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THE IMPACT OF TEMPOL ON GUT MICROBIAL FERMENTATION AND SHORT-
CHAIN FATTY ACIDS

RYAN D. LYNCH
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Reviewed and approved* by the following:

Andrew Patterson
Assistant Professor of Molecular Toxicology
Thesis Supervisor

Lester C. Griel Jr.
Professor & Program Coordinator of VBS Undergraduate
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) is an antioxidant that is being developed as a therapy for the prevention and treatment for two of the most prevalent killers throughout the United States: cardiovascular disease and cancer. Recent studies have demonstrated tempol to be an effective scavenger of reactive oxygen species (ROS) that is capable of restoring normal function throughout the cardiovascular system in injured tissue due to ischemia and reperfusion. This report focuses on the ability of tempol (dose = 100 mg/kg by gavage) to prevent obesity in C57Bl/6 male mice (n = 7). Previous research has reported that tempol supplementation has prevented obesity in high-fat diet-fed mice through modulation of their gut microbiome, including pronounced phylum shifts in bacteria from *Firmicutes* to *Bacteroidetes*. This report expanded on this previous research by determining whether tempol supplementation affected the metabolism of short-chain fatty acids in addition to its effects on the gut microbiome. Thus, it was hypothesized that tempol supplementation would have significant effects on the gut microbial fermentation and short-chain fatty acid metabolism. NMR and GC-MS analysis was consistent and confirmed the hypothesis because there was a significant difference ($P < 0.05$) in the amount of short-chain fatty acids (butyrate, acetic acid, and propionate) in the cecal content of the tempol supplemented mice (n = 4) versus the non-tempol treated mice (n = 3). This experiment indicates that tempol has potential to prevent obesity in humans but more research is necessary to determine optimal dosage.

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Chapter 1: Introduction

1.1 Introduction to Tempol:

The heterocyclic compound tempol (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) has attracted the interest of the biomedical community for its versatile application in treating cardiovascular disease as well as its radioprotective effects (Li et al 2013, Mitchell et al 2012). For example, when mice were exposed to total body radiation and treated with tempol, it was reported that the mice not only lost weight without toxic effects but also lived longer (Mitchell et al 2012). It should not come as a surprise that the tempol treated mice lived longer because the antioxidant treated two of the most prolific causes of death: cardiovascular disease and cancer. In fact, according to the Center for Disease Control (CDC), in 2010 heart disease (24.2%) and cancer (23.3%) accounted for nearly half of the deaths in the United States (Murphy et al 2010).

Recent studies have highlighted the ability of tempol to counteract obesity in high-fat diet rodents. A study published in the *Nature Communications* reported that tempol reduced obesity in mice by modifying the gut microbiome through reducing the genus of bacteria in the gut in the *Firmicutes* phylum (particularly the *Lactobacillus* genus) in favor of the *Bacteroidetes* phylum (Li et al 2013). This phylum shift of gut bacteria simultaneously reduced the amount of bile salt hydrolase (BSH) activity in the bacterial community of the tempol treated mice compared to the control mice, which plays a significant role in breaking down tauro- β -muricholic acid (T- β -MCA). Thus, the

accumulation of T- β -MCA, a farnesoid X receptor (FXR) nuclear receptor antagonist, disrupted FXR signaling (Li et al 2013).

Other studies have also focused on the role of tempol in disrupting the FXR signaling pathway as a means to prevent obesity. For instance, a 2002 study published in *Science* analyzed the way in which a certain tree extract, called guggulsterone [4,17(20)-pregnadiene-3,16-dione], was able reduce LDL (low-density lipoprotein) cholesterol in high-cholesterol diet mice (Urizar et al 2002). The experiment showed that the extract was an inhibitor of the FXR nuclear signaling pathway, similar to T- β -MCA (Urizar et al 2002). Furthermore, a 2013 study published in *Molecular and Cellular Endocrinology* developed the understanding of how crucial the FXR-signaling pathway is to the regulation of bile acid synthesis in the digestive tract and to the communication between the liver and small intestine (Matsubara et al 2013). Through deactivation of this FXR-signaling pathway, substances like tempol have been shown to limit glucose intolerance, increase insulin sensitivity, and reduce overall body fat, body fat to overall body mass ratio, and body fat to lean mass ratio in high-fat diet-fed tempol supplemented mice as compared to the control mice (Li et al 2013).

