a vastly larger and more varied number of compounds than more simple fluids such as blood or urine, making analysis more difficult. The contributions of the microbiota, which can vary greatly even between animals of the same species, add to this complexity, and make cataloguing the compounds of the fecal microbiome difficult. Currently the human and murine fecal metabolomes are best understood, however progress is advancing in understanding the bovine metabolome. Very limited metabolomics studies have been conducted on poultry, however at least one study using ultra-high-performance liquid chromatography on broiler fecal samples has been conducted (Zhou et al., 2020).

Once NMR is run on a sample, the compounds in a sample are viewable as overlapping spectral data. Analysis such as principal component analysis (PCA) can be used to visualize differences in metabolome composition in a large number of samples. Compound identification and quantification is possible by comparing to references in databases. There exist both general libraries of chemicals as well as species-specific libraries such as the Human Metabolome Database and Bovine Metabolome Database. As each compound produces a unique signal, when

a known single chemical is processed through NMR, it can be used as a reference for mixed samples. Reference libraries are available both online and through analysis software. Consideration of experimental conditions must be taken when identifying compounds, as factors such as solvent, pH, and frequency used, can produce different spectral patterns for the same compound.

Metabolomics is closely related to the fields of genomics and proteomics; combining the three can help to better understand how biological pathways function. Metabolites can be mapped to biological pathways and connected to changes in gene and protein expression. While the genome of an organism is relatively stable, the metabolome is volatile, and can have large fluctuations as the organism ages, the environment around the organism changes, or even on an hour-to-hour basis as digestion progresses. The metabolome of an animal is influenced by both the animal and the microbiome of the animal. Changes in metabolite levels may be the result of either or both factors. Thus, analysis of the metabolome can be used to provide insight into the combined animal system at the molecular level. In the following experiment, we used fecal NMR to analyze the metabolome of cecal samples of chickens fed an enzyme mixture, fruit extracts, or the two combined.

5.2 Objective

The objective of the following experiment was to determine whether cranberry or blueberry pomace, with or without an enzyme mixture, influence chicken microbial metabolites. Analysis of metabolites was conducted on cecal samples using Nuclear Magnetic Resonance (NMR).

5.3 Hypothesis

The addition of dietary supplements, in the form of digestive enzymes, fruit extracts, or the two combined, would influence the poultry digestive tract and microbiome to produce changes in levels of metabolites found in the cecum.

5.4 Methodology

5.4.1 Management and Treatment

A total of 3150 1-day-old Cobb chickens were raised in a floor pen barn. The birds were randomly assigned to 10 dietary treatments with bacitracin methylene disalicylate (BMD), wild cranberry pomace (CRP) (0.5% and 1%), wild blueberry pomace (BLP) (0.5% and 1%) alone or in combination with a mixture of enzymes (7 pens/treatment, 45 chicks/pen) over a 5-week experimental period. Chicks and feed were obtained from identified sources (Agri-Marche). The blueberry pomace (about 40 kg) and cranberry pomace (40 kg) have been prepared by Kelly Ross (Summerland, BC). A mixture of enzymes (cellulase: minimum 2800 CMC units/g, mannanase: minimum 400 MAN units/g, galactanase: minimum 50 GAL units/g, xylanase: minimum 1000 XYL units/g, glucanase: minimum 600 GLU units/g, amylase: minimum 2500 FAA units/g, protease: minimum 200 HUT units/g) was obtained from a dependable commercial source (Canadian Bio-System, Calgary, Alberta, Canada). The temperature was initially set at 34°C and then gradually reduced by 2°C each week to reach 24°C at 35 days of age. Chicks were exposed to light for 24 h on the 1st day, 23 h on the 2nd, 18 h on the 3rd day, and 16 h thereafter. Starter (d1-14), grower (14-28) and finisher (28-35) diets were formulated with wheat, barley and corn as the principal cereals and soya meal as protein concentrates to meet the Cobb nutritional requirements. All experimental procedures performed in this study were approved by the Animal Care Committee of the Centre de recherche en sciences animales de Deschambault

(protocol # 1920-AV-397, CRSAD, Deschambault, QC, Canada) according to guidelines described by the Canadian Council on Animal Care (CCAC, 1993; Canadian Council on Animal Care, Ottawa, Ontario, Canada).

Cecal samples were collected on September 24-25 and October 7-8, 2019, when the birds were 21 and 35 days old, respectively. Samples were collected from the cecum of the birds. Fecal samples were stored at -80C until processing.

5.4.2 NMR Sample Processing

NMR analysis of fecal sample was done following the procedure outlined in Kim et al., 2018. In brief: samples were thawed and prepared as follows. The sample (250mg) was weighed and placed into a microcentrifuge tube along with PowerBeads and 1250uL of pH 7.4 buffer solution. Samples were vortexed for 1-2 minutes and further blended for 20-30 minutes or until fecal slurry became homogenous. Samples were centrifuged at 16,000 g for 15 minutes at a temperature of 4C. The supernatant (800 ul) was removed and stored at -80C until NMR analysis. A pool was created by mixing 20uL of each sample as an internal control. Samples were processed by Dr. Andree Gravel at the Drug Discovery Platform, Research Institute of the McGill University Health Center. Spectral processing (fourier transformation, phase correction, baseline correction, and calibration) was done automatically using TopSpin 4.4 software, integration calculations were done in TopSpin 4.4, and quantification and curve fitting was done in Chenomx NMR Suite 9.0.

5.4.3 Statistical Analysis

Data were analyzed in IBM SPSS Statistics 27, under a general linear model using univariate analysis for both time points combined. To compare time points individually, a one-way ANOVA and Welch's test were performed to test for significance, followed by Duncan's

post hoc test. Analysis was performed twice; first with all samples included and after with outliers removed. The software splits outliers into two outlier types: mild and extreme. Mild outliers are ones which fall outside the range of Q1 – 1.5*IQR or Q3 + 1.5*IQR. Extreme outliers are ones which fall outside the range of Q1 – 3*IQR or Q3 + 3*IQR.

5.5 Results

5.5.1 Relative Concentration Determined Through Curve Integration

There are multiple programs and methods by which compound concentrations can be determined using an NMR spectrum. In the processed NMR spectrum, the area under the curve of a peak (integral value) is representative of the number of protons at that location. Thus, if the NMR resonance frequency (spectral pattern) of a compound is known, and if a standard of known concentration is added to the sample, the peaks can be compared to estimate a relative concentration of a compound. In ^{1}H NMR, the area under the peak is directly representative to the number of protons (hydrogen atoms) giving rise to the signal, thus we must correct for the number of protons in a single molecule of the compound of interest. For our first experiment, we analyzed the relative concentration of acetate in each sample using TopSpin 4.4.1. Figure 10 shows a portion of a sample spectrum viewed in TopSpin. Acetate (4 proton compound) was chosen for two reasons: its NMR spectral pattern consists of a single peak at a frequency not shared by many other compounds (approximately 1.9ppm); and it tends to be the SCFA present in the highest concentration. This produces a large, easily recognizable peak. Our internal standard was 4 mM sodium 3-trimethylsilyl (2,2,3,3-d4) propionate (TSP) in D2O (99.9% D). The integral value of the acetate curve and the integral value of the TSP curve can be compared for each sample to derive a value. The following formula was used to determine the molar ratio of the two:

$$\frac{Mx}{My} = \frac{Ix}{Iy} \cdot \frac{Ny}{Nx}$$

Where Mx/My is the molar ratio, I is the integral, and N is the number of protons giving rise to the signal

Example calculation (For the spectrum shown in Figure 10):

TSP has 13 protons, while acetate has 4 protons.

Integral value of TSP (y): 1 (set to 1 via calibration option)

Integral value of Acetate (x): 13.1821

Ratio of x/y = $\frac{(13.1821)(4)}{(1)(13)}$ = 4.0560

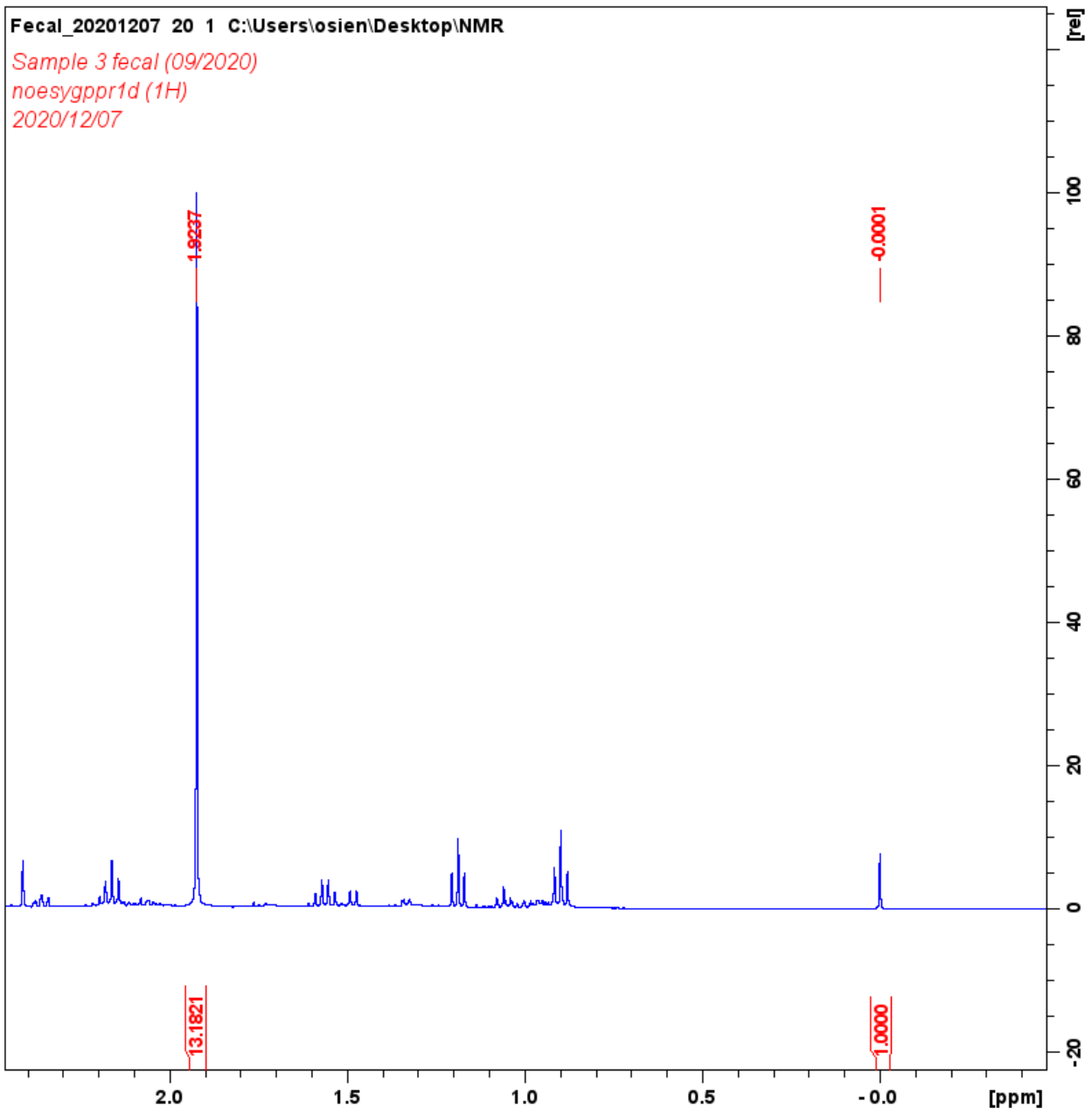


Figure 10: Sample showing acetate at approximately 1.9ppm with integral value of 13.1821, and TSP at 0.0ppm with integral value of 1.0000.

The results of the relative acetate quantities for all 10 groups, at day 21 and day 35, are summarized in Table 4. At the day 21 sampling point, very little group variability was observed, suggesting the dietary treatments have no significant effect on acetate levels. By day 35, there was still no significant differences between groups however the individual variation between

samples was greater. There were no outlies on the day 35, while eight outliers existed for day 21, suggesting the need for removal of outliers.

a. Before Removal of Outliers

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	2.658	0.417
	35	6	2.430	0.681
Cranberry 0.5% + Enzyme	21	7	3.118	0.558
	35	6	3.086	0.626
Cranberry 1% + Enzyme	21	4	2.539	0.296
	35	7	3.612	0.425
Blueberry 0.5% + Enzyme	21	6	2.649	0.514
	35	6	3.396	0.389
Blueberry 1% + Enzyme	21	7	2.884	0.344
	35	6	3.687	0.391
Control	21	6	3.035	0.2183
	35	7	2.992	0.397
Cranberry 0.5%	21	7	2.533	0.438
	35	5	3.856	0.278
Cranberry 1%	21	6	2.839	0.594
	35	6	3.384	0.525
Blueberry 0.5%	21	7	2.638	0.158
	35	6	3.8007	0.430
Blueberry 1%	21	6	2.226	0.175
	35	5	4.053	0.429

Table 4: Summary table of relative concentrations of acetate during the two sampling points, before removal of outliers.

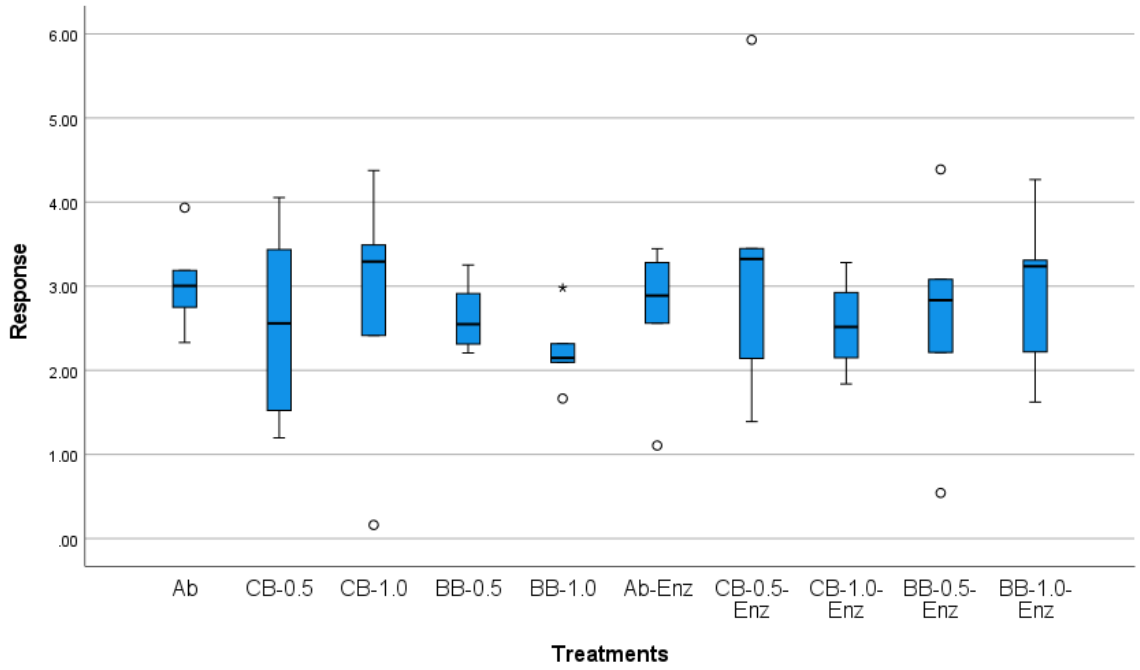


Figure 11: Relative concentrations of acetate at day 21. The chart shows data visualized from Table 4. Relative levels of acetate were determined by comparison to an internal standard of a known concentration. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

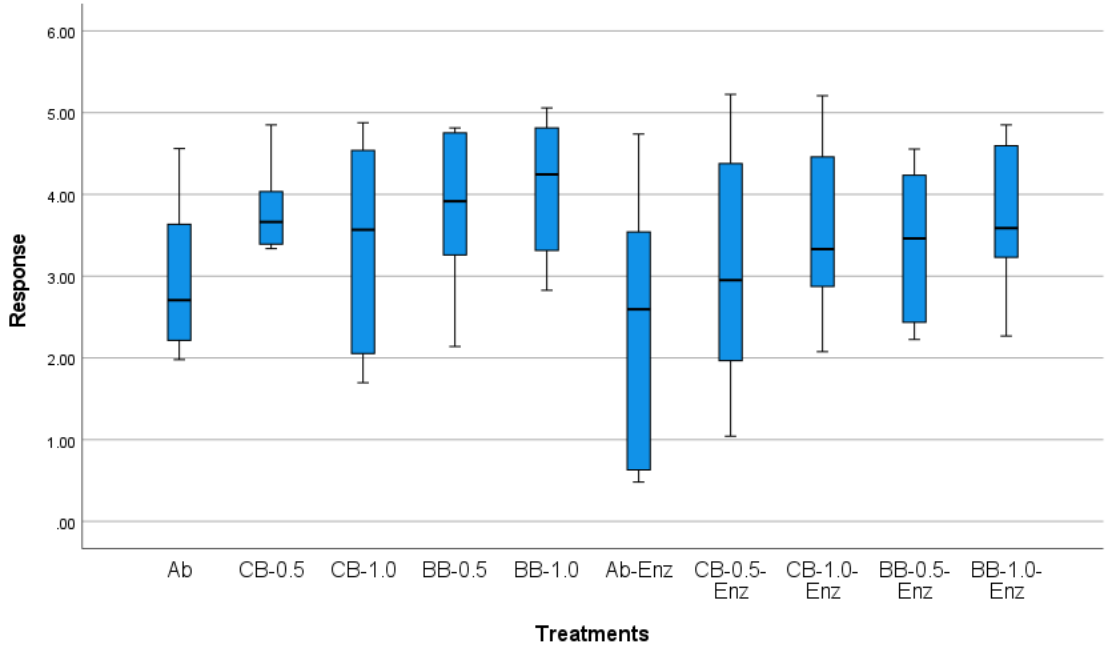


Figure 12: Relative concentrations of acetate at day 35. The chart shows data visualized from Table 4. Relative levels of acetate were determined by comparison to an internal standard of a

known concentration. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

b. After Removal of Outliers

The results of the relative acetate quantities after removal of outliers for all 10 groups, at day 21, are summarized in Table 5. The results from this table are visualized in Figure 13. For day 21, after removal of 8 outliers, significant differences between groups were detected through the Welch's test. Here, only two groups were significantly different from one another; the CB 1.0 group had a significantly higher concentration than the BB 1.0 group; however neither of these groups were significantly different from any of the other groups including the control, suggesting that none of the treatment groups have significant effects on acetate levels when compared to a traditional antibiotic treatment.

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	4	3.044	0.199
Cranberry 0.5% + Enzyme	21	6	2.649	0.358
Cranberry 1% + Enzyme	21	4	2.539	0.296
Blueberry 0.5% + Enzyme	21	4	2.741	0.189
Blueberry 1% + Enzyme	21	7	2.884	0.344
Control	21	5	2.856	0.152
Cranberry 0.5%	21	7	2.533	0.438
Cranberry 1%	21	5	3.374	0.314
Blueberry 0.5%	21	7	2.638	0.158
Blueberry 1%	21	4	2.178	0.049

Table 5: Relative concentration of acetate after removal of outliers.

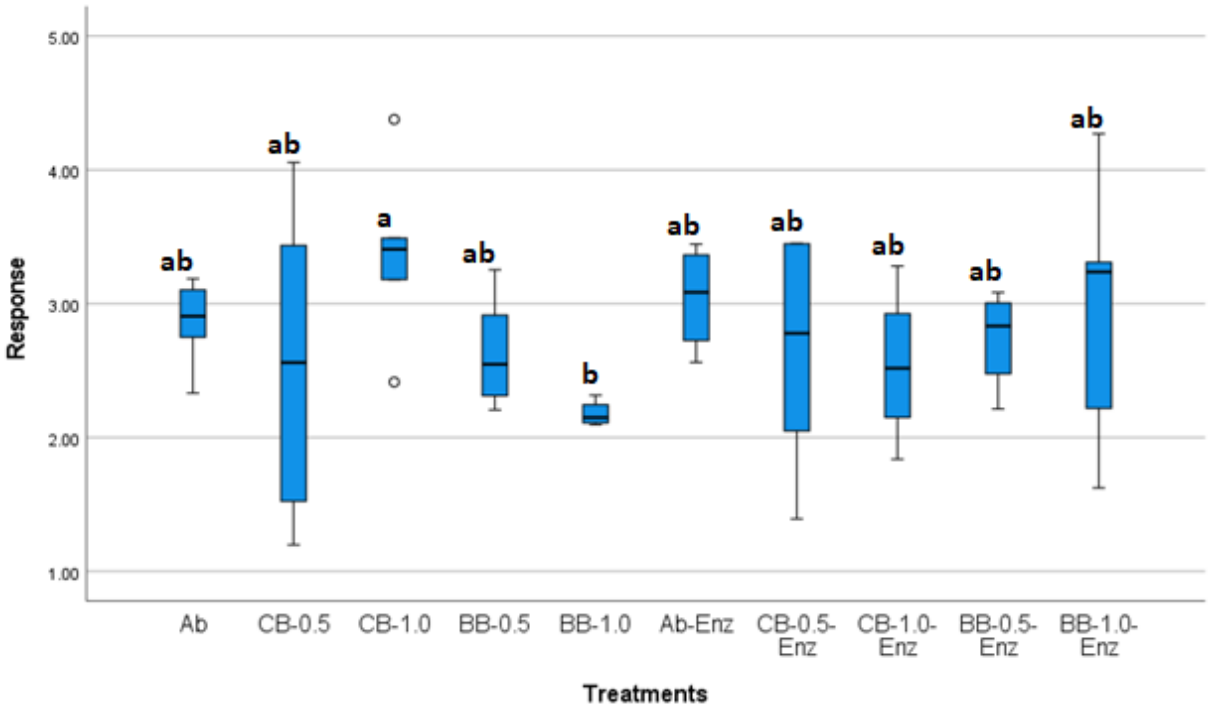


Figure 13: Relative concentrations of acetate at day 21, after removal of 8 outlier points. The chart shows data visualized from Table 5. Relative levels of acetate were determined by comparison to an internal standard of a known concentration. Groups that share letters indicate no significant differences. After removal of the initial outliers, two new mild outliers were formed in the CB-1.0 group. Mild outliers are marked by a °. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

5.5.2 Quantification Through Curve Fitting

The previous method works well for compounds with a small number of recognizable peaks in fluids with low complexity. Fecal samples however can contain hundreds of compounds which can have dozens of peaks. This creates a complicated spectrum which is best handled with a combination of software and human analysis. We used Chenomx software to create a table of compounds which had spectral patterns matching sample spectra and chose eight metabolites which were present in significant quantities and relevant to microbial activity and digestive health: acetate, butyrate, ethanol, glutamate, propionate, valerate, glucose, and glucose-6-phosphate. Chenomx sorts compounds with clusters which can be singlet, doublet, triplet, etc;

the ppm location is the center of each cluster determined by the software. The software-determined cluster centers of our chosen metabolites of interest are shown in Table 6.

Metabolite	Cluster Centers (ppm)
Acetate	**1.90**
Butyrate	0.88175, 1.54562, 2.14602
Ethanol	**1.17265**, 3.64860
Glutamate	**2.03890, 2.12471, 2.32104, 2.36432**, 3.74798
Propionate	**1.04316**, 2.17134
Valerate	0.87602, 1.28796, **1.51318**, 2.16921
Glucose	3.23452, 3.39160, 3.40368, 3.45608, 3.48243, 3.52659, 3.70545, 3.71647, 3.75569, 3.81808, 3.83659, 3.89193, **5.22546**
Glucose-6-Phosphate	3.26597, 3.49516, 3.49741, 3.56495, 3.57349, 3.57665, 3.71888, 3.87380, 3.91435, 3.98656, 4.03605, **5.22342**

Table 6: Cluster centers of metabolites of interest identified by Chenomx. Bold values indicate chosen clusters of focus during fitting process.

Using Chenomx, we overlayed metabolites of interest to identify regions of high and low overlap. The software automatically determines the maximum concentration of each compound spectrum identified; however these maximum concentrations do not account for spectral overlap, which may cause artificial inflation of values. We decided on an order to fit compounds on the sum spectrum and performed the fitting strategy on each sample in the same way. Figures 14, 15, 16, and 17 show the peaks of interest of our chosen metabolites highlighted with their positions on the X-axis visible.

Order of fitting:

1. Fit Glutamate
2. Fit Butyrate
3. Fit Propionate
4. Fit Ethanol
5. Fit Valerate
6. The list was cleared to fit the final three compounds

7. Fit acetate

8. Fit glucose & glucose-6-phosphate

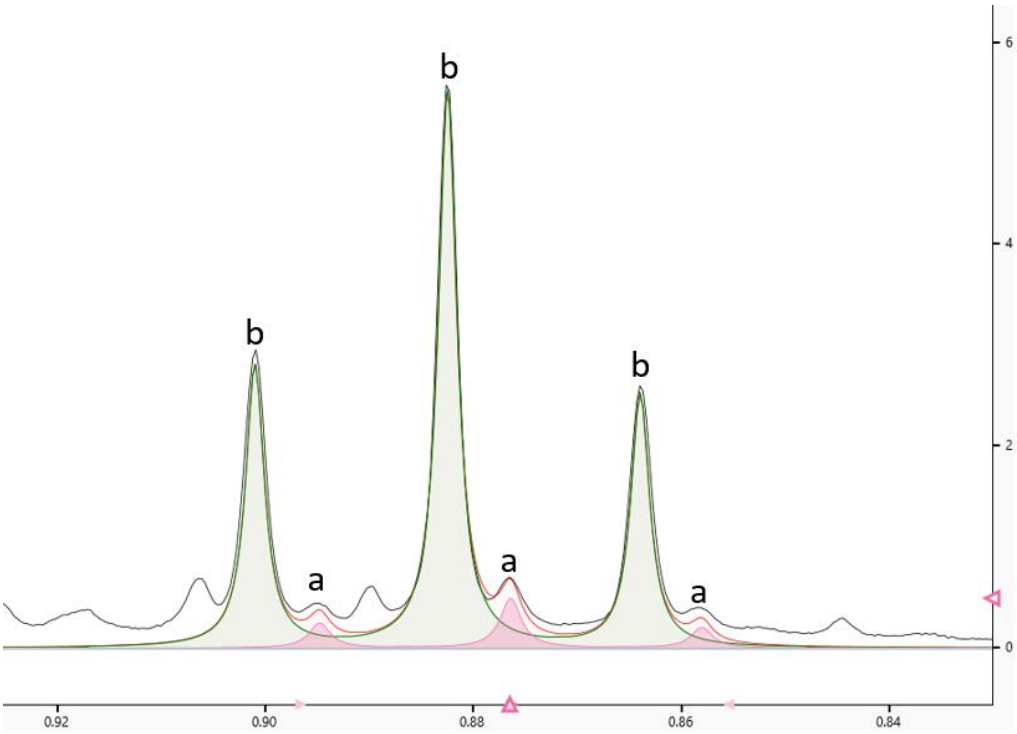


Figure 14: The fitting pattern of butyrate (b) and valerate (a).

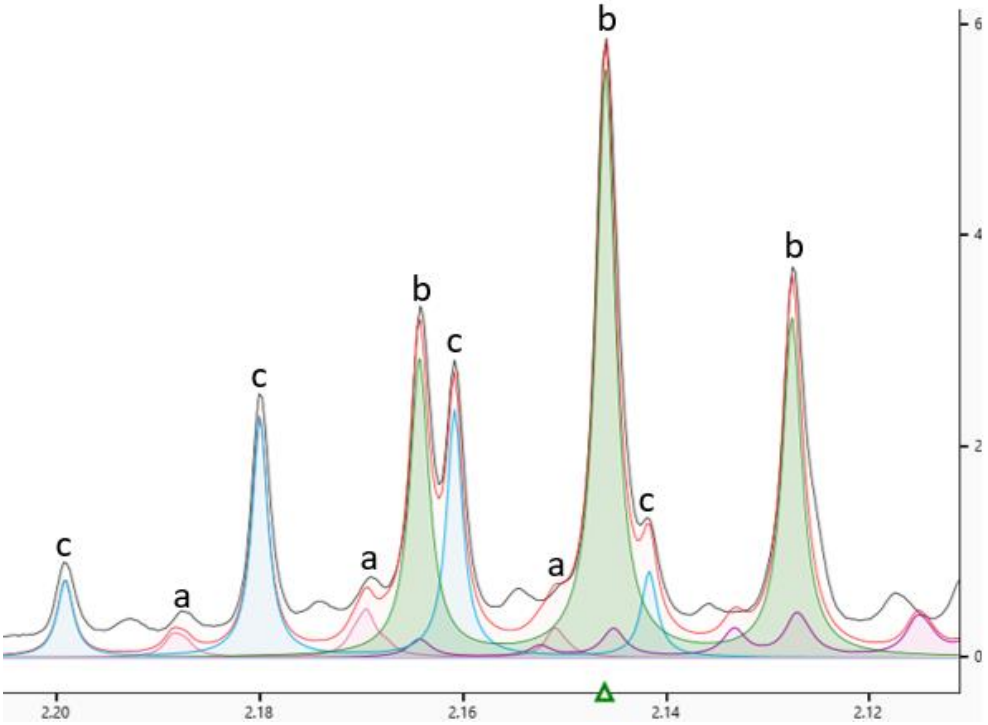


Figure 15: Fitting of butyrate (b), valerate (a), propionate (c).

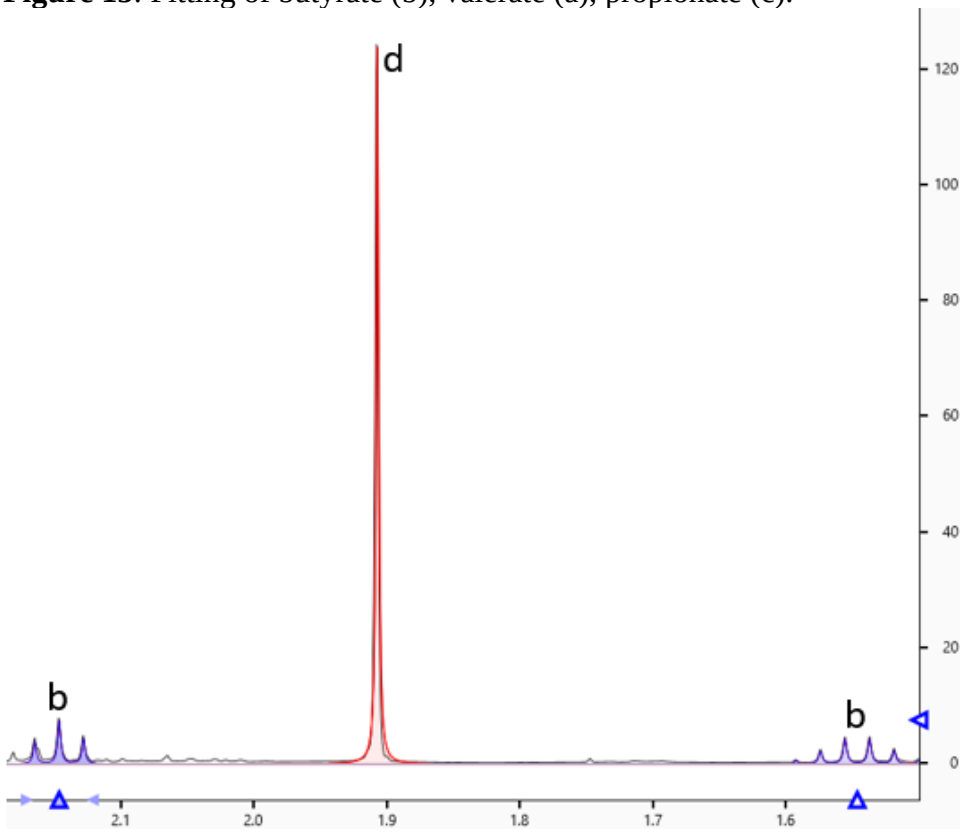


Figure 16: Fitted acetate (d) with concentration 202.7540mM, contrasted with butyrate (b)

concentration 44.1769mM; concentrations are determined by the total area under the curve of all peaks a compound consists of.

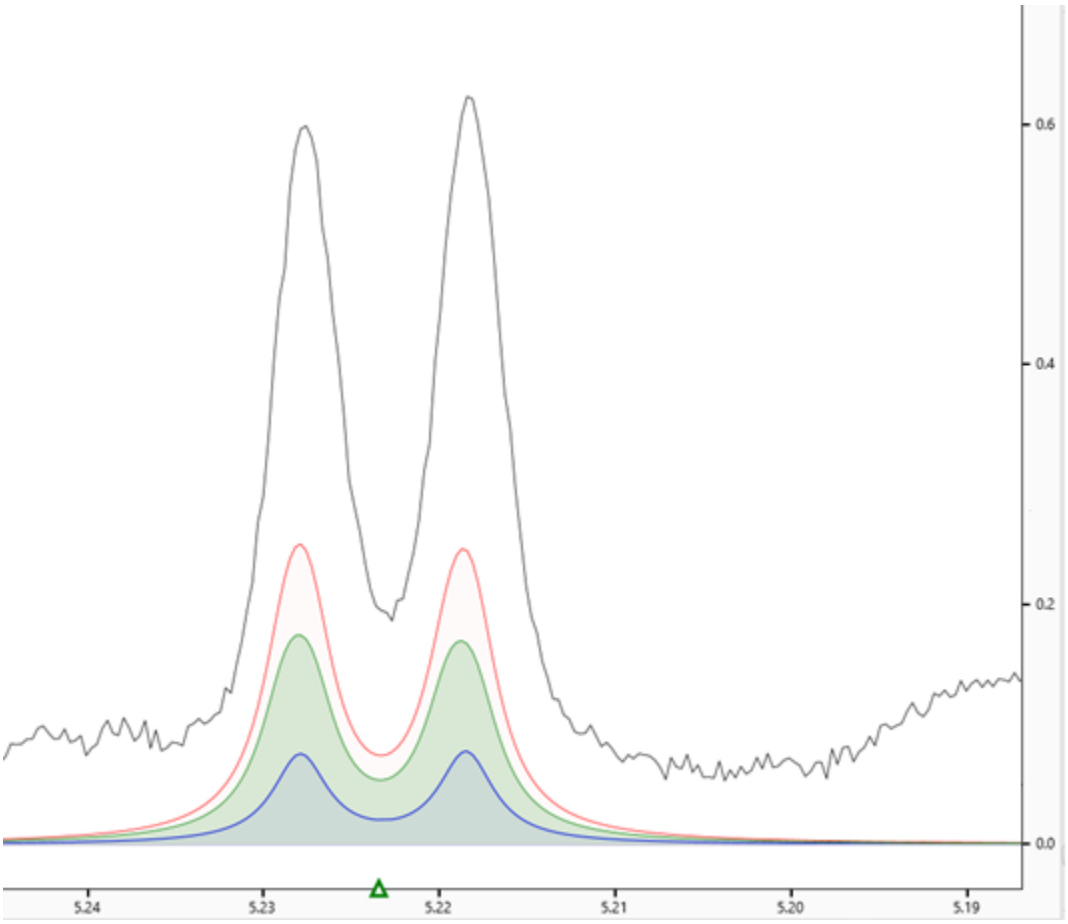


Figure 17: This doublet is shared by glucose-6-phosate and glucose; but not by the other named glucose compounds in the table. The blue line represents glucose while the green line represents glucose-6-phosphate. The red line is the sum of the two doublets. Both compounds have been matched to under their maximum, by looking in the other regions with signal and making sure they do not go above the spectrum line in their sum fit. The spectrum line is the produced signal: because in this case it is so much higher than the sum line, this means that signal is being produced by other compounds. Thus, the fitted sum line can only serve as an educated estimate.

For the initial statistical analysis, levels of each metabolite were calculated for two time points for 10 groups. A one-way ANOVA, followed by a Welch's unequal variances test was conducted to determine statistical significance. Duncan's test was used to determine significant differences between groups. The analysis was repeated a second time after removal of outliers

detected by the software. In SPSS, an outlier is defined as a value outside of 1.5X the interquartile range (IQR).

For the most part, removal of outliers did not change statistical results; however, the following groups showed statistically different results between groups: Acetate (September), Ethanol (Day 21), and Glucose (Day 35). None of the three showed significant differences from the control (antibiotic) group, however differences between other treatments were observed. In the Acetate group for the month of September, the blueberry (BB) 1% group was significantly lower than the cranberry (CB) 1% and antibiotic + enzyme groups. These results are similar to the differences detected in the relative quantification of acetate done previously. In the Ethanol group at the day 21 time point, the CB 1%, BB 0.5%, and CB 1% + enzyme groups were characterized by a large sample variance with a range that included higher values; the other groups tended to show lower concentrations of ethanol with less sample variance. In the glucose group at day 35, the BB 0.5% + enzyme group, CB 0.5% + enzyme, and antibiotic group showed a larger sample variance than other treatments; the BB 0.5% + enzyme group was significantly different from CB 0.5%, CB 1%, BB 0.5%, BB 1%, Antibiotic + enzyme, CB 1% + enzyme, and BB 1% + enzyme groups.

Next, a full model analysis was performed using a general linear model. The following formula was used to define the model:

$Y = u + trt + time + trt * time + e$

Where u= average, trt= treatment group, time= time point, e= error

A time effect was observed for some metabolites. Acetate, butyrate, propionate, and valerate were all significantly higher day 35 than day 21. Ethanol levels were significantly higher

at day 21 and decreased by day 35. Since there was strong effect of time, the differences among treatments were also investigated for each time point, using the following model:

$Y = u + trt + e$

Where u=average trt= treatment group, e=error.

a. Acetate

The mean concentration of acetate for all ten groups at two time points is visualized in Figure 18. Individual time point analysis was conducted by splitting the two time points and doing separate analysis. These results are summarized in Table 7 and are visualized in Figures 19 and 19. No significant differences between groups were detected in acetate levels during either time point. Seven outliers were removed, and these results are summarized in Table 8. After removal of outliers, differences between groups were observed at day 21 (Figure 21). Here, we saw that the CB 1.0 and Ab + Enz groups had significantly higher concentration than the BB 1.0 group, however none of these groups were significantly different from the antibiotic control, suggesting that none of the treatment groups have significant effects on acetate levels when compared to a traditional antibiotic treatment. At day 35, no significant differences between groups were found, and no outlier points were identified. The day 21 results mirror the previous results for acetate which were determined using TopSpin.

i. General Linear Model Analysis

Y= u + trt + time + trt *time + e

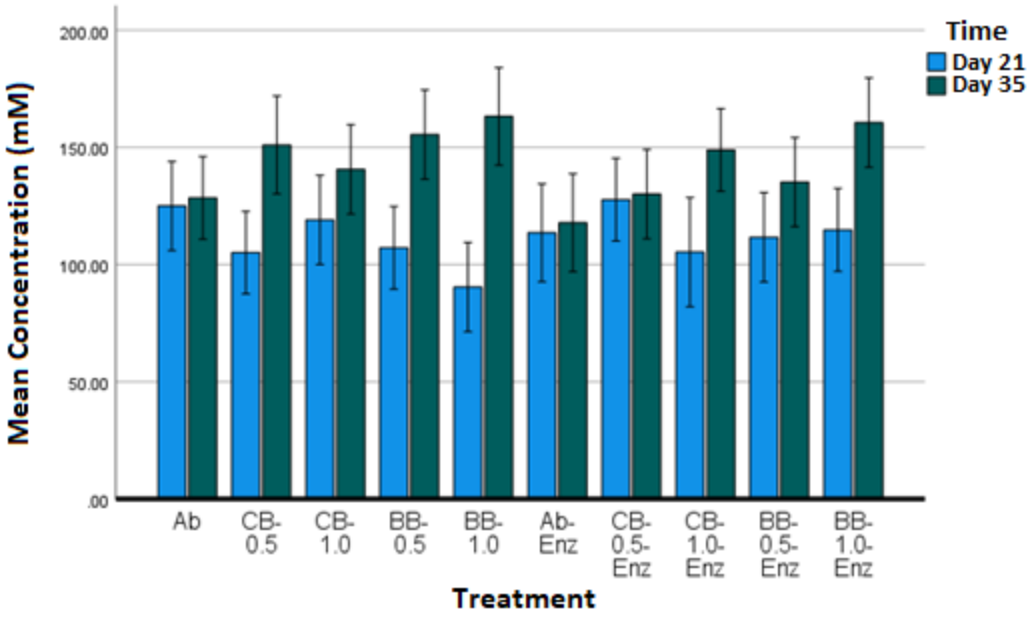


Figure 18: Mean concentrations of acetate at day 21 and day 35. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

ii. Individual Time Point Analysis (Complete)

Using ANOVA and Welch's tests, no significant differences between groups were observed for either time point.