1.2 Introduction to the Microbiome:

It is no coincidence that the intestinal tract is home to both a significant portion of the immune system and a substantial community of symbiotic microorganisms. In fact, the human intestinal tract contains ten times as many cells belonging to microorganisms than it does human cells (Zimmer et al 2010). Furthermore, the microbiome contains over 100 times as many genes as the human cell (Gill et al 2006). The microbiome is the

collection of the genomes of the microorganisms inhabiting the human intestinal tract (Backhead et al 2005). Similarly, the microbiota is the group of the microorganisms found throughout the intestinal tract (Backhead et al 2005).

It is not until as of late that the scientific community has come to appreciate how crucial the colonies of bacteria in the gut are to host immunity and overall health. Surprisingly, there is a strong connection between the microbiota and cognitive health or disorders. For example, mice with lowered levels of *Lactobacillus*, a phylum of bacteria that is abundant in healthy mammals, exhibited signs of depression (Gareau et al 2007). Disruption of proper gut microbiota has also been linked to anxiety disorders (Mikocka-Walus et al 2007). Nonetheless, proper immunity and cardiovascular health is also associated with a functional microbiota.

1.3 Tempol and Reactive Oxygen Species:

In addition to the anti-obesity effects experienced with tempol administration, tempol has attracted the interest of the biomedical community due to its protection against reactive oxygen species (ROS) as an antioxidant. In a 2008 study analyzing the effects of tempol on hypertensive rats, the antioxidant was shown to significantly reduce the levels of 8-Iso prostaglandin F₂α (8-ISO), a reliable *in vivo* marker of hypertension (Schnackenberg et al 1999). The same study went on to demonstrate that short-term administration of tempol mitigated oxidative stress as a free radical scavenger (Schnackenberg et al 1999). Other research has supported the theory that tempol provides radio-protection against reactive oxygen species (Hahn et al 1992).

Tempol may also combat tissue damage that resulted from exposure to ROS. For example, a pair of studies in *Kidney International* and *Brain Research* reported that tempol significantly restored function in renal and neural tissue that was damaged due to ischemia-reperfusion injury, which developed from extensive ROS exposure. These effects were likely mediated by the antioxidant effects of tempol (Chatterjee et al 2000, Cuzzocrea et al 2000).

Just as the study from *Brain Research* indicates above, the membrane-permeable ROS-scavenger effects of tempol may be more comprehensive than just eliminating free radicals. Supplementary research has concentrated on tempol as a treatment for various other diseases associated with obesity and the cardiovascular system. Not only does tempol supplementation combat cardiovascular disease through a shift in gut bacteria (which modulates fatty acid, glucose, and bile acid metabolism) but also through eliminating harmful ROS produced by excessive fat tissue found in and around the cardiovascular system (Fleenor et al 2014). A 2013 study published in *Aging Cell* identified tempol as a treatment for eliminating ROS produced by aortic perivascular adipose tissue (PVAT) in elderly subjects, relieving arterial stiffness and obesity (Fleenor et al 2014). Another study in *Hypertension Journal* has proposed that acute tempol treatment resulted in lowered heart rate (HR), mean arterial pressure (MAP), and renal sympathetic nerve activity (RSNA) without lowering vascular oxygen levels (Xu et al 2004). This study proposed that this was accomplished through direct sympathetic nerve activity inhibition in addition to its ROS-scavenging capabilities (Xu et al 2004).

1.4 Tempol and Nitroxide Activity:

In addition to being an effective antioxidant, tempol is also one of the most well-known nitroxides. Tempol administration rids the body of ROS and lowers blood pressure, but studies have shown that it may also affect the cardiovascular system through vasodilation and increased nitroxide activity (Wilcox et al 2008). Tempol may also affect the nervous system through reduction in sympathetic nervous system activity at central and peripheral sites and enhanced potassium channel conductance in blood vessels and neurons (Wilcox et al 2008). Furthermore, the report demonstrated that these benefits were observed at both acute and prolonged doses of tempol to the subjects (Wilcox et al 2008). Due to the fact that oxidative stress and nitroxide deficiency contribute to many complications with cardiovascular disease, tempol treatment has been proven to be an effective treatment because it addresses both of those underlying causes (Carlström et al 2013).