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	113.579	18.431
	35	5	117.908	30.101
Cranberry Low + Enzyme	21	7	127.735	24.424
	35	6	130.102	28.855
Cranberry High + Enzyme	21	4	105.357	13.926
	35	7	148.928	17.712
Blueberry Low + Enzyme	21	6	111.681	22.264
	35	6	135.240	16.138
Blueberry High + Enzyme	21	7	114.821	14.024
	35	6	160.623	19.073

Control	21	6	125.032	9.687
	35	7	128.495	15.156
Cranberry Low	21	7	105.179	19.133
	35	5	151.052	9.238
Cranberry High	21	6	119.093	25.599
	35	6	140.656	24.448
Blueberry Low	21	7	107.208	8.404
	35	6	155.512	16.608
Blueberry High	21	6	90.438	8.497
	35	5	163.257	15.421

Table 7: Levels of acetate during two time points, before removal of outliers.

Results from Y= u + trt + e for day 21 and day 35, separately.

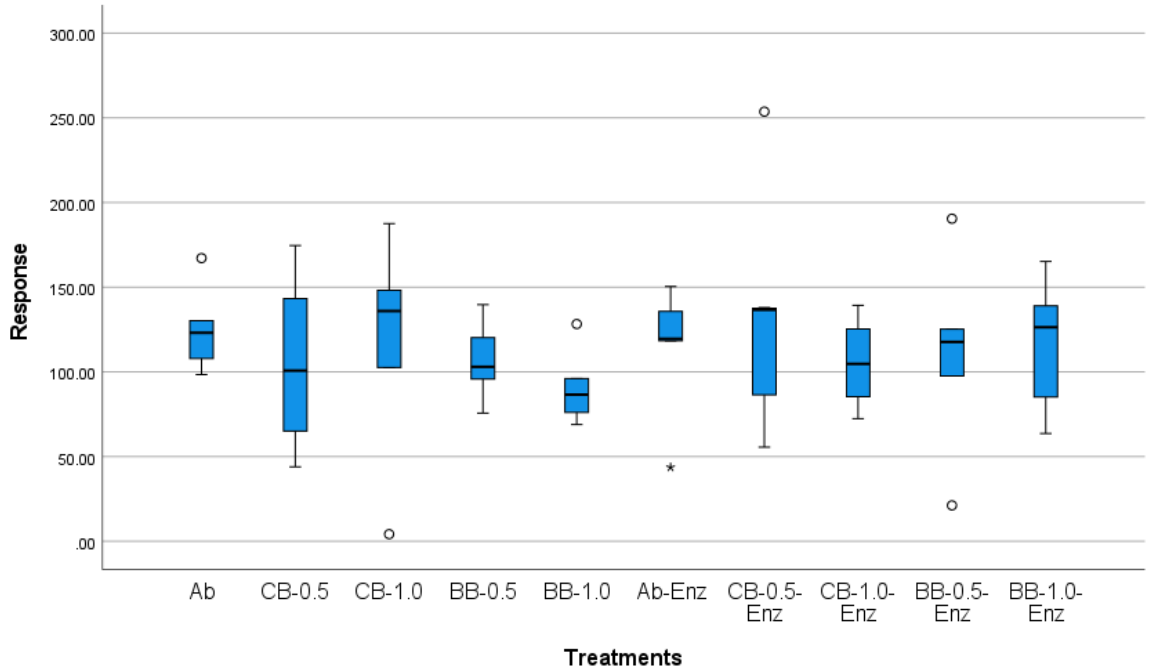


Figure 19: Concentrations of acetate, in mM, at day 21. The chart shows data visualized from Table 7. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

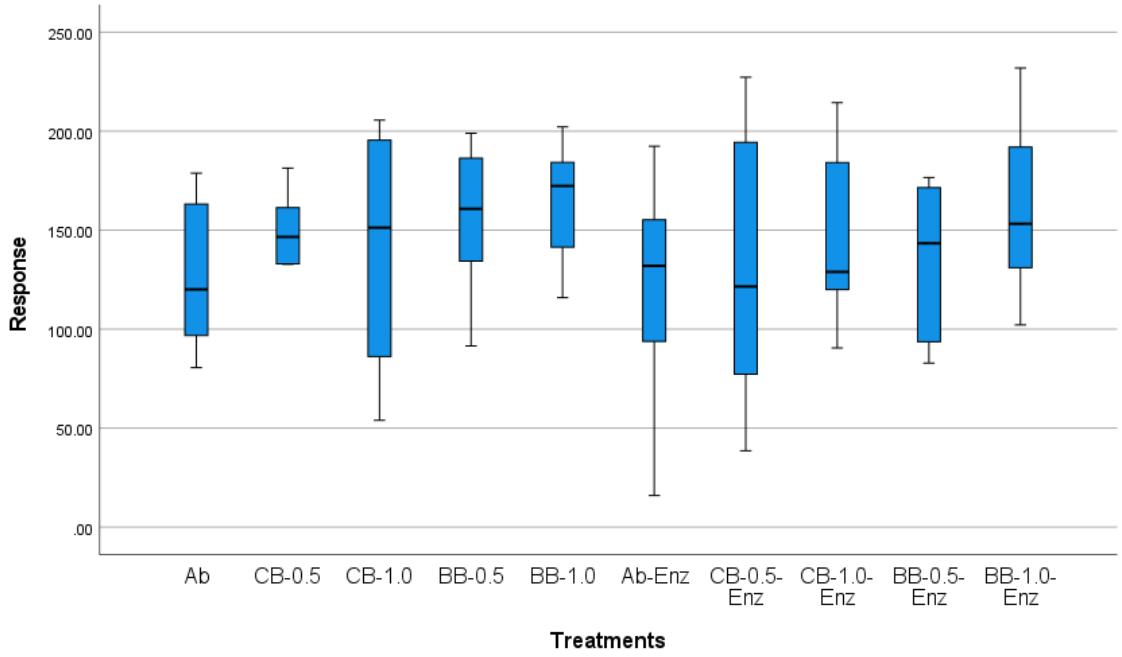


Figure 20: Concentrations of acetate, in mM, at day 35. The chart shows data visualized from Table 7. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

iii. Individual Time Point Analysis (Outliers Removed)

No outliers detected at day 35 so analysis was not done. Significant differences found at day 21 through Welch's test.

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	4	131.0450	7.58976
Cranberry Low + Enzyme	21	6	106.7417	14.77243
Cranberry High + Enzyme	21	4	105.3550	13.92619
Blueberry Low + Enzyme	21	4	114.5975	6.08162
Blueberry High + Enzyme	21	7	114.8229	14.02458
Control	21	5	116.6000	5.83927
Cranberry Low	21	7	105.1771	19.13401
Cranberry High	21	5	142.0660	13.83576
Blueberry Low	21	7	107.2071	8.40342
Blueberry High	21	4	82.8680	4.72529

Table 8: Acetate concentrations after the removal of outliers.

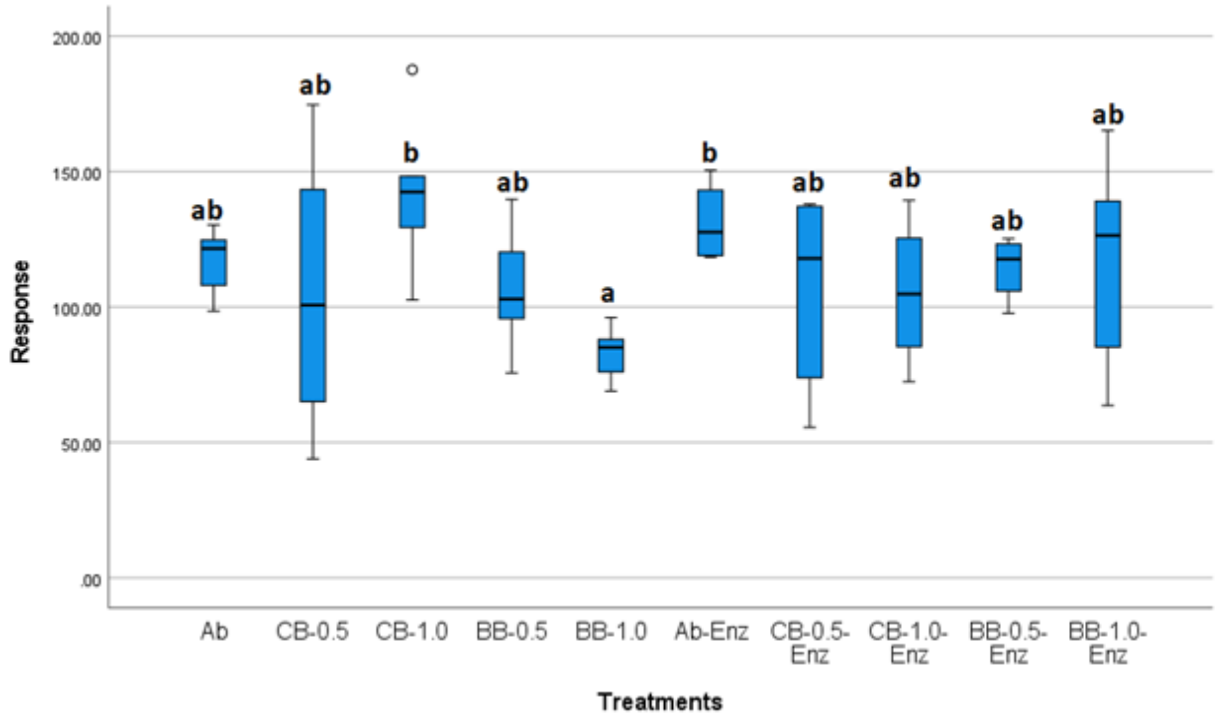


Figure 21: Concentrations of acetate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 8. Groups that share letters indicate no significant differences.

After removal of the initial outliers, one new outlier was formed in the CB-1.0 group. Mild outliers are marked by a °. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

b. Butyrate

The mean concentration for all ten groups at two time points is visualized in Figure 22. Individual time point analysis was conducted by splitting the two time points and doing separate analysis. These results are summarized in Table 9 and are visualized in Figures 23 and 24. Before removal of outliers, there were no significant differences between groups detected. The results after removal of 3 (day 21) and 2 (day 35) outliers are summarized in Table 10.No significant differences between groups were detected at either day 21 (Figure 25) nor day 35 (Figure 26). This suggests that none of the treatment groups have significant effects on butyrate levels when compared to a traditional antibiotic treatment.

i. General Linear Model Analysis

$Y = u + trt + time + trt * time + e$

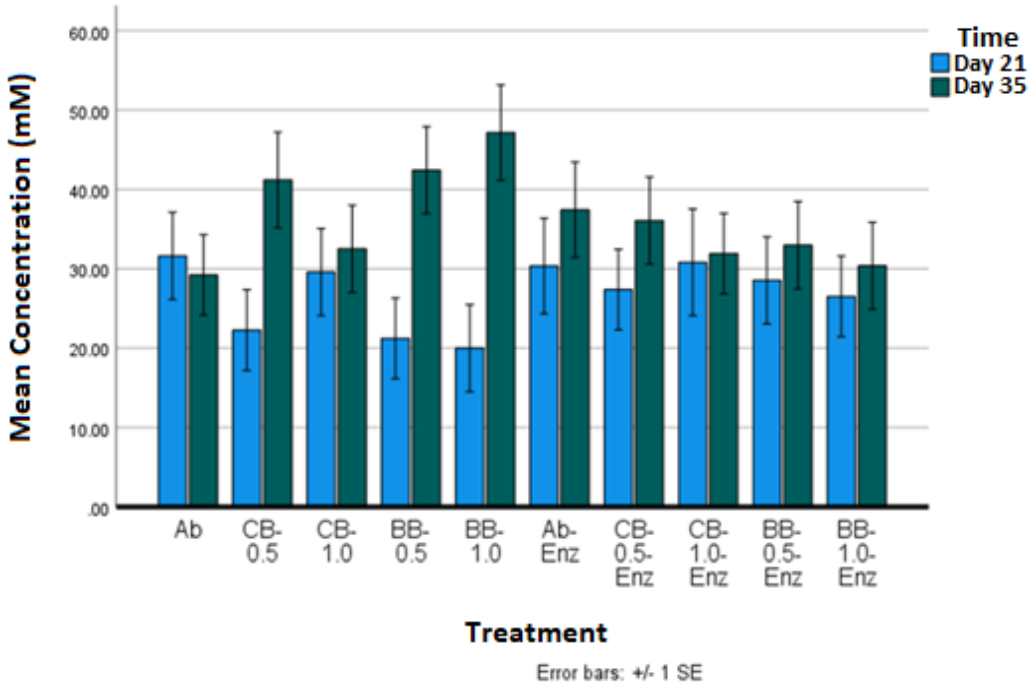


Figure 22: Mean concentrations of butyrate at day 21 and 35. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

ii. Individual Time Point Analysis (Complete)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	30.3465	5.74093
	35	5	37.4471	10.98253
Cranberry Low + Enzyme	21	7	27.3856	5.72967
	35	6	36.0861	10.02866
Cranberry High + Enzyme	21	4	30.8190	7.57281
	35	7	31.9252	4.31801
Blueberry Low + Enzyme	21	6	28.5563	6.26062
	35	6	32.9790	5.31502
Blueberry High + Enzyme	21	7	26.5251	3.50194
	35	6	30.3848	4.64510
Control	21	6	31.6269	2.36210
	35	7	29.2170	3.90165
Cranberry Low	21	7	22.2766	4.35838

	35	5	41.2032	2.77701
Cranberry High	21	6	29.5852	6.22834
	35	6	32.5261	5.90285
Blueberry Low	21	7	21.2425	2.87873
	35	6	42.4294	5.54489
Blueberry High	21	6	20.0032	1.47186
	35	5	47.1769	5.70648

Table 9: Concentrations of butyrate for two time points.

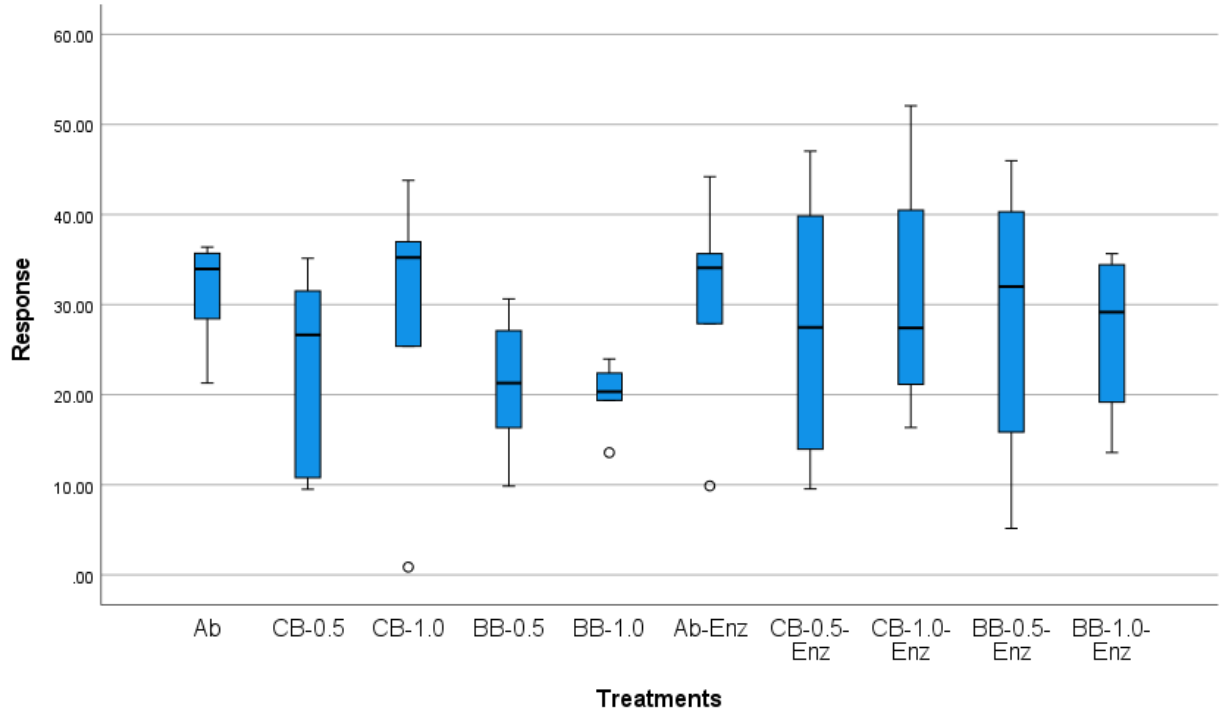


Figure 23: Concentrations of butyrate, in mM, at day 21. This chart shows data visualized from Table 9. Mild outliers are marked by a °. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

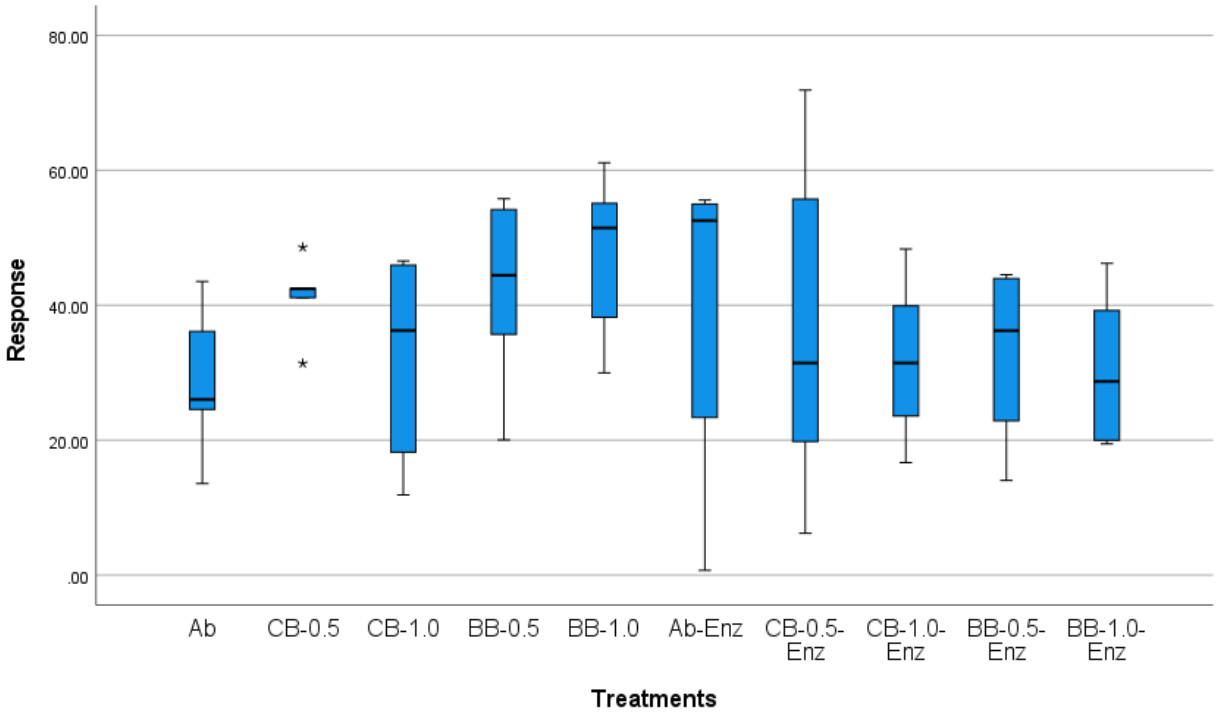


Figure 24: Concentrations of butyrate, in mM, at day 35. This chart shows data visualized from Table 9. Extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

iii. Individual Time Point Analysis (Outliers Removed)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	4	35.4625	3.36422
	35	5	37.4471	10.98253
Cranberry Low + Enzyme	21	7	27.3871	5.72945
	35	6	36.0861	10.02866
Cranberry High + Enzyme	21	4	30.8200	7.57402
	35	7	31.9252	4.31801
Blueberry Low + Enzyme	21	6	28.5567	6.26087
	35	6	32.9790	5.31502
Blueberry High + Enzyme	21	7	26.5257	3.50242
	35	6	30.3848	4.64510
Control	21	6	31.6267	2.36310
	35	7	29.2170	3.90165
Cranberry Low	21	7	22.2771	4.35868

	35	3	42.0134	.44956
Cranberry High	21	5	35.3280	2.95170
	35	6	32.5261	5.90285
Blueberry Low	21	7	21.2443	2.87864
	35	6	42.4294	5.54489
Blueberry High	21	5	21.2860	.87676
	35	5	47.1769	5.70648

Table 10: Butyrate concentrations for two time points after the removal of outliers.

While significance was determined by ANOVA and Welch for September, no significance was found in the post-hoc test. The program created two new outliers in this data automatically after initial outliers were removed. No significant differences for October were observed.

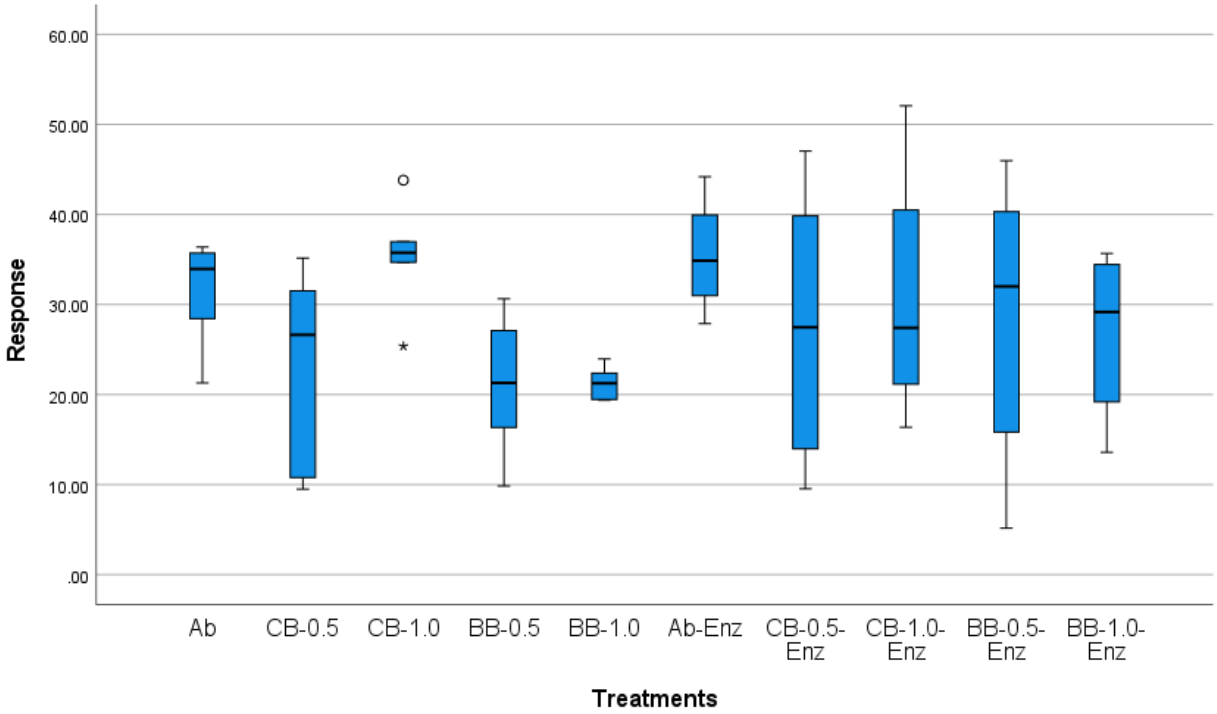


Figure 25: Concentrations of butyrate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 10. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

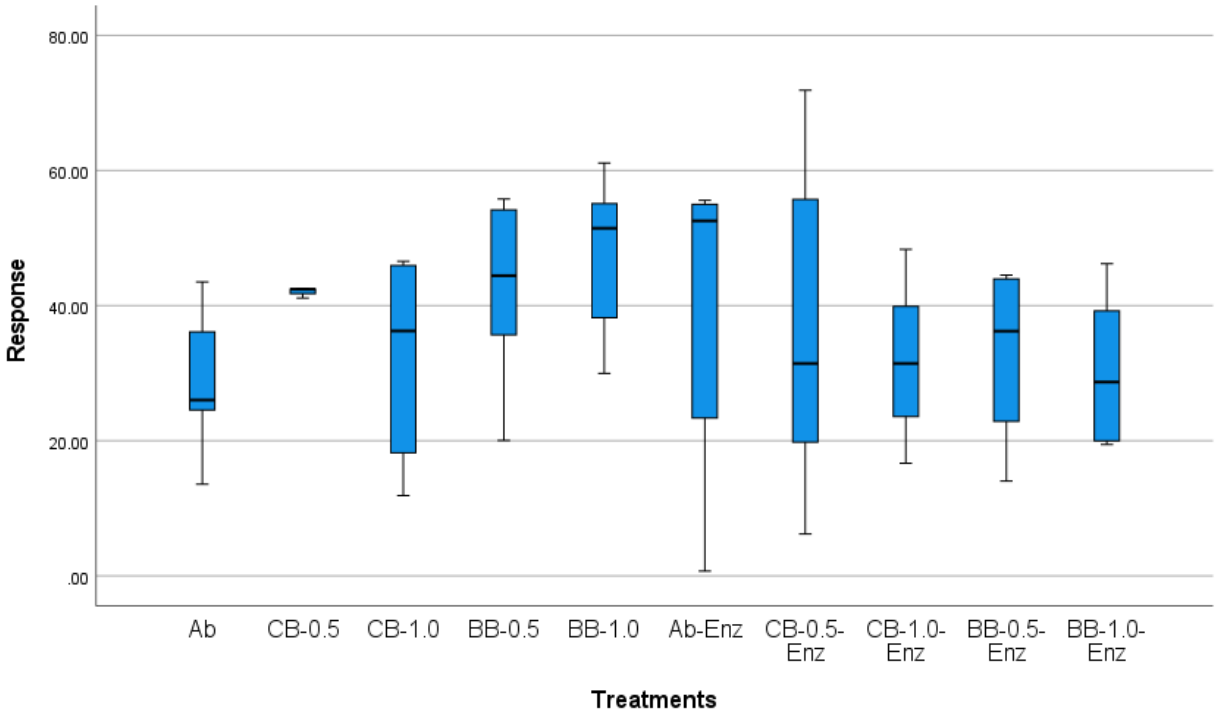


Figure 26: Concentrations of butyrate, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 10. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

c. Ethanol

The mean concentrations for all ten groups at two time points is visualized in Figure 27. Individual time point analysis was conducted by splitting the two time points and doing separate analysis. These results are summarized in Table 11 and are visualized in Figures 28 and 29. Before removal of outliers, there were no significant differences between groups detected. The results after removal of 3 (Day 21) and 4 (Day 35) outliers are summarized in Table 12. There were no significant differences between groups observed at day 35 (Figure 31). Significant differences between groups were observed at day 21 only. Here, we saw that the CB 0.5, CB 1.0, BB 0.5, and CB 1.0 + Enz had significantly higher ethanol concentrations than the BB 1.0, Ab + Enz, CB 0.5 + Enz, and BB 0.5 + Enz groups (Figure 30). No groups were significantly different

from the antibiotic control however, suggesting that none of the treatment groups have significant effects on ethanol levels when compared to a traditional antibiotic treatment.

i. General Linear Model Analysis

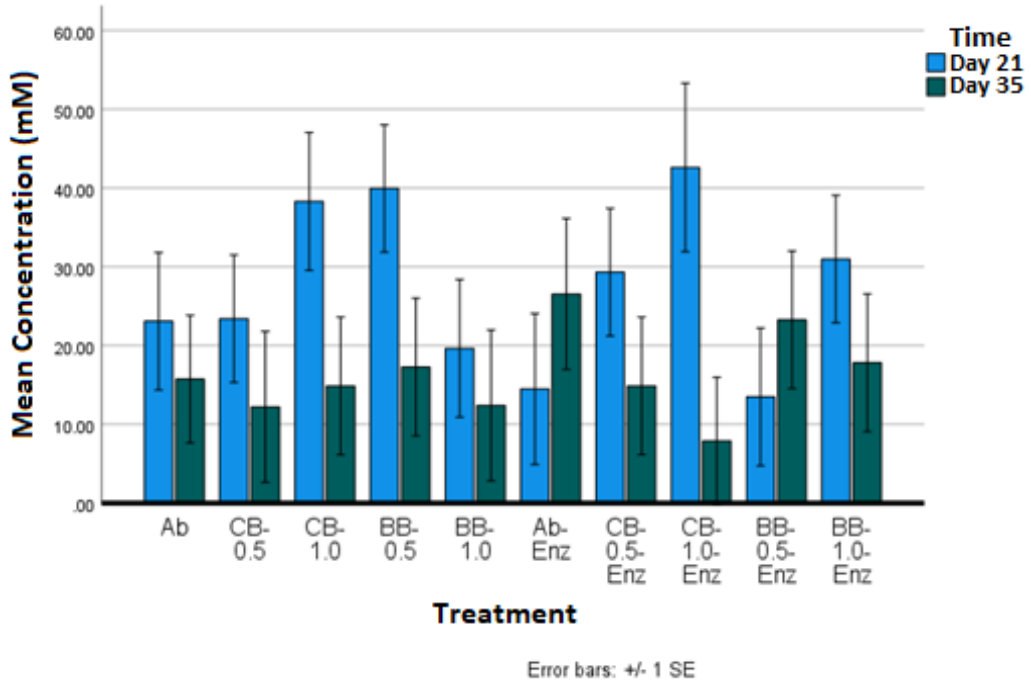


Figure 27: Mean concentrations of ethanol at day 21 and day 35. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

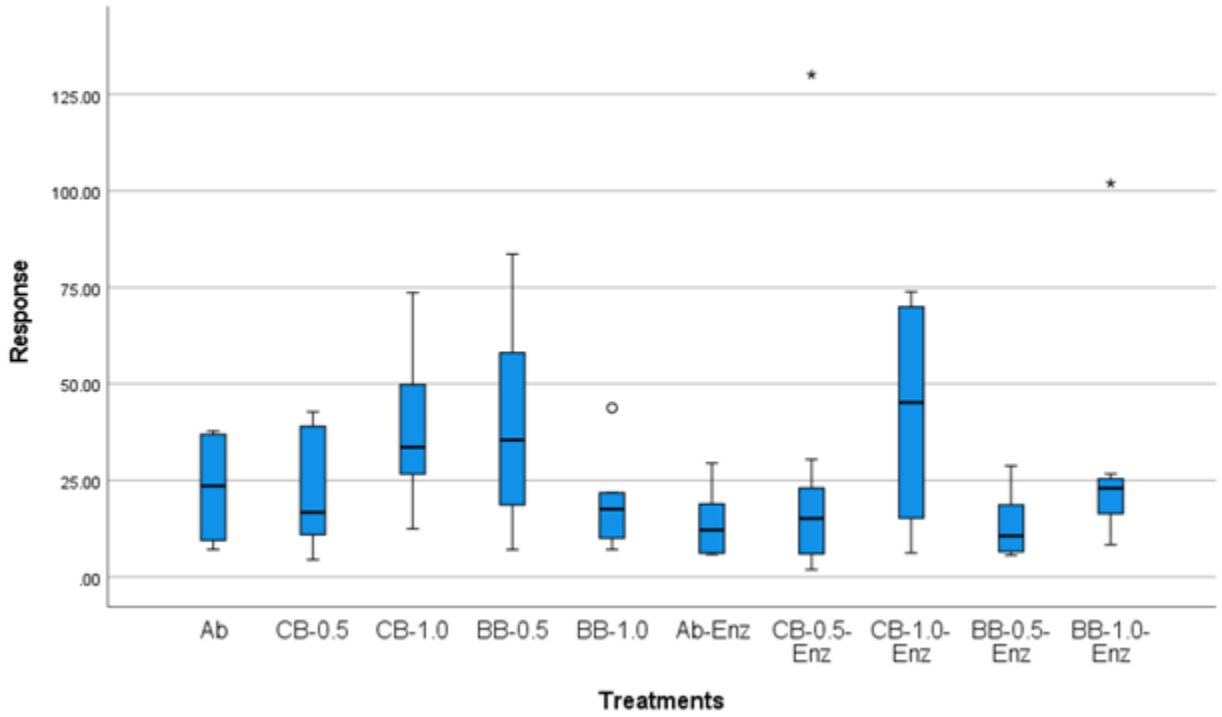
ii. Individual Time Point Analysis (Complete)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	14.4778	4.44120
	35	5	26.5454	16.68012
Cranberry Low + Enzyme	21	7	29.3124	17.17732
	35	6	14.8728	3.91558
Cranberry High + Enzyme	21	4	42.6116	16.29936
	35	7	7.8725	1.31441
Blueberry Low +	21	6	13.4683	3.63649

Enzyme	35	6	23.2675	7.64879
Blueberry High +	21	7	30.9772	12.09136
Enzyme	35	6	17.8056	4.34487
Control	21	6	23.0837	5.32407
	35	7	15.7307	5.28811
Cranberry Low	21	7	23.4082	6.24127
	35	5	12.2094	3.56289
Cranberry High	21	6	38.3029	8.66992
	35	6	14.8644	5.29249
Blueberry Low	21	7	39.9513	11.49137
	35	6	17.2726	6.44983
Blueberry High	21	6	19.6370	5.32365
	35	5	12.3900	3.80049

1572 **Table 11:** Concentrations of ethanol during two time points.

1573



1574

1575 **Figure 28:** Concentrations of ethanol, in mM, at day 21. This chart shows data visualized from

1576 Table 11. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab =

antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

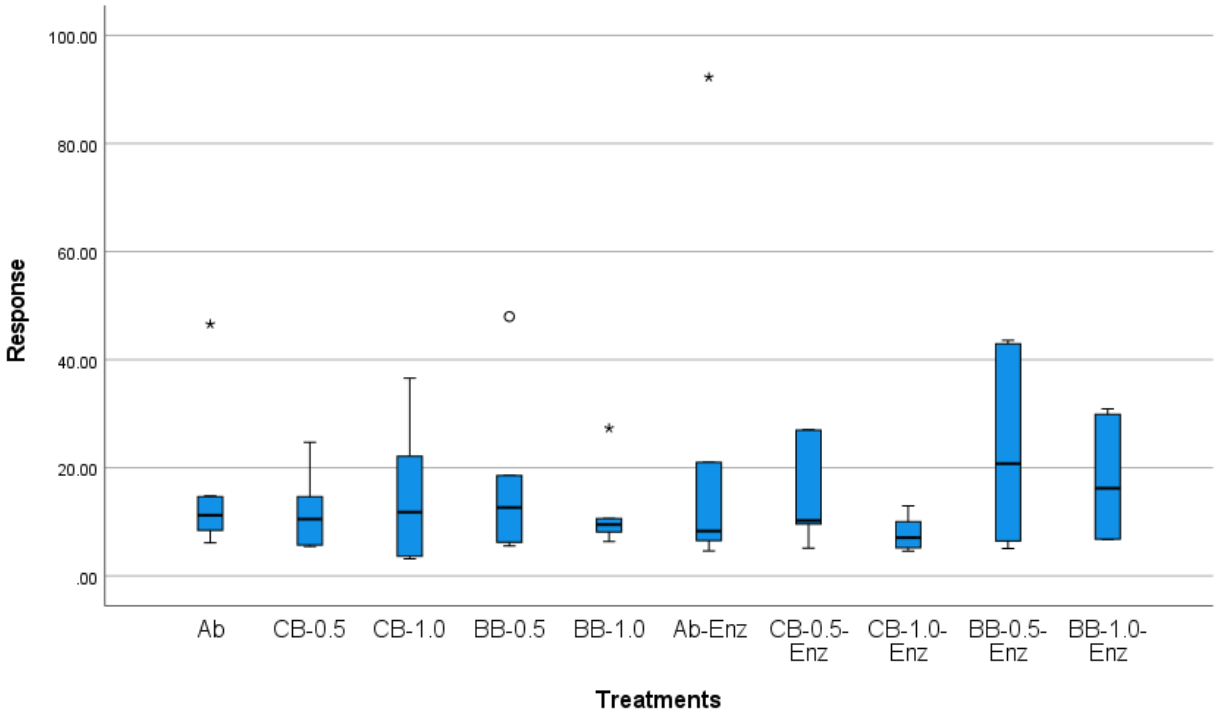


Figure 29: Concentrations of ethanol, in mM, at day 35. This chart shows data visualized from Table 11. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

iii. Individual Time Point Analysis (Outliers Removed)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	14.4778	4.44120
	35	4	10.1142	3.70614
Cranberry Low + Enzyme	21	6	12.5167	4.26001
	35	6	14.8728	3.91558
Cranberry High + Enzyme	21	4	42.6116	16.29936
	35	7	7.8725	1.31441
Blueberry Low + Enzyme	21	6	13.4683	3.63649
	35	6	23.2675	7.64879

Blueberry High + Enzyme	21	6	19.1497	2.97223
	35	6	17.8056	4.34487
Control	21	6	23.0837	5.32407
	35	6	10.5887	1.46044
Cranberry Low	21	7	23.4082	6.24127
	35	5	12.2094	3.56289
Cranberry High	21	6	38.3029	8.66992
	35	6	14.8644	5.29249
Blueberry Low	21	7	39.9513	11.49137
	35	5	11.1309	2.41259
Blueberry High	21	5	14.8071	2.74207
	35	4	8.6562	.91508

1586 **Table 12:** Concentrations of ethanol for two time points, after removal of outliers.