1.5 Tempol Shift on Gut Bacteria Phyla:

Although tempol's capacities to resolve oxidative stress and nitroxide deficiency significantly contribute to its effectiveness in treating cardiovascular disease and obesity, its induced shift in gut bacteria phyla also plays a large role. As previously mentioned, tempol treatment has been shown to induce a phylum shift in gut bacteria from *Firmicutes* to *Bacteroidetes* (Li et al 2013). Aside from the fact that this shift inhibits the FXR-signaling pathway and therefore may promote fatty acid metabolism, another reason this shift may prevent high-fat diet-induced obesity is that it fosters the digestion and

absorption of high-molecular weight molecules, particularly proteins and carbohydrates, like oligosaccharides, in favor of fatty acids (Thomas et al 2011).

Another factor that must be considered as a contributor to cardiovascular diseases is the inflammation resulting from immunological disorders. The intestinal tract is the most prominent site of interaction between the host's immune system, foreign bodies, and bacteria that can be either symbiotic or harmful (Round et al 2009). In fact, studies have shown that disruption of proper host-symbiotic bacteria interaction has led to chronic inflammation and host pathological disorders, indicating that proper host immune system function is partially dependent on the regulation of the microbiota (Round et al 2009). For example, "aberrant microbiota," or unfavorable changes to the gastrointestinal tract's microbial composition, has been shown to bring about an increased energy harvest and increase in toxic metabolites, which leads to intestinal inflammation and obesity (Chassaing et al 2014). Therefore, any substance like tempol that may alter this environment may also alter the regulation and function of the immune system (Round et al 2009).

1.6 Potential Drawbacks and Negative Effects of Tempol:

Though many of tempol's potential benefits have been thoroughly described above, it is important to note that like many substances tempol is not without its own potential drawbacks or toxic effects. It was previously mentioned that proper microbiota environment is vital for a functional immune system (Round et al 2009). It was also previously stated that tempol treatment fosters a shift of bacteria phyla from *Lactobacillus* to *Bacteroidetes* (Li et al 2013). However, it has been hypothesized that

probiotic *Lactobacillus* supplementation in infants is important for them to develop sound immune systems throughout their gastrointestinal tracts in order to prevent allergic diseases (Cox et al 2010). Moreover, various strains of *Lactobacillus* have exhibited antigenotoxic, or cancer-fighting, properties in regards to colon cancer (Cox et al 2010). Thus, any substance that potentially shifts away from this phylum of bacteria may very well prevent cardiovascular disease, but at the risk of exposing the host to a variety of other illnesses.

1.7 Tempol and Short-Chain Fatty Acids:

The metabolites of gut bacteria can be useful markers of cardiovascular disease as well. For example, it has been shown that type-II diabetes patients were associated with a decrease in bacteria that produce butyrate, the ideal energy source for colonic epithelial cells and an important proponent of the immune system (Qin et al 2012). Researchers developed a protocol titled, “metagenome-wide association study (MGWAS)” in order to determine the gut microbial content in subjects suffering from diabetes (Qin et al 2012). The NMR and GC-MS data collection for the results section of this experiment should be interesting to analyze the concentrations of products like butyrate in the tempol treated versus vehicle samples. A significant difference in concentrations between the two has potential to be a dependable predictor of diabetes-susceptibility in the mice, with anticipation that the tempol treated mice will exhibit higher levels of butyrate and therefore lower risk of developing cardiovascular disease.

Metagenome-wide association studies have also been used to help shed light on the genetic component related to diseases associated with obesity. Though the quality of

diet and supplementation of substances like tempol may heavily contribute to obesity and its complications, the genetic component must not be ignored. One study claims that, gene disorders are responsible for 5% of obesity cases (Bouchard et al 2009). The report continues on to state that although several different genetic loci have been linked to obesity, there still has not been one that is found in common with all obesity cases (Bouchard et al 2009). This goes to show that many factors, even genetic, can influence and predispose individuals for obesity, not just their diet and behaviors.

Analysis of patients' fecal matter is also being used to predict susceptibility to diseases associated with obesity, particularly type-II diabetes. Scientists are using a system called "shotgun sequencing," or breaking large strands of DNA into smaller ones for sequencing before reassembling them, in order to analyze the level of glucose metabolism and whether or not it appears to be "normal, impaired, or type-II diabetes-like (Karlsson et al 2013)." The researchers were even able to identify specific alternations in the fecal metagenomes of the Type II Diabetes subjects and thus were able to reasonably predict diabetes-like metabolism in the women subjects (Karlsson et al 2013). This study analyzes the cecal content of the mice subjects for similar purposes.