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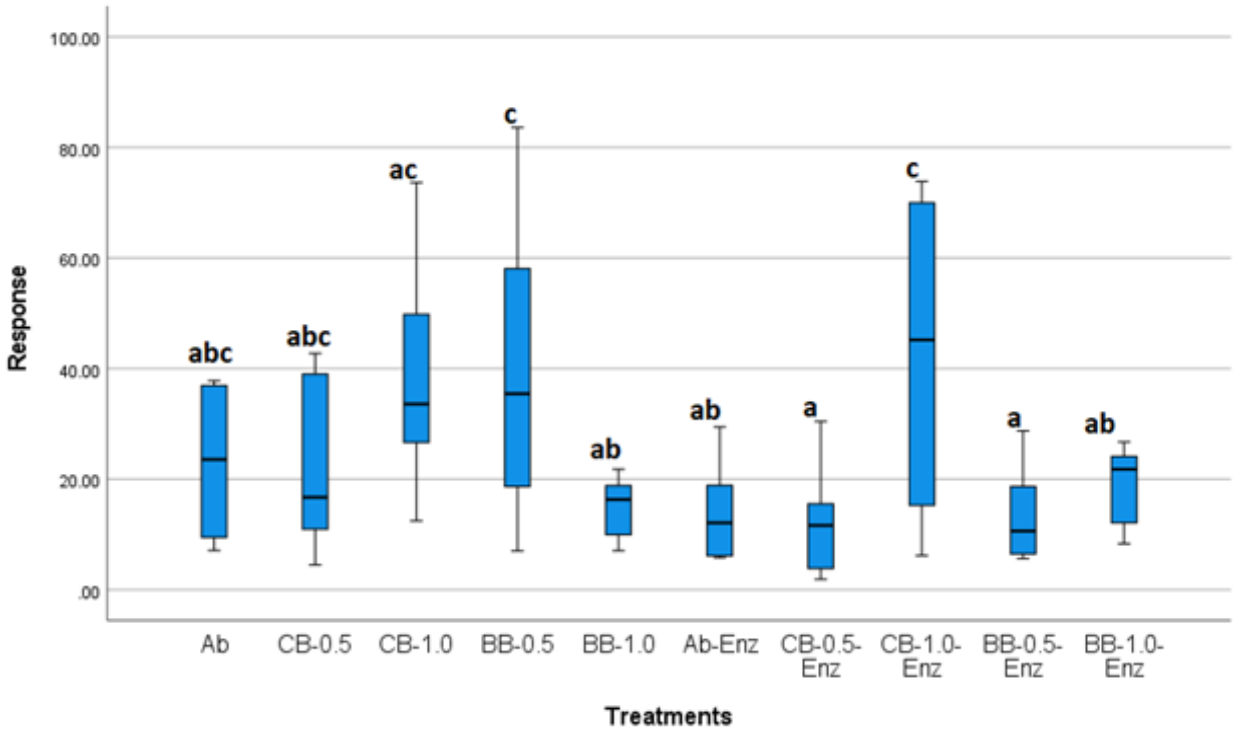


1590

1591 **Figure 30:** Concentrations of ethanol, in mM, at day 21, after removal of outliers. This chart

1592 shows data visualized from Table 12. Groups that share letters indicate no significant

differences. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

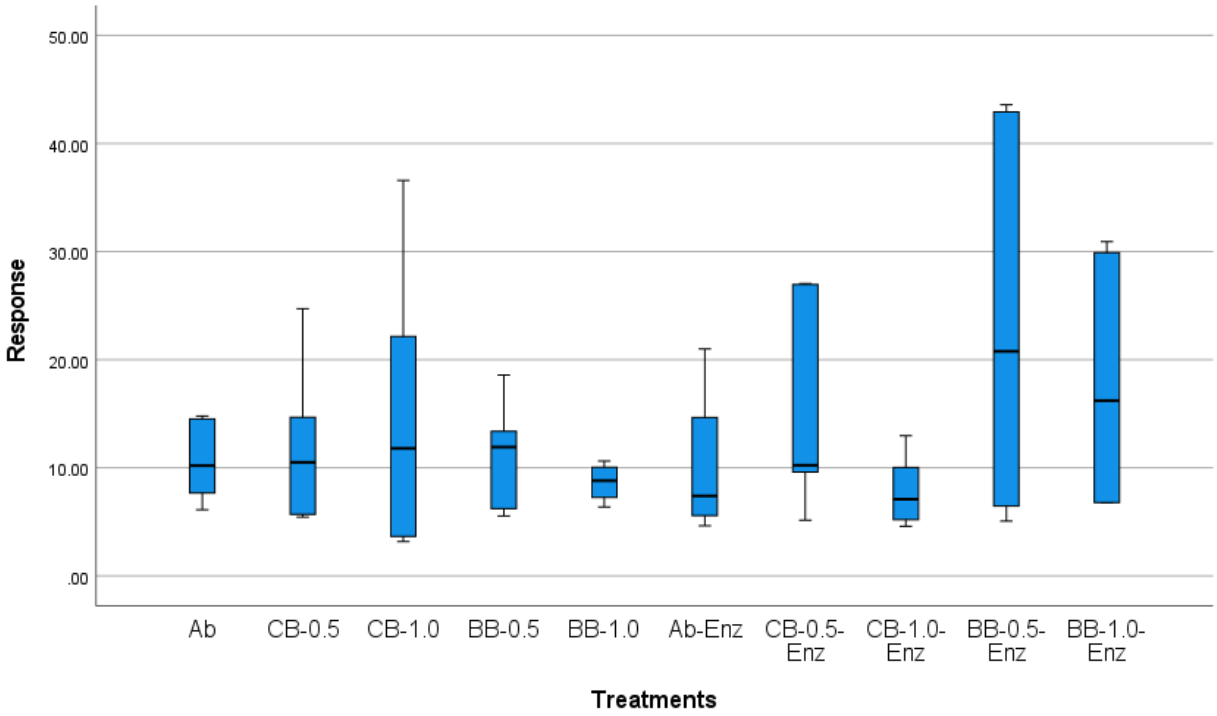


Figure 31: Concentrations of ethanol, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 12. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

d. Glutamate

The mean concentration for all ten groups at two time points is visualized in Figure 32. Individual time point analysis was conducted by splitting the two time points and doing separate analysis. These results are summarized in Table 13 and are visualized in Figures 33 and 34. Before removal of outliers, there were no significant differences between groups detected. The results after removal of 3 (Day 21) and 7 (Day 35) outliers are summarized in Table 14. No significant differences between groups were detected at either day 21 (Figure 35) nor day 36 (Figure 36). This suggests that none of the treatment groups have significant effects on glutamate levels when compared to a traditional antibiotic treatment.

i. General Linear Model Analysis

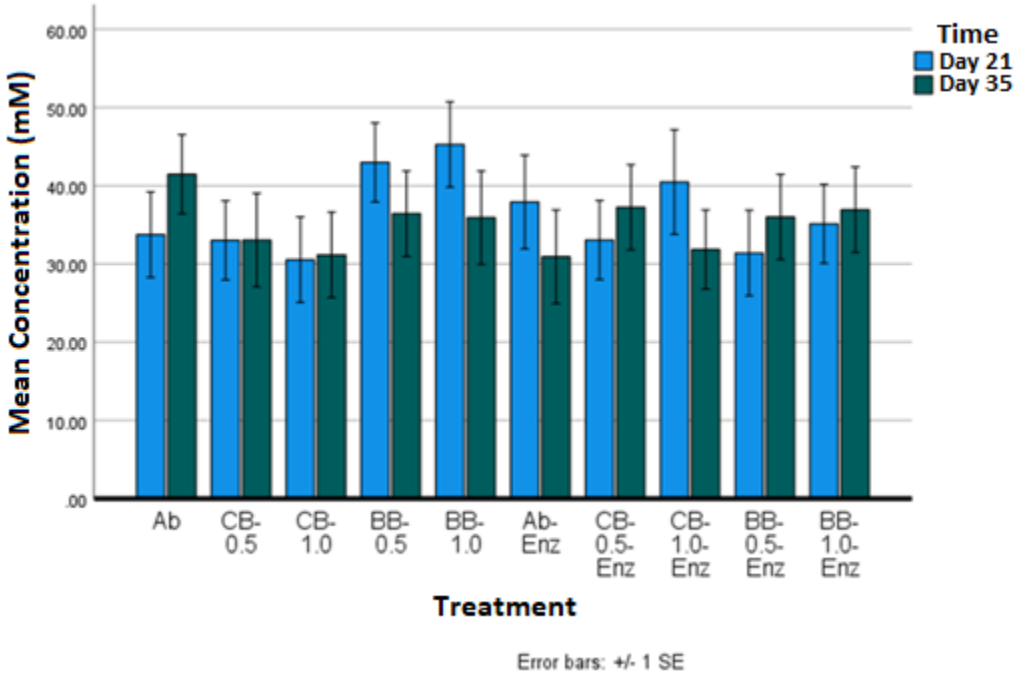


Figure 32: Mean concentrations of glutamate at day 21 and day 35. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

ii. Individual Time Point Analysis (Complete)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	37.9298	5.79358
	35	5	30.9269	5.80701
Cranberry Low + Enzyme	21	7	33.0788	3.46393
	35	6	37.2542	5.37959
Cranberry High + Enzyme	21	4	40.4812	6.24601
	35	7	31.8471	3.57179
Blueberry Low + Enzyme	21	6	31.4107	4.04846
	35	6	36.0119	2.27663
Blueberry High + Enzyme	21	7	35.1482	3.92470
	35	6	36.9359	3.69767
Control	21	6	33.7496	3.68378

	35	7	41.4792	6.67067
Cranberry Low	21	7	33.0293	4.06335
	35	5	33.0656	6.06851
Cranberry High	21	6	30.5465	4.61007
	35	6	31.1795	2.95930
Blueberry Low	21	7	42.9903	10.40412
	35	6	36.4486	4.39279
Blueberry High	21	6	45.2883	8.73505
	35	5	35.9366	4.61590

Table 13: Concentrations of glutamate for two time points.

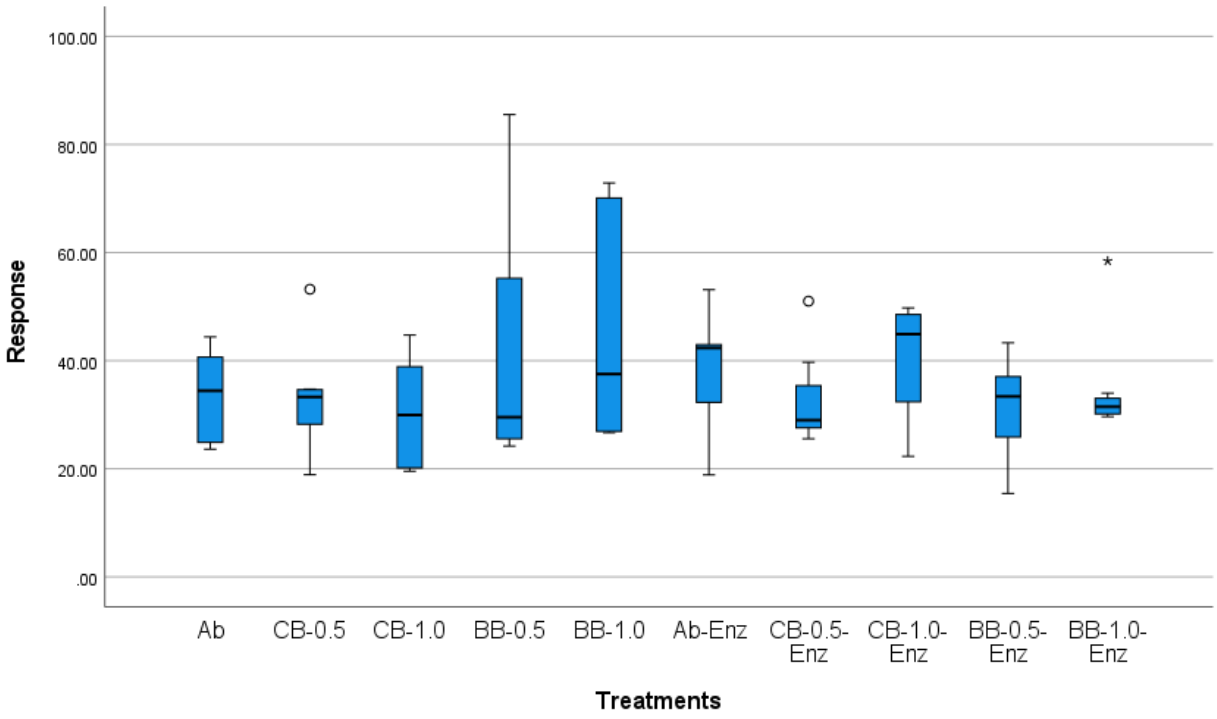


Figure 33: Concentrations of glutamate, in mM, at day 21. This chart shows data visualized from Table 13. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

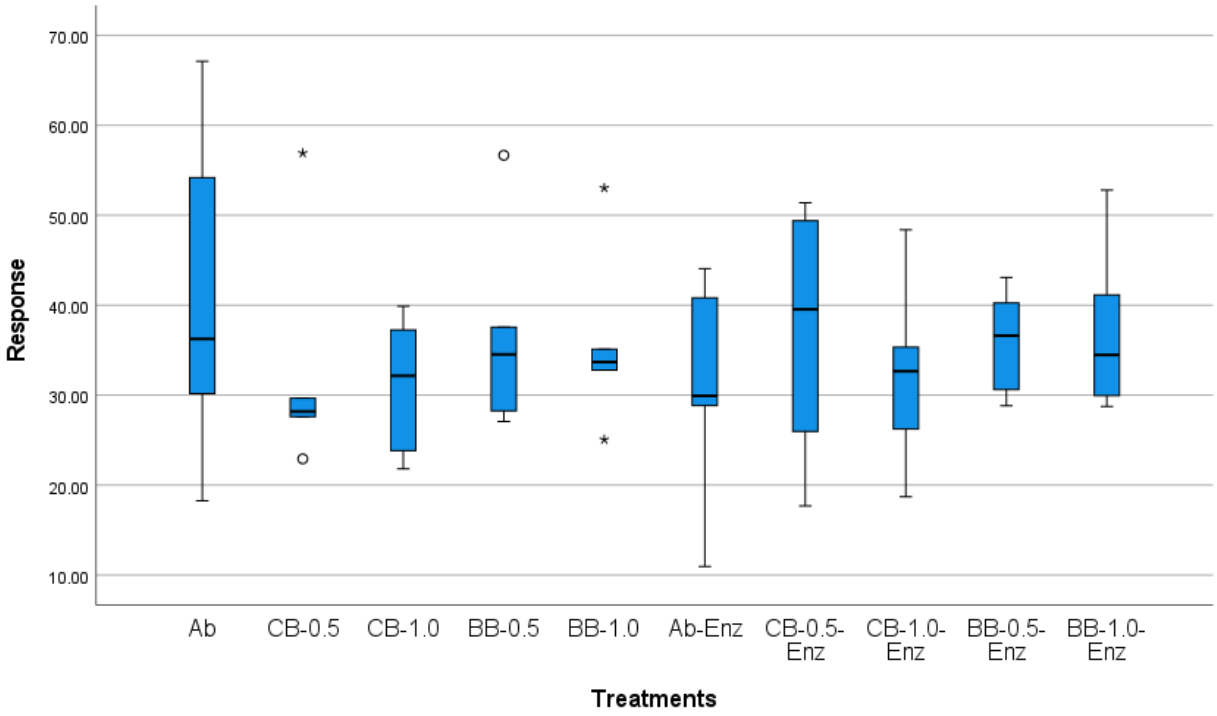


Figure 34: Concentrations of glutamate, in mM, at day 35. This chart shows data visualized from Table 13. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

iii. Individual Time Point Analysis (Outliers Removed)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	37.9298	5.79358
	35	5	30.9269	5.80701
Cranberry Low + Enzyme	21	6	30.0887	2.06905
	35	6	37.2542	5.37959
Cranberry High + Enzyme	21	4	40.4812	6.24601
	35	7	31.8471	3.57179
Blueberry Low + Enzyme	21	6	31.4107	4.04846
	35	6	36.0119	2.27663
Blueberry High + Enzyme	21	6	31.2639	.66510
	35	6	36.9359	3.69767
Control	21	6	33.7496	3.68378
	35	7	41.4792	6.67067

Cranberry Low	21	6	29.6639	2.69429
	35	3	28.4952	.61554
Cranberry High	21	6	30.5465	4.61007
	35	6	31.1795	2.95930
Blueberry Low	21	7	42.9903	10.40412
	35	5	32.4034	2.09775
Blueberry High	21	6	45.2883	8.73505
	35	3	33.8594	.67713

Table 14: Concentrations of glutamate after removal of outliers.

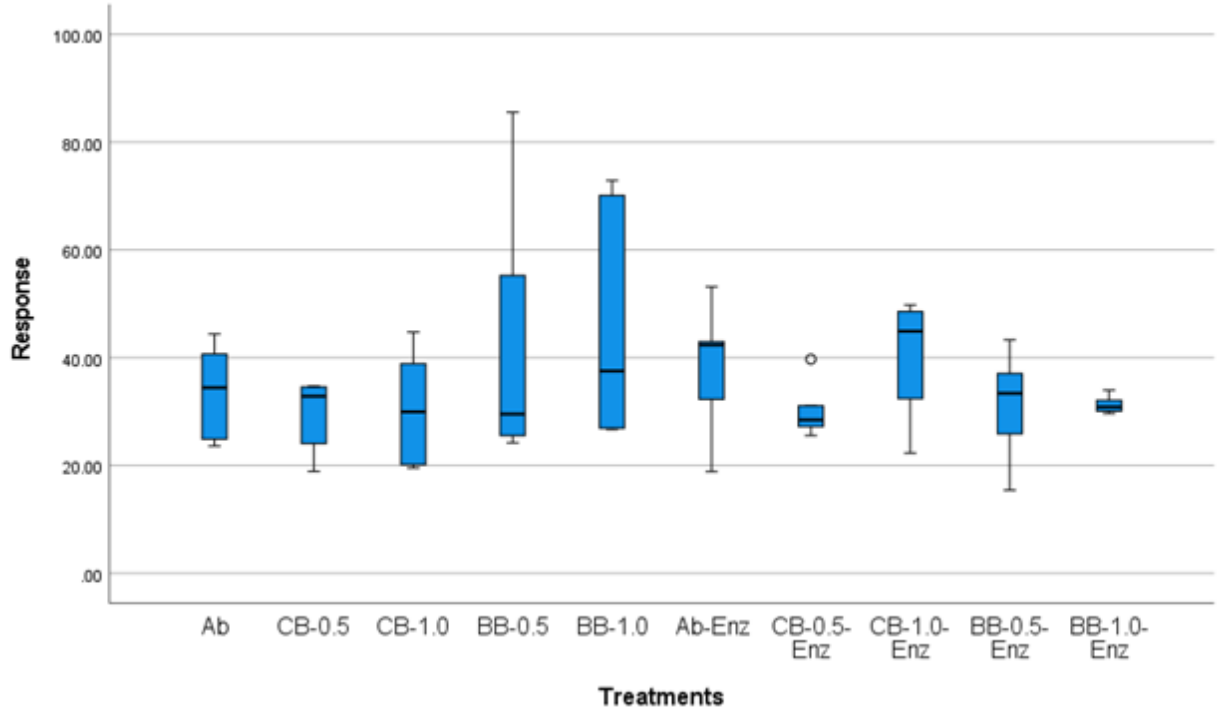


Figure 35: Concentrations of glutamate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 14. After removal of initial outliers, one new outlier was formed in the CB-0.5 group. Mild outliers are marked by a °. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

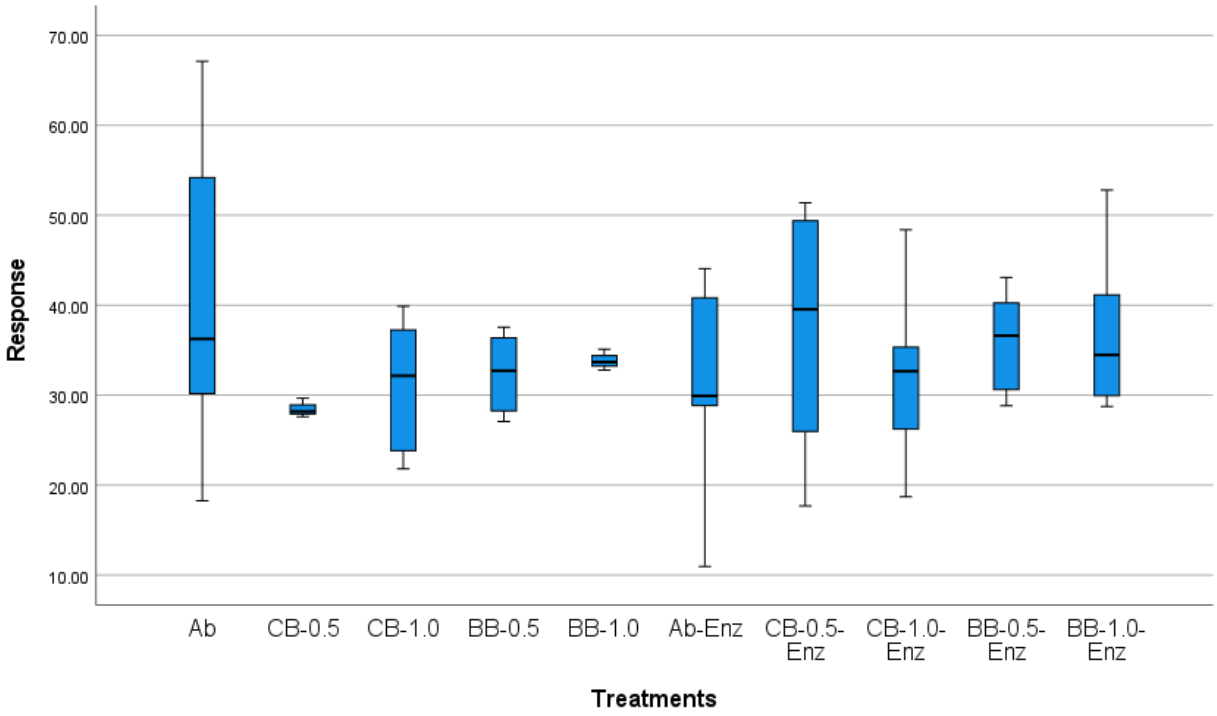


Figure 36: Concentrations of glutamate, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 14. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

e. Propionate

The mean concentration for all ten groups at two time points is visualized in Figure 37. Individual time point analysis was conducted by splitting the two time points and doing separate analysis. These results are summarized in Table 15 and are visualized in Figures 38 and 39. Before removal of outliers, there were no significant differences between groups detected. The results after removal of 5 (day 21) and 3 (day 35) outliers are summarized in Table 16. No significant differences between groups were detected at either day 21 (Figure 40) nor day 35 (Figure 41). This suggests that none of the treatment groups have significant effects on propionate levels when compared to a traditional antibiotic treatment.

i. General Linear Model Analysis

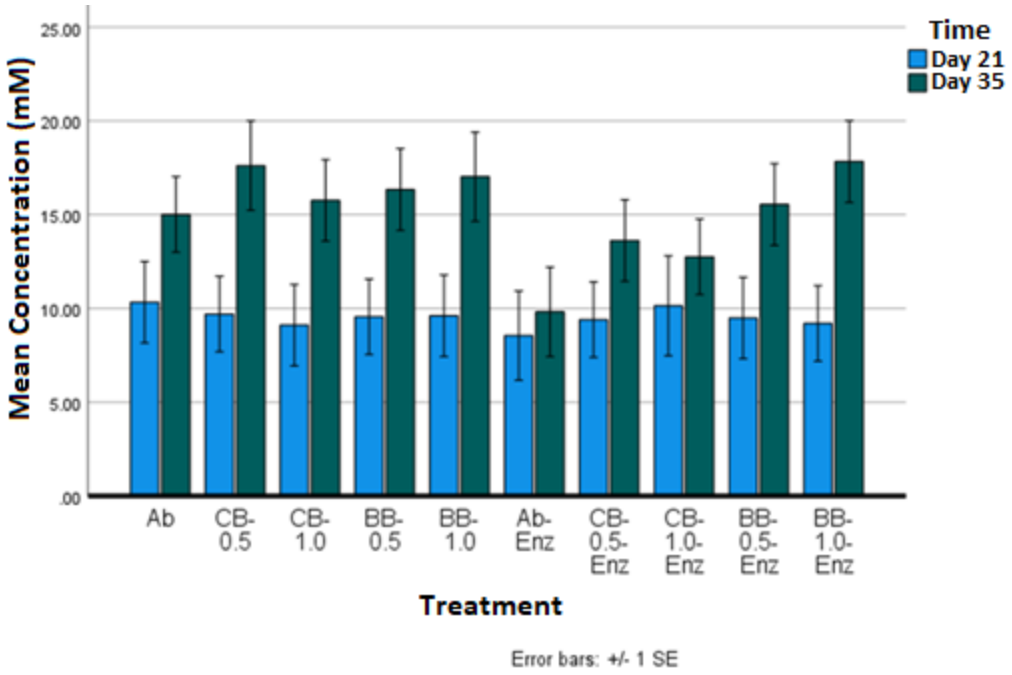


Figure 37: Mean concentrations of propionate at day 21 and day 35. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

ii. Individual Time Point Analysis (Complete)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	8.5531	2.24943
	35	5	9.8237	3.32568
Cranberry Low + Enzyme	21	7	9.4057	1.29480
	35	6	13.6259	3.18847
Cranberry High + Enzyme	21	4	10.1357	1.33610
	35	7	12.7557	1.27233
Blueberry Low + Enzyme	21	6	9.4891	2.44952
	35	6	15.5478	2.20200
Blueberry High + Enzyme	21	7	9.2026	1.31035
	35	6	17.8383	4.27980
Control	21	6	10.3371	1.69744
	35	7	15.0180	2.54639
Cranberry Low	21	7	9.7028	1.90060

	35	5	17.6211	2.91516
Cranberry High	21	6	9.1144	2.31428
	35	6	15.7665	1.81307
Blueberry Low	21	7	9.5572	.78721
	35	6	16.3495	1.71865
Blueberry High	21	6	9.6102	1.04770
	35	5	17.0304	.85471

Table 15: Concentrations of propionate for two time points.

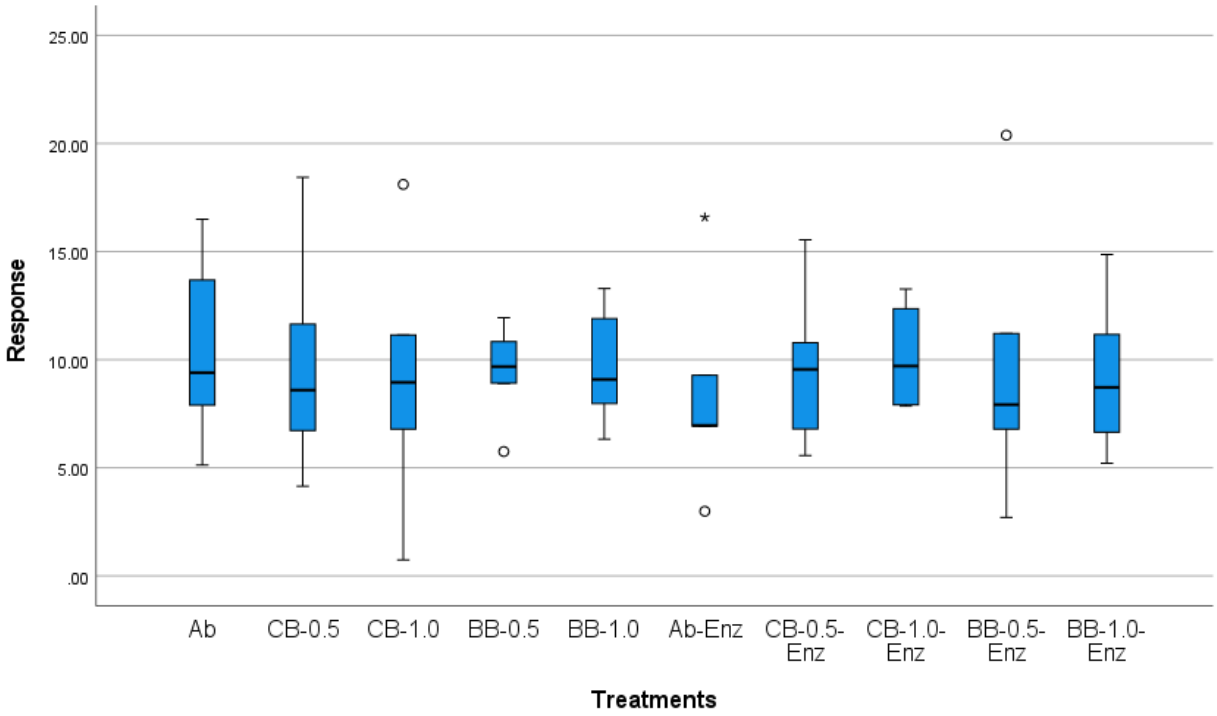


Figure 38: Concentrations of propionate, in mM, at day 21. This chart shows data visualized from Table 15. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

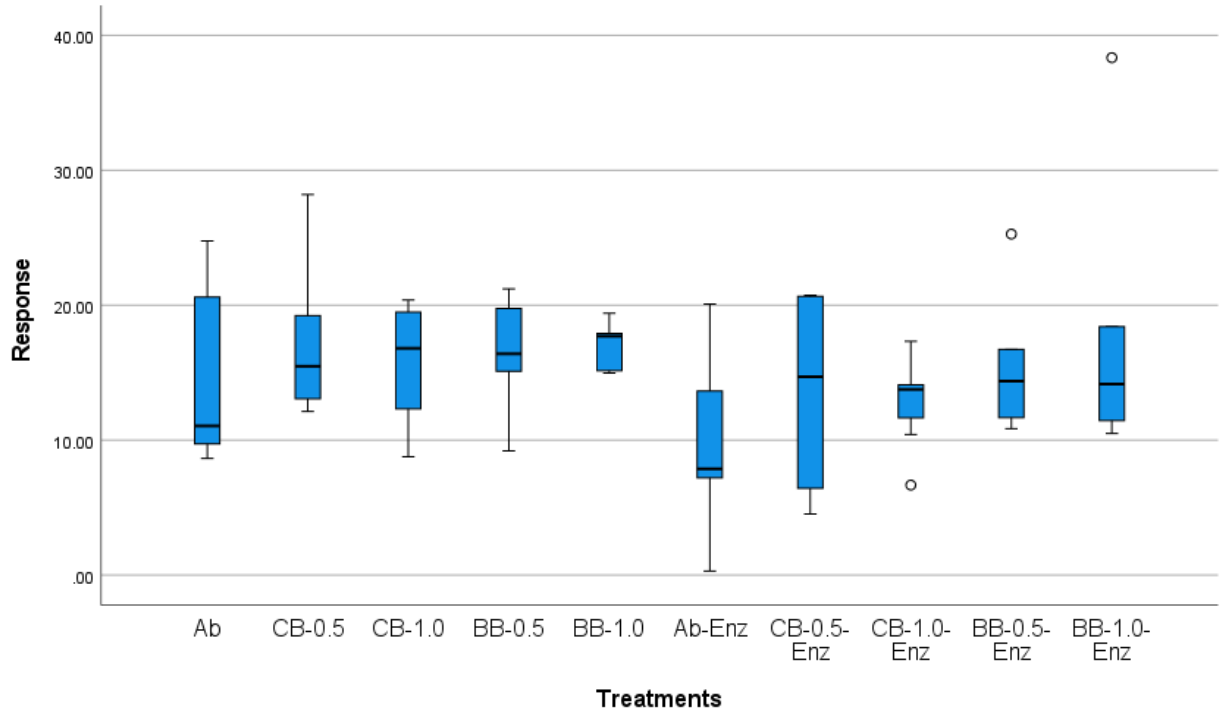


Figure 39: Concentrations of propionate, in mM, at day 35. This chart shows data visualized from Table 15. Mild outliers are marked by a °. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

iii. Individual Time Point Analysis (Outliers Removed)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	3	7.7279	.77768
	35	5	9.8237	3.32568
Cranberry Low + Enzyme	21	7	9.4057	1.29480
	35	6	13.6259	3.18847
Cranberry High + Enzyme	21	4	10.1357	1.33610
	35	6	13.7694	.90985
Blueberry Low + Enzyme	21	5	7.3095	1.36894
	35	5	13.6020	1.26267
Blueberry High + Enzyme	21	7	9.2026	1.31035
	35	5	13.7362	1.49486
Control	21	6	10.3371	1.69744
	35	7	15.0180	2.54639
Cranberry Low	21	7	9.7028	1.90060

	35	5	17.6211	2.91516
Cranberry High	21	5	7.3156	1.78342
	35	6	15.7665	1.81307
Blueberry Low	21	6	10.1912	.55206
	35	6	16.3495	1.71865
Blueberry High	21	6	9.6102	1.04770
	35	5	17.0304	.85471

Table 16: Concentrations of propionate for two time points, after removal of outliers.

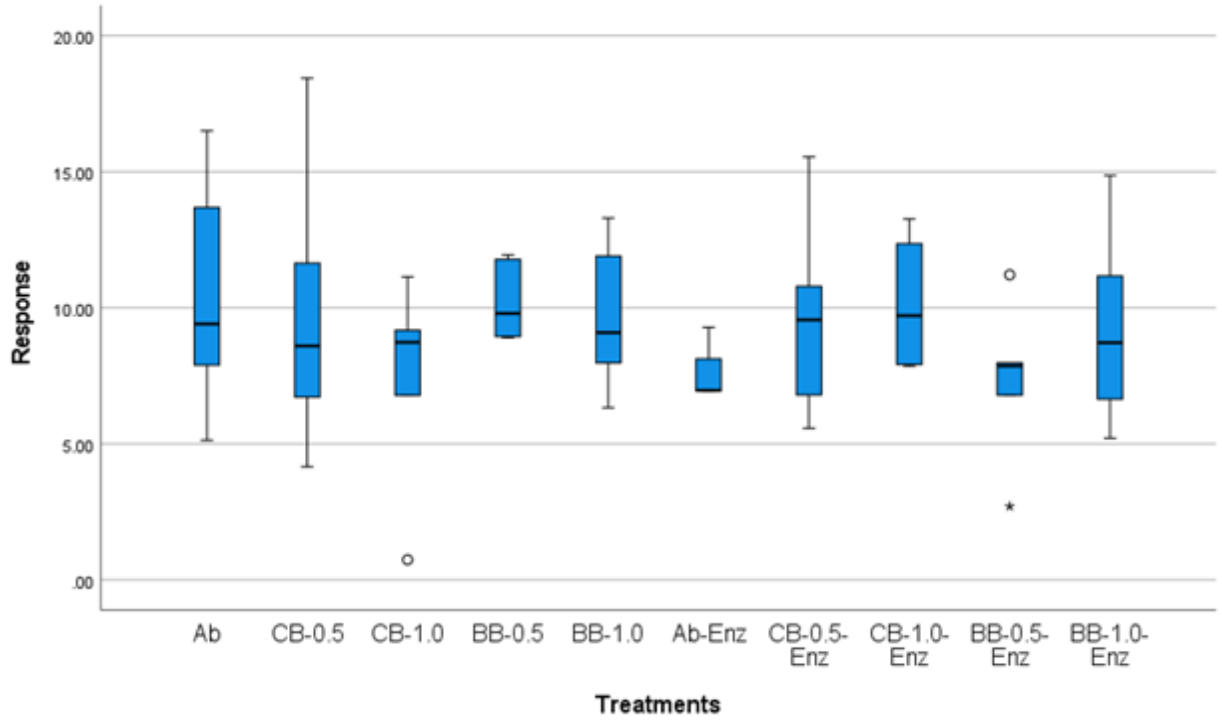


Figure 40: Concentrations of propionate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 16. After removal of initial outliers, three new outliers formed: one in group CB-1.0, and two in group BB-0.5-Enz. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

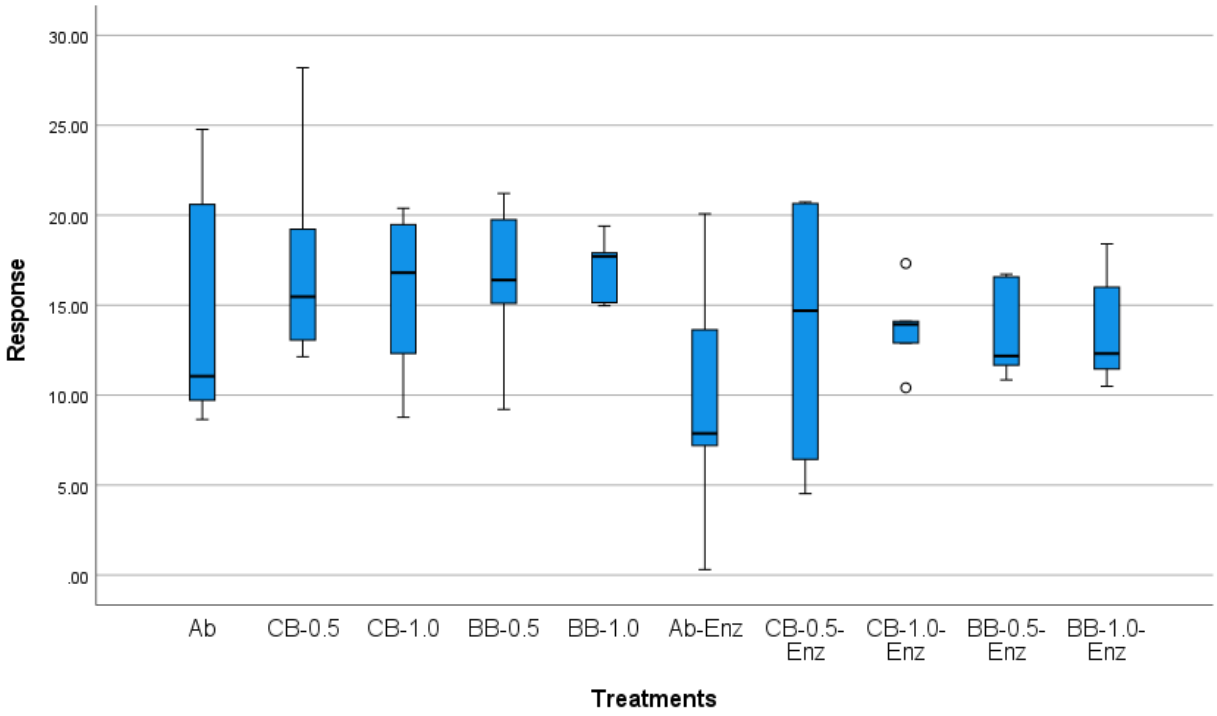


Figure 41: Concentrations of propionate, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 16. After removal of initial outliers, two new outliers formed in the CB-1.0-Enz group. Mild outliers are marked by a °. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

f. Valerate

The mean concentration for all ten groups at two time points is visualized in Figure 42. Individual time point analysis was conducted by splitting the two time points and doing separate analysis. These results are summarized in Table 17 and are visualized in Figures 43 and 44. Before removal of outliers, there were no significant differences between groups detected. The results after removal of 3 (Day 21) and 5 (Day 35) outliers are summarized in Table 18. Once again no significant differences between groups were detected at either day 21 (Figure 45) nor day 35 (Figure 46). This suggests that none of the treatment groups have significant effects on concentrations of valerate when compared to a traditional antibiotic treatment.