The focus of this study aimed to develop an understanding for how tempol supplementation in mice affected the host's short-chain fatty acid (SCFA) metabolism. SCFAs are 4, 5, or 6-carbon molecules with aliphatic tails and have recently been identified as important factors in digestive function and overall health and immunity (Brody et al 2009, Tan et al 2014). SCFAs are produced by the bacteria in the gut either during lipid metabolism or chemical breakdown of a partially indigestible substance (i.e., fiber) (Brody et al 2009, Tan et al 2014). These fatty acids are primarily digested in the

large intestine where they then enter the bloodstream and are processed or stored by the liver, if not utilized in the digestive tract environment (Tan et al 2014). SCFAs can also bind to and activate G-coupled protein-receptors, specifically the GPR43, GPR41, and GPR109A receptors (Tan et al 2014). It was previously stated that inflammation throughout the gastrointestinal tract is a very detrimental to health and immune system function; binding of these G-coupled protein-receptors has been linked to proper immune system functioning and prevention of inflammation (Tan et al 2014). Therefore, if tempol treatment results in increased production of SCFAs, then it may be more strongly implicated as a proponent of immunity throughout the digestive tract.

Increased production of SCFAs may also promote overall health through protection against ROS. By altering the metabolism and production of ROS, SCFAs, particularly butyrate and propionate, exhibit a chemoprotective ability that defends against cancer while fostering proper cellular proliferation and programmed-deaths (Giardina et al 1998). It is the location of SCFAs actions in the large intestine that their supplementation has been associated with prevention of colon cancer (McIntyre et al 1993).

However, there a couple of theories as to how SCFAs like butyrate fend off colon cancer. One theory suggests that butyrate stops cancerous cells from proliferating or growing by programming them for cellular death, or apoptosis; while another theory claims that the SCFAs is a crucial component of cellular growth for epithelial cells found in the colon (Singh et al 1997).

All of this information above strongly indicates that tempol administration may have several positive benefits for the host organism. Not only may this antioxidant foster

a shift in gut micro-bacteria that modulates host metabolism, but it also may fend off ROS, promote proper digestive and immune health, and offer protection from diseases related to obesity. Given the research pertinent to tempol, it is hypothesized that dietary exposure to the antioxidant tempol will have a significant effect on gut microbial fermentation of SCFAs.

Chapter 2: Methods & Materials

2.1 Diet and Mice Preparation:

This experiment included seven male mice (n=7), all six weeks of age, that were of the C57BL/6 strain, a common strain of mice used in metabolomics experiments. Three of the seven mice were in the control group and were not given any tempol supplementation. Four of the seven mice were in the experimental group and were given a 100 mg/kg of body weight dosage of tempol orally for 5 days. All mice were given a normal diet during this period of time. After the 5 days, the cecal contents, or the pouches of bacteria in the intestines, of the seven mice were extracted for analysis via NMR and GC-MS. All studies were approved by the Penn State Animal Care and Use Committee.

2.2. NMR Sample Preparation and Analysis:

NMR extraction was performed using PBS. More specifically, 0.1 M K_2HPO_4 : Na_2HPO_4 (4:1) containing D_2O and TSP was used. 50 mg of cecal contents were weighed into a homogenization tube. 6-8 beads were added to the tissue in addition to 1 mL PBS. The tissue was then vortexed for 30 seconds and homogenized for 30 seconds at 6500 rpm. The samples were freeze-thawed three separate times using liquid nitrogen. Following the last thaw, the tissue was spun at 11,800 x G for 20 minutes. After, about 600 microliters of supernatant was transferred to an Eppendorf tube. An additional 600 microliters was added to re-suspend the pellet. The sample was homogenized at 6500

rpm for 30 seconds and then vortexed for another 30 seconds. The sample was then spun at 11,800 x G for 20 minutes and the supernatant was transferred to the previous Eppendorf tube. The previous two steps were repeated twice in order to generate a combination of 3 supernatants in the one Eppendorf tube. These supernatants were vortexed and spun at maximum speed for 20 minutes. Finally, 600 microliters was transferred to the NMR tube for analysis.

2.3 ^1H NMR Spectroscopy:

^1H NMR spectra of aqueous liver and fecal extracts were recorded at 298 K on a Bruker Avance III 600 MHz spectrometer (operating at 600.08 MHz for ^1H) equipped with a Bruker inverse cryogenic probe (Bruker Biospin, Germany). Typical one-dimensional NMR spectrum was acquired for each of all samples employing the first increment of NOESY pulse sequence (NOESYPR1D). A weak continuous wave irradiation was applied to the water peak during recycle delay (2 s) and mixing time (100 ms) in order to suppress the water signal. The 90° pulse length was adjusted to approximately 10 μs for each sample and 64 transients were collected into 32 k data points for each spectrum with spectral width of 20 ppm.