i. General Linear Model Analysis

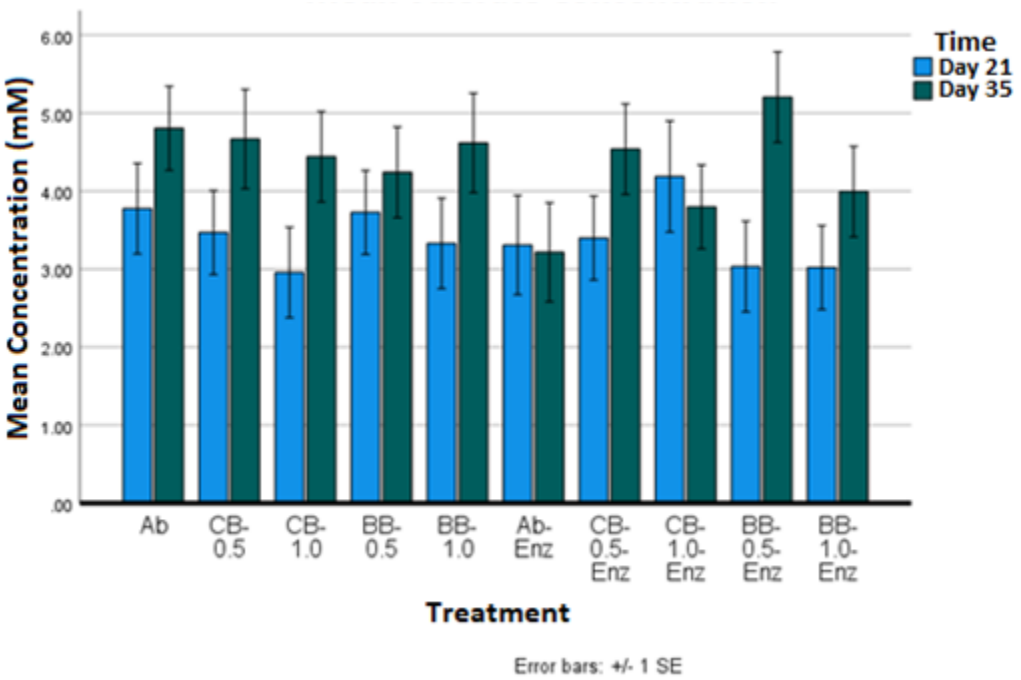


Figure 42: Mean concentration of valerate at day and day 35. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

ii. Individual Time Point Analysis (Complete)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	3.3091	.59207
	35	5	3.2170	.67066
Cranberry Low + Enzyme	21	7	3.3998	.41960
	35	6	4.5382	1.13582
Cranberry High + Enzyme	21	4	4.1882	.71529
	35	7	3.7988	.34825
Blueberry Low + Enzyme	21	6	3.0334	.58132
	35	6	5.2056	.52184
Blueberry High + Enzyme	21	7	3.0202	.26222
	35	6	3.9935	.18681
Control	21	6	3.7768	.46046

	35	7	4.8071	.77218
Cranberry Low	21	7	3.4687	.32293
	35	5	4.6675	.80908
Cranberry High	21	6	2.9572	.43780
	35	6	4.4421	.60036
Blueberry Low	21	7	3.7271	.82478
	35	6	4.2418	.40315
Blueberry High	21	6	3.3308	.34038
	35	5	4.6176	.47909

Table 17: Concentration of valerate for two time points.

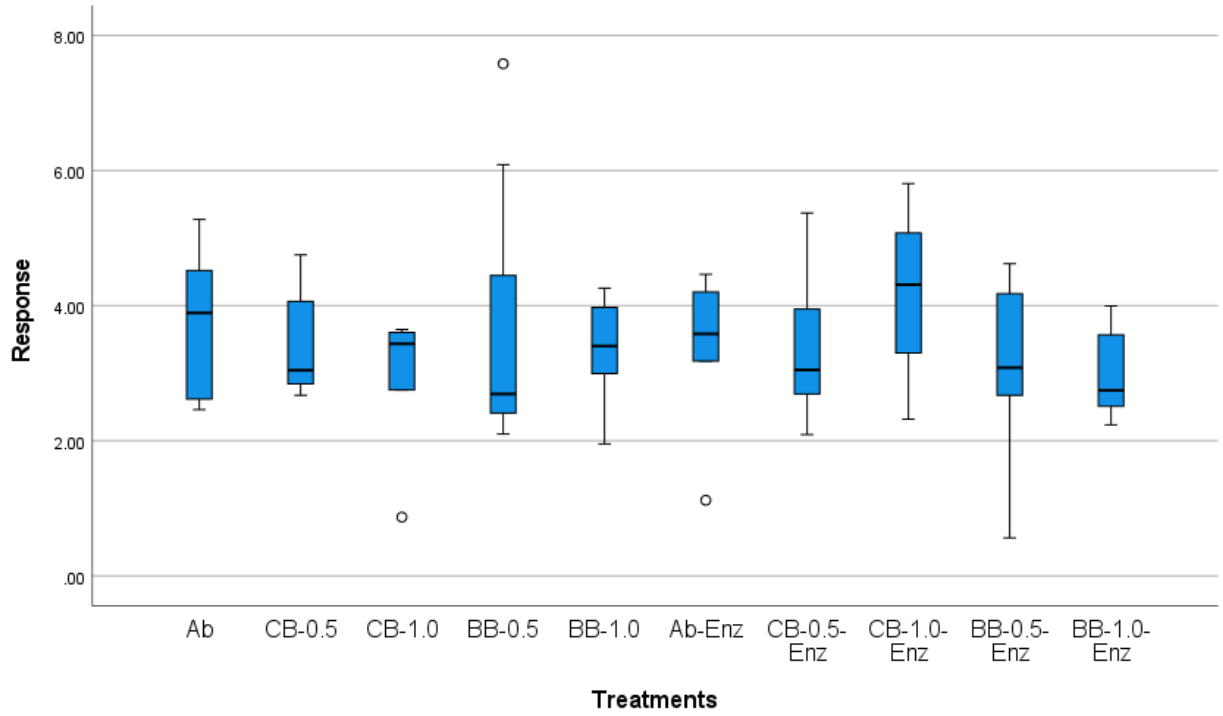


Figure 43: Concentrations of valerate, in mM, at day 21. This chart shows data visualized from Table 17. Mild outliers are marked by a °. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

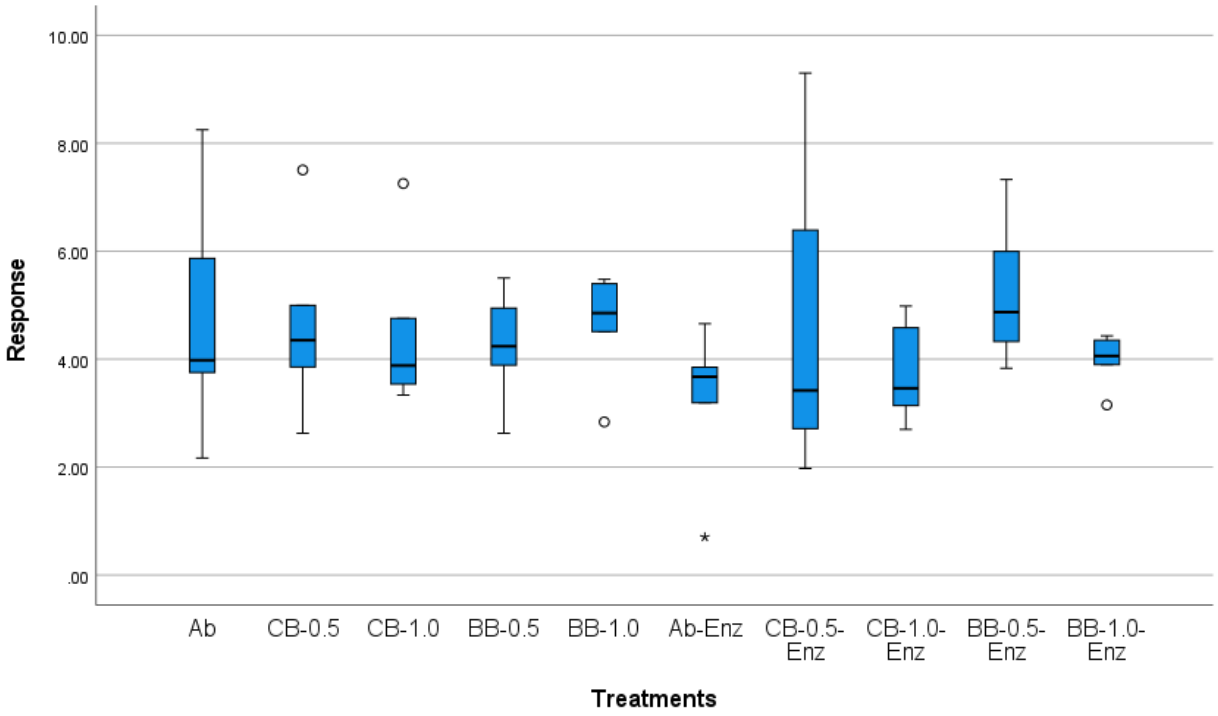


Figure 44: Concentrations of valerate, in mM, at day 35. This chart shows data visualized from Table 17. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

iii. Individual Time Point Analysis (Outliers Removed)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	4	3.8564	.29159
	35	4	3.8447	.30468
Cranberry Low + Enzyme	21	7	3.3998	.41960
	35	6	4.5382	1.13582
Cranberry High + Enzyme	21	4	4.1882	.71529
	35	7	3.7988	.34825
Blueberry Low + Enzyme	21	6	3.0334	.58132
	35	6	5.2056	.52184
Blueberry High + Enzyme	21	7	3.0202	.26222
	35	5	4.1616	.09984
Control	21	6	3.7768	.46046

	35	7	4.8071	.77218
Cranberry Low	21	7	3.4687	.32293
	35	4	3.9578	.50151
Cranberry High	21	5	3.3746	.16170
	35	5	3.8794	.25620
Blueberry Low	21	6	3.0848	.61222
	35	6	4.2418	.40315
Blueberry High	21	6	3.3308	.34038
	35	4	5.0625	.22975

Table 18: Concentration of valerate at two time points, with outliers removed.

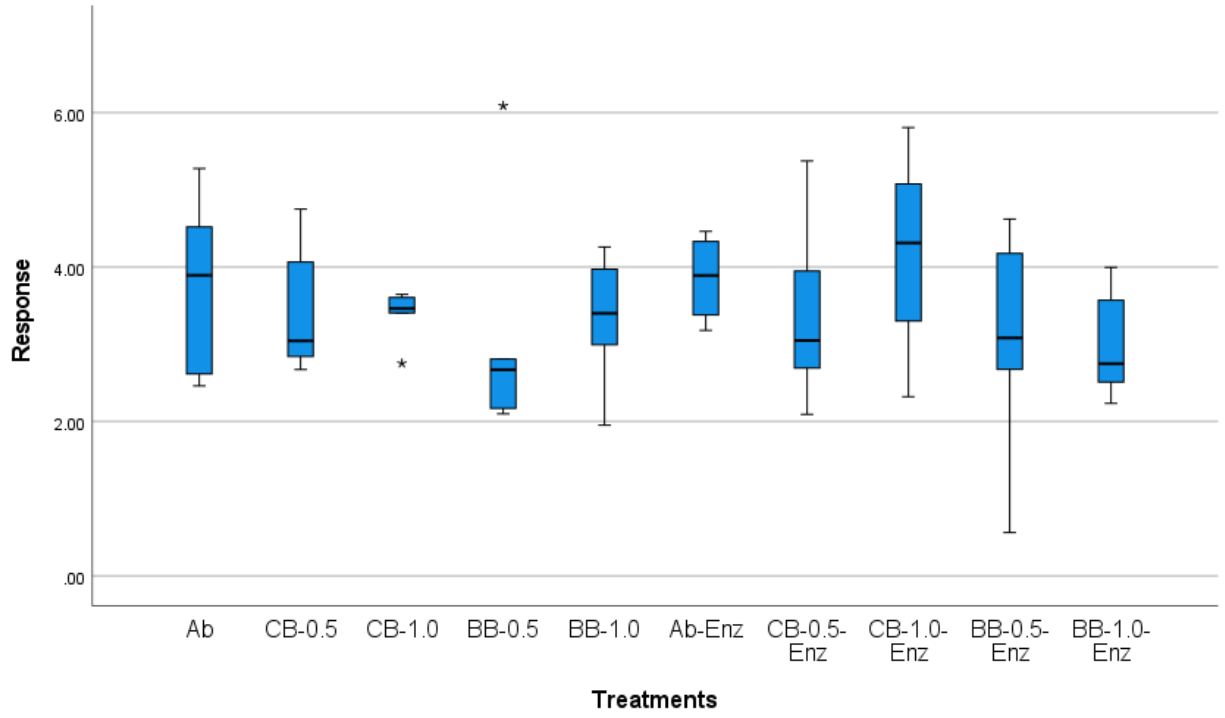


Figure 45: Concentrations of valerate, in mM, at day 21, after removal of outliers. This chart shows data visualized from Table 18. After removal of initial outliers, two new ones formed; one in group CB-1.0, and one in group BB-0.5. Extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

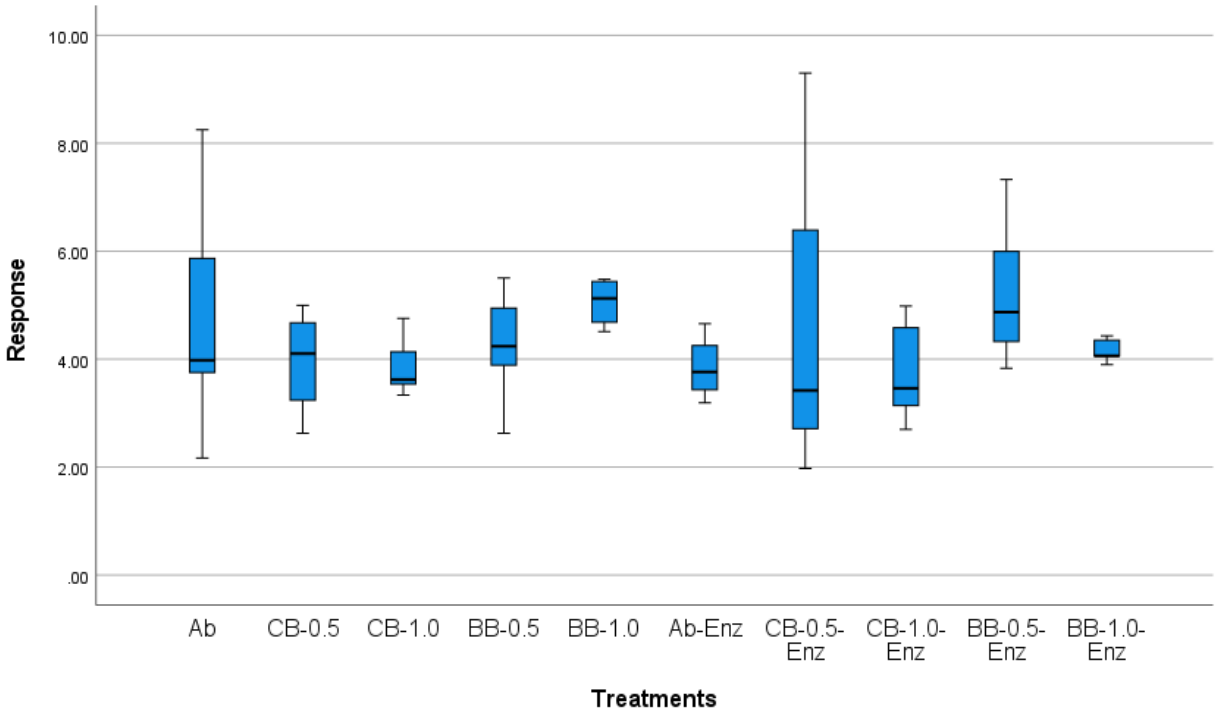


Figure 46: Concentrations of valerate, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 18. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

g. Glucose

The mean concentration for all ten groups at two time points is visualized in Figure 47. Individual time point analysis was conducted by splitting the two time points and doing separate analysis. These results are summarized in Table 19 and are visualized in Figures 48 and 49. Before removal of outliers, there were no significant differences between groups detected. The results after removal of 3 (Day 21) and 2 (Day 35) outliers are summarized in Table 20. No significant differences were found at day 21 (Figure 50), while some significant differences were found at day 35 (Figure 51). Namely, the BB 0.5 + Enz group had significantly higher concentrations than all treatments besides the CB 0.5 + Enz group and the control group. While these three groups trended towards higher glucose levels, all three also had a considerably larger range in concentrations, suggesting higher variability in these groups as opposed to solely a higher average concentration.

i. General Linear Model Analysis

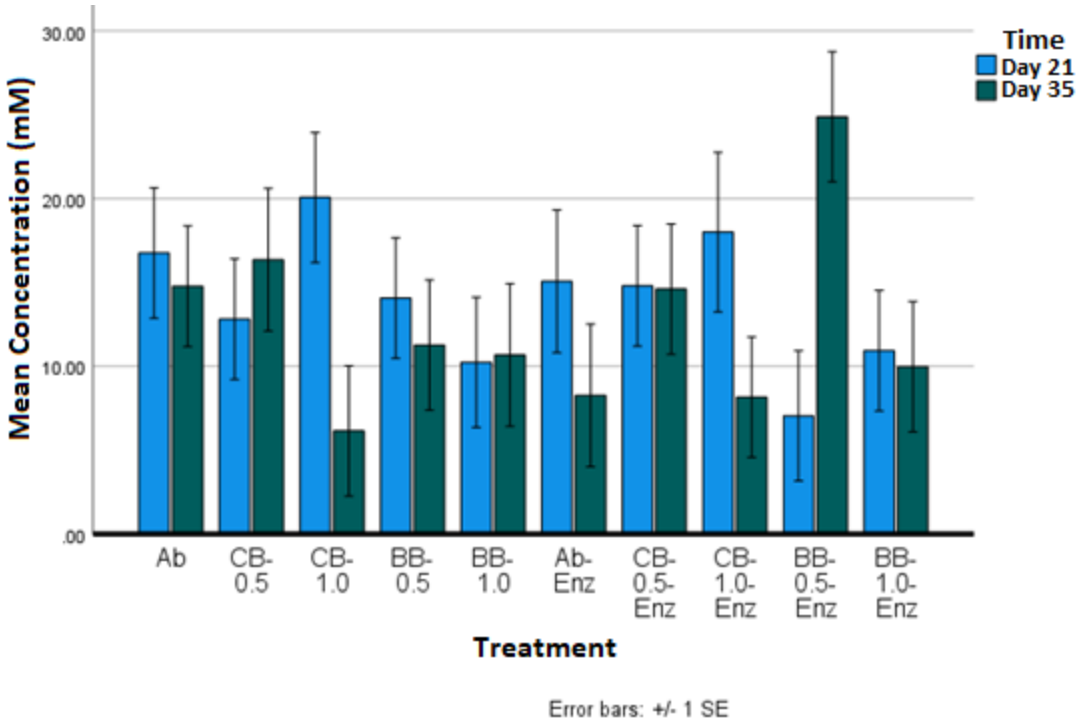


Figure 47: Mean concentration of glucose at day 21 and day 35. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

ii. Individual Time Point Analysis (Complete)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	5	15.0635	5.64658
	35	5	8.2636	3.77762
Cranberry Low + Enzyme	21	7	14.7956	3.63519
	35	6	14.6084	4.07916
Cranberry High + Enzyme	21	4	18.0054	6.99901
	35	7	8.1577	1.66339
Blueberry Low + Enzyme	21	6	7.0461	1.55879
	35	6	24.8786	8.33503
Blueberry High + Enzyme	21	7	10.9339	1.97373
	35	6	9.9724	2.28526
Control	21	6	16.7538	4.87256

	35	7	14.7782	3.34318
Cranberry Low	21	7	12.8091	3.07597
	35	5	16.3575	3.75987
Cranberry High	21	6	20.0671	4.82214
	35	6	6.1516	1.53780
Blueberry Low	21	7	14.0671	3.67762
	35	6	11.2671	3.81175
Blueberry High	21	6	10.2371	2.37187
	35	5	10.6713	2.01331

Table 19: Concentrations of glucose at two time points.

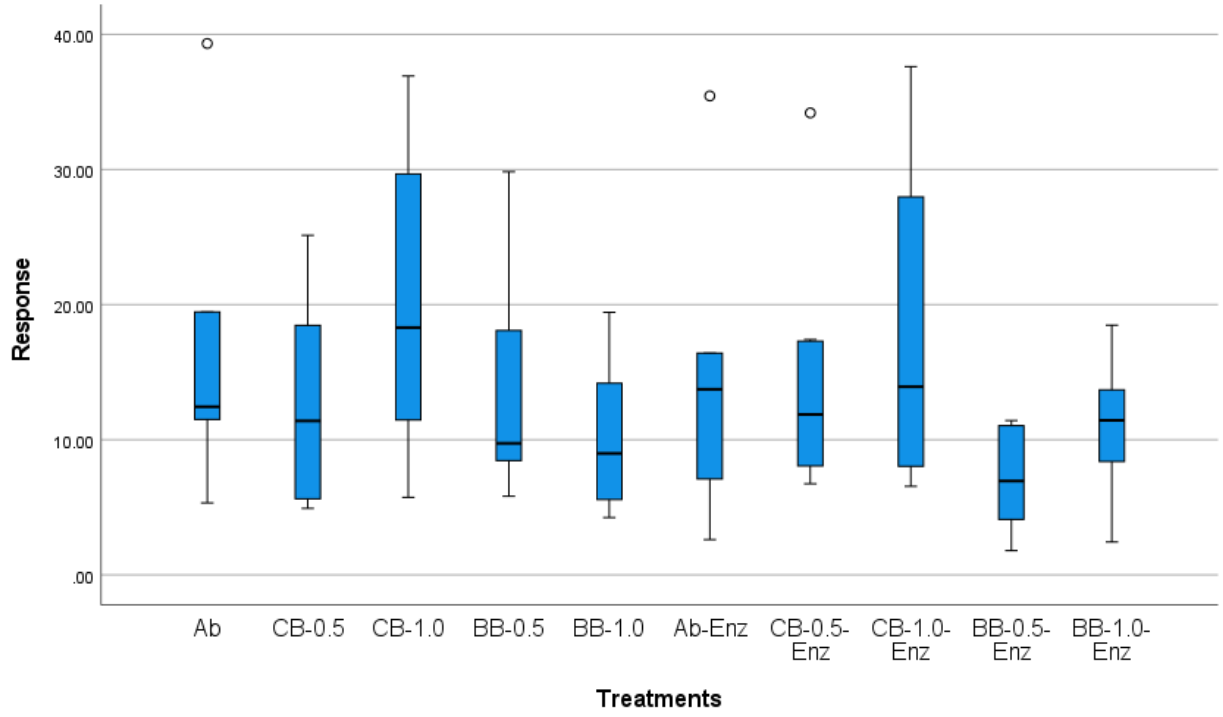


Figure 48: Concentrations of glucose, in mM, at day 21. This chart shows data visualized from Table 19. Mild outliers are marked by a °. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

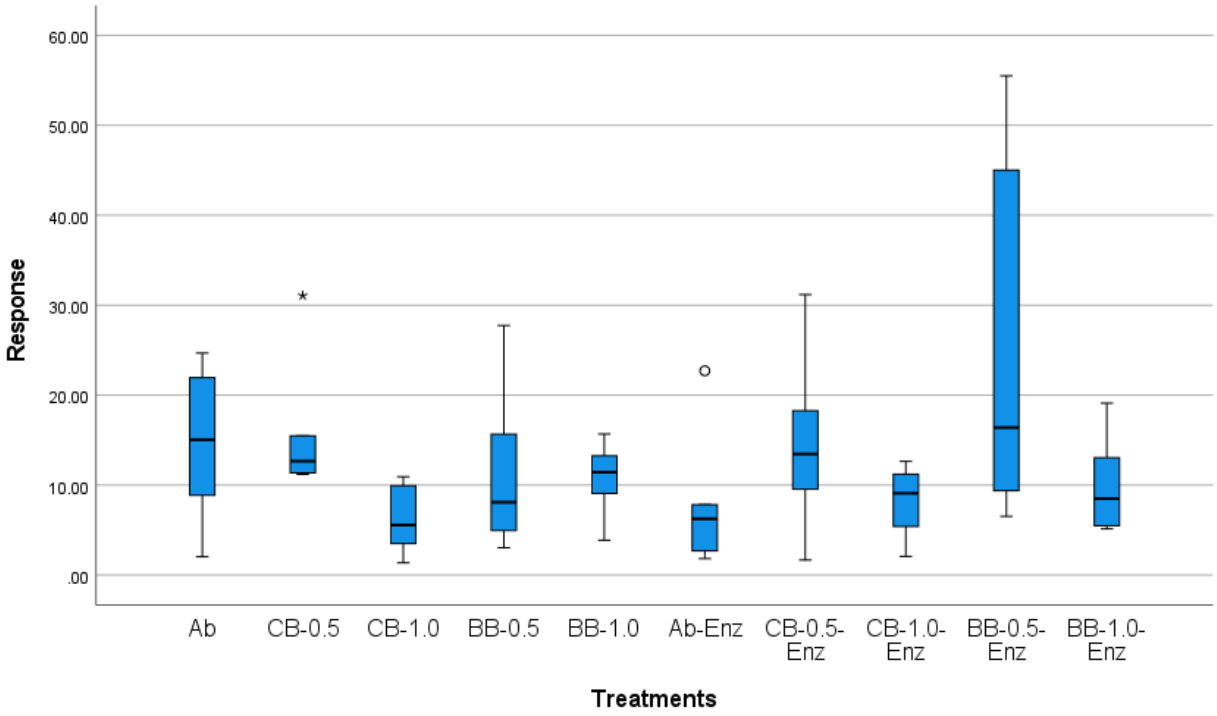


Figure 49: Concentrations of glucose, in mM, at day 35. This chart shows data visualized from Table 19. Mild outliers are marked by a °, while extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

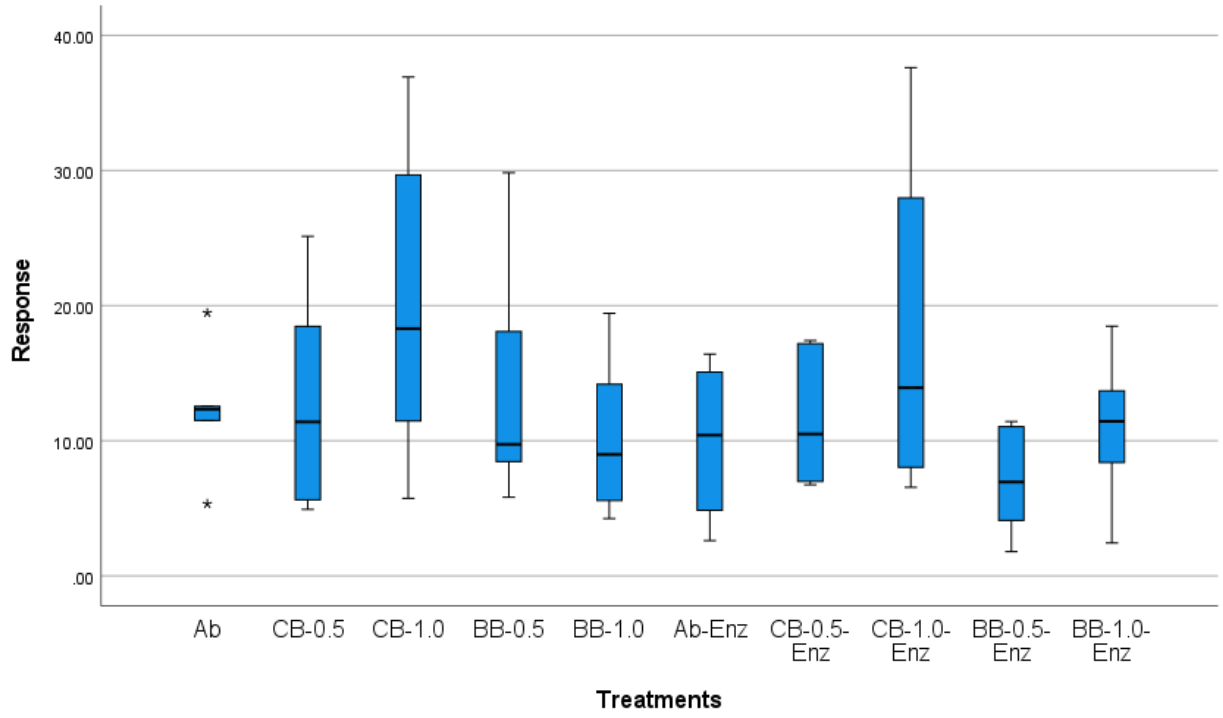
iii. Individual Time Point Analysis (Outliers Removed)

Group	Age (Days)	N	Mean	Standard Error
Enzyme	21	4	9.9671	3.13866
	35	4	4.6516	1.42821
Cranberry Low + Enzyme	21	6	11.5625	1.96640
	35	6	14.6084	4.07916
Cranberry High + Enzyme	21	4	18.0054	6.99901
	35	7	8.1577	1.66339
Blueberry Low + Enzyme	21	6	7.0461	1.55879
	35	6	24.8786	8.33503
Blueberry High + Enzyme	21	7	10.9339	1.97373
	35	6	9.9724	2.28526
Control	21	5	12.2386	2.24304

	35	7	14.7782	3.34318
Cranberry Low	21	7	12.8091	3.07597
	35	4	12.6768	.99044
Cranberry High	21	6	20.0671	4.82214
	35	6	6.1516	1.53780
Blueberry Low	21	7	14.0671	3.67762
	35	6	11.2671	3.81175
Blueberry High	21	6	10.2371	2.37187
	35	5	10.671	2.013

1750 **Table 20:** Concentrations of glucose at two time points, after removal of outliers.

1751



1752

1753 **Figure 50:** Concentrations of glucose, in mM, at day 21, after removal of outliers. This chart
1754 shows data visualized from Table 20. After removal of initial outliers, two new outliers formed
1755 in the Ab group. Extreme outliers are marked by a *. Ab = antibiotic control; Enz = Enzymes
1756 added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

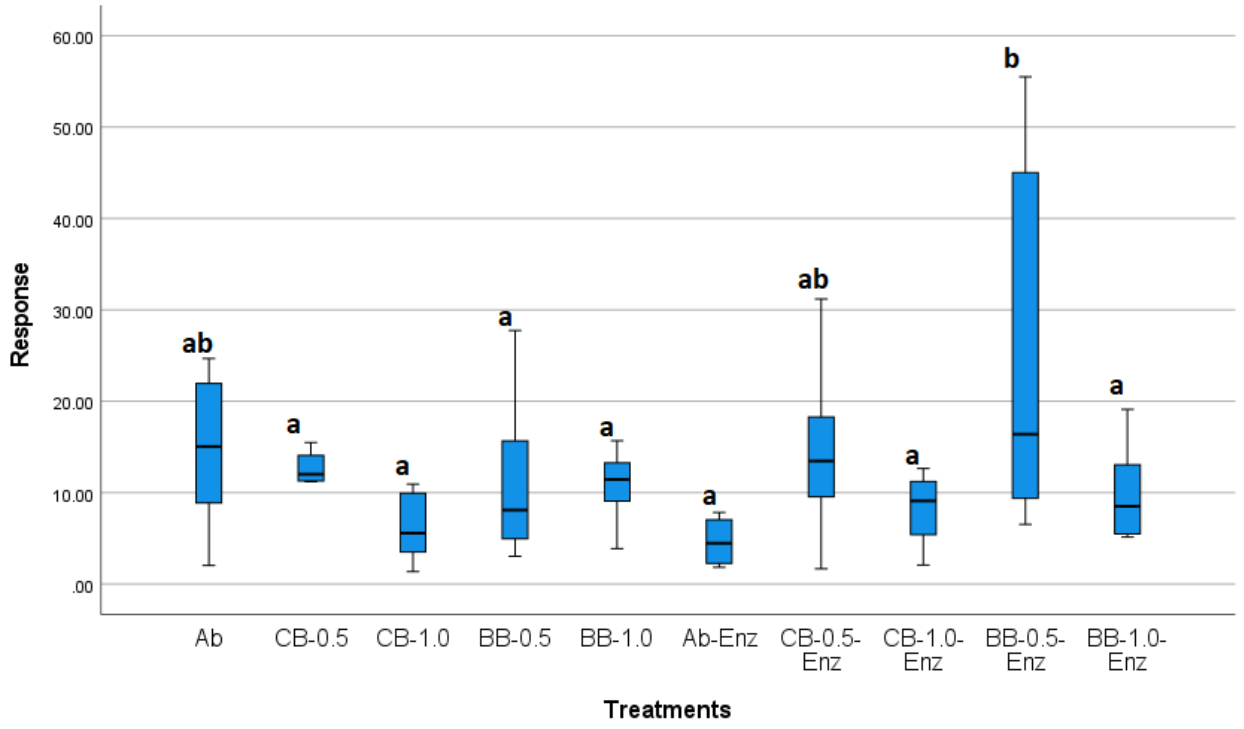


Figure 51: Concentrations of glucose, in mM, at day 35, after removal of outliers. This chart shows data visualized from Table 20. Groups which share a letter have no significant differences. Ab = antibiotic control; Enz = Enzymes added; CB = Cranberry; BB = Blueberry; 0.5 = 0.5% Concentration; 1.0 = 1.0% Concentration.

Metabolite	Change with Age
Acetate	Increased
Butyrate	Increased
Propionate	Increased
Valerate	Increased
Glutamate	No change
Ethanol	Decreased
Glucose	No change

Table 21: Summary showing the time effect differences observed in certain metabolites as birds mature.

5.6 Discussion

For this portion of the thesis, the goal was to investigate if fruit extracts, with or without digestive enzymes, would alter the cecal metabolome of growing broilers in any significant ways. Cecal samples were collected at days 21 and 35 of broiler growth. Using NMR analysis to examine fecal metabolites is a relatively new yet expanding topic, as NMR analysis has traditional been reserved for liquid materials, however dissolution of fecal matter using solvents allows for this type of metabolic analysis. This type of work is relatively novel in chickens, with few publications at the time of writing.

During the quick life of a growing broiler, from hatch until processing, vast changes occur in the gut microbial community which reflect in the metabolome and offer insight into health status as well as efficiency of energy metabolism. On the day the bird hatches, the gut contains a low density of bacteria and the gut is quickly colonized, reaching a significant level of maturity by the second week of life (Bilal et al., 2021). Alongside this development of microbiome, the metabolome of birds also changes rapidly in the first few weeks of life. In birds grown under standard conditions, acetate, propionate, butyrate, and valerate all increase from the first day of life, stabilizing around 21 days of age (Liao et al., 2020). This aligns with the maturation of the microbiome and offers explanation into why our analysis found few differences looking at days 21 and 35 of life only. Slight changes were observed in most metabolites between these two time points, summarized in Table 21.

Our analysis showed no significant changes between groups for any of the metabolites examined: acetate, butyrate, ethanol, glutamate, propionate, valerate, or glucose. This was due to large sample variation, which may have been, in part, caused by variability in how dissolvable each sample was in the chosen solvent. The physical properties of cecal contents vary depending

on the stage of digestion the bird was in before sampling, creating a difficult to control variable. One potential method to avoid this before NMR analysis would be to cut samples which had higher amounts of non-dissolvable materials. Often the same sample corresponded to low values in each metabolite, indicating the sample had less fecal slurry dissolved for analysis. Thus, another option could have been to add the concentrations of all the metabolites in a sample together and determine a percent composition of each metabolite per sample. We attempted to reduce the effects of sample variability during statistical analysis by cutting out samples which had high variability (outside the 1.5X interquartile range).

Overall, the levels of short chain fatty acids were not affected by any treatment. Acetate continuously composes the majority of SCFA in the cecum, with our treatments showing relatively stable levels among all groups at both time points. Altering the metabolic profile of chickens may offer positive or negative consequences in terms of performance, so an understanding of the role each metabolite plays host health is essential. Addition of fishmeal to broiler diets was found to reduce both acetate and butyrate concentrations in the cecum (Wu et al., 2016), which may offer insight into a potential reason fishmeal has often been cited as a potential factor in necrotic enteritis in birds. Butyrate is known to support health of endothelial cells and supplemental butyric acid has been found to reduce signs of NE in chickens (Wu et al., 2016). However, Wu et al. (2016) concluded that fishmeal alone was not enough to predispose birds to necrotic enteritis reliably. The addition of tea polyphenols into broiler chickens was previously found to have increased levels of both acetate and butyrate in the cecum, suggesting these polyphenols may promote gut health in birds (Terada et al., 1992). While found in lower concentrations in the gut, valerate and propionate also play roles in various metabolic pathways. It was previously noted that inoculation with *Eimera* and *C. perfringens* increased relative

propionate levels in the cecum in chickens; while the addition of fishmeal to the diet increased levels of valerate (Wu et al., 2016). Further investigation into such patterns in these lower concentration metabolites in chickens may prove useful to understanding factors which predispose birds to, or contribute to, various diseases. Our results present stable levels of acetate, butyrate, propionate, and valerate between all treatment groups indicating that while not ameliorated, these vital components of metabolic health were also not negatively disturbed by any of our treatments.

We saw a trending metabolic shift between 21 and 35 days with the decrease in ethanol concentration in eight out of ten groups; the enzyme only and blueberry low + enzyme groups had higher ethanol concentrations at day 35 (Figure 27; Table 11). It is possible that higher ethanol levels later in life coincide with lower amounts of energy being converted into muscle tissue, as the trial also found that the feeding of blueberry pomace alongside the feed enzymes resulted in significantly lower yields of breast meat (Xu et al., 2020). However, there is a slight contradiction in the pattern as the high dose of blueberry with enzyme added did follow the general pattern of a reduction in ethanol levels by day 35 (Figure 27; Table 11). Glutamate and glucose are both key components of energy capture from digestion. Glutamate levels remained stable in all groups between days 21 and 35 (Figure 32). Glucose levels were only affected at day 35 after the removal of outliers. Here we saw some significant differences, however no groups differed from the control group. The control, CB-0.5-Enz, and BB-0.5-Enz groups had a larger range in values, potentially trending to higher glucose levels than other treatment groups.

As some differences were seen looking at other areas, such as breast meat yield, there may be a point of interest to continue investigating how the extracts relate to certain metabolites, as well as justification to examine how the microbiome and metabolic pathways are impacted by

the addition of the extracts. Further studies could also include NMR analysis of matter from the small intestine, as pathogens such as *C. perfringens* tend to impact the small intestine more than the cecum, and the cranberry extract in particular has been noticed to improve the integrity of the small intestine in broilers.

5.7 Conclusion

Overall, cranberry and blueberry pomace, with or without feed enzymes added, did not produce any significant changes in the metabolome of growing broilers at day 21 or 35 of life. This suggests that at the metabolic level, none of these products had any negative impact on broiler health. The use of NMR to study the broiler metabolome is relatively novel and this thesis only examined a portion of the identifiable compounds in the spectra. Chenomx software had identified over 50 chemical compounds which were present in a concentration higher than 4mM, of which only eight were chosen for examination in the scope of this thesis. This opens up the possibility for more analysis with the dataset used. In future studies, more care should be taken to use only samples which are able to homogenize completely at the preparation stage; alternatively larger sample sizes would be needed if a large number of outliers are expected to occur due to the heterogenous nature of the cecal samples.

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