2.4 Spectral Data Processing and Multivariate Data Analysis:

All free induction decays (FID) were multiplied by an exponential function with a 1 Hz line broadening factor prior to Fourier transformation. ^1H NMR spectra were corrected manually for phase and baseline distortions and spectral region δ 0.5-9.5 was integrated into regions with equal width of 0.004 ppm (2.4 Hz) using AMIX software

package (V3.8, Bruker-Biospin, Germany). A few regions of the spectral data, however, had to be removed. For example, region δ 4.45-5.20 was discarded by imperfect water saturation and regions δ 1.15-1.23 and δ 3.62-3.69 were also removed for ethanol contaminations in the cecal contents during mice dissection process. Each bucketed region was then normalized to the total sum of the spectral integrals to compensate for the overall concentration differences prior to statistical data analysis.

Multivariate data analysis was carried out with SIMCAP+ software (version 13.0, Umetrics, Sweden). Principle Component Analysis (PCA) was initially carried out on the NMR data to generate an overview and to assess data quality. Orthogonal Projection to Latent Structures with Discriminant Analysis (OPLS-DA) was subsequently conducted on the NMR data. To facilitate interpretation of the results, back-transformation (Cloarec et al., 2005) of the loadings generated from the OPLS-DA was performed prior to generating the loadings plots, which were color-coded with the Pearson linear correlation coefficients of variables (or metabolites) using an in-house developed script for MATLAB (The Mathworks Inc.; Natwick, MA). The color-coded correlation coefficient indicates the significance of the metabolite contribution to the class separation, with a “hot” color (e.g., red) being more significant than a “cold” color (e.g., blue). In other words, the closer the loading plot was to red, and the further it was from blue, the more significant the difference was between the tempol treated and vehicle groups.

2.3. GC-MS Sample Preparation and Analysis:

In order to acquire the best identification of short-chain fatty acids from the cecal contents of the mice, GC-MS analysis was performed. To initiate this process,

derivatives were extracted from the samples at 4 degrees Celsius. 5-10 acrylic beads, 50 mg of each sample, and 1 mL of 5 mM aqueous NaOH containing 5 micrograms/mL D3-caproic acid were all combined in a 2 mL homogenized tube. The internal standard, D3-caproic acid, was used in a known quantity and added to the components of interest so that the signals of the components could be compared to the signal of the internal standard to determine how much of the components were present. Next, the samples were homogenized at 6500 rpm, 1 cycle, for 60 seconds. The samples were then centrifuged at 4 degrees Celsius for 20 minutes at max speed. After centrifuging was finished, 500 microliters from the supernatant of each of the tubes was transferred to 20 mL glass scintillation vials.

Finally, derivitization was performed for each of the samples. 500 microliters of 1-propanol: pyridine (3:2 ratio) solution was added to the samples. 100 microliters of propyl chloroformate was slowly added to the samples to avoid an aversive reaction and vortexed gently, periodically opening and closing the caps to relieve pressure from the reaction. Samples were heated at 60 degrees Celsius for 1 hour and transferred to glass GC vials. 300 microliters of hexanes were added to the derivatized samples and vortexed for 1 minute as part of the extraction process. The samples were then centrifuged at 2000 x G for 5 minutes. Following this, 300 microliters of the supernatant from the samples were transferred to glass auto-sampler vials. Another 200 microliters of hexanes was added to the remaining derivatized samples, not the supernatants which were just transferred. The process of vortexing and centrifuging was repeated for the remaining derivatized samples and 200 microliters of the resulting supernatants were added to the

files containing the previously deposited 300 microliters of supernatant. Finally, the samples were crimped and stored at -20 degrees Celsius.

The GC-MS procedure was performed using the 25 minute PSPLIT method. The samples were run in an Agilent HP-5ms column and reached a maximum temperature of 325 °C.

After the samples had been run, they were analyzed via the ChemStation program. The substances were identified and labeled for each sample by their known retention times, peak areas, and fragmentation patterns compared to the standard used.

Chapter 3: Results

3.1 NMR Analysis Results:

Figure 1: ^1H NMR Spectroscopy Data of Tempol vs. Vehicle

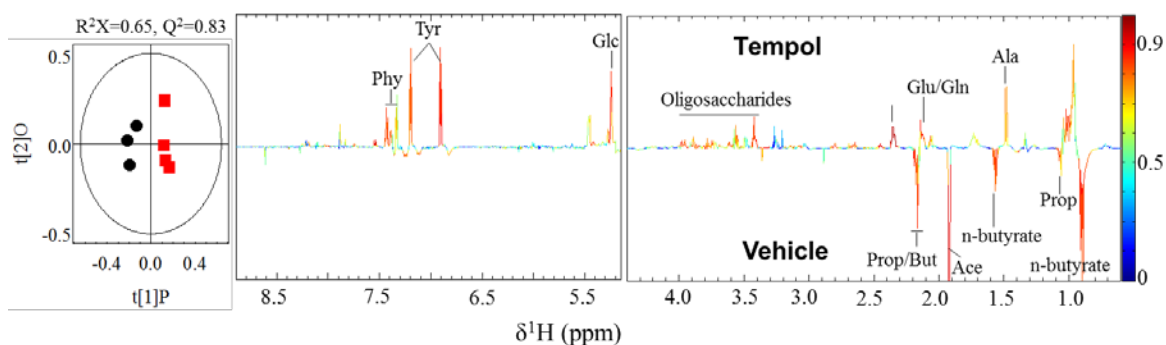


Figure 1 Legend. Tempol Reduces the Bacterial Fermentation Process. OPLS-DA scores (left) discriminated between the vehicle (black circles) and tempol treated mice (red dots). The two axes of the OPLS-DA graph, $t[1]P$ and $t[2]O$, represent the principle components and the R^2X and the Q^2 values measured the model fit and model's predictive value, respectively. On the right of the figure are coefficient-coded loadings plots for the models from NMR spectra of cecal content extracts. The substances were identified by their chemical shifts (in ppm) on the x-axis of the figure. The larger the plot of a substance and the closer the color is to red, the more significant difference in amount there is of the substance between the vehicle and tempol treated mice. A plot that is colored blue denotes a difference in amount of SCFA between the vehicle and tempol treated groups of mice that is not significant. Thus, concentrations of “n-butyrate,” “Ace,” and “Prop/But” are significantly reduced in the tempol treated mice, as evident by their larger peaks and red colors. [Abbreviations: Prop = propionate, Ala = alanine, Ace

= acetic acid, Glu/Gln = glutamate/glutamine, Prop/But = propionate/butyrate, Glc = glucose, Tyr = tyrosine, and Phy = phenyl groups]

3.2. GC-MS Analysis Results: (significant differences highlighted in yellow)

Table 1a-c: GC- MS Peak Areas of SCFAs Relative to Internal Standard Area

1a.

Ret Time		643 Tempol	644 Tempol	645 Tempol	646 Tempol
2.5	Acetic Acid	2.0	2.2	1.8	1.6
3.8	Propionic Acid	0.8	0.7	0.8	0.8
5.3	Butyric Acid	1.1	1.8	1.2	0.7

1b.

Ret Time		670 Control	671 Control	672 Control
2.5	Acetic Acid	2.9	2.9	2.4
3.8	propionic Acid	0.9	1.1	1.1
5.3	Butyric Acid	2.0	1.7	1.3

1c.

Ret Time		Average	t test
2.5	Acetic Acid	2.8	0.02
3.8	propionic Acid	1.0	0.01
5.3	Butyric Acid	1.7	0.18

Table 1 Legend. Mice 643, 644, 645, and 646 were tempol treated. Mice 670, 671, and 672 were control mice that were not given tempol. T-test values that are less than 0.05 are considered significant. [Units: (Peak Area of SCFA)/(Peak Area of Internal Standard)]

Table 2a-b: GC-MS Peak Areas of SCFAs

2a.

Ret Time		Blank	643 Tempol	644 Tempol	645 Tempol	646 Tempol
2.5	Acetic Acid	0	14887395	18670420	12891946	10621330
3.8	propionic Acid	0	6168638	6077881	5716106	5307620
5.3	Butyric Acid	0	7750700	14791686	8081203	4787564
10.8	D3-Caproic Acid	8517784	7322643	8306522	6973718	6713172

2b.

Ret Time		Blank	670 Control	671 Control	672 Control
2.5	Acetic Acid	0	20658248	19714392	16027813
3.8	propionic Acid	0	6778133	7274017	7134448
5.3	Butyric Acid	0	14072068	11545517	8271552
10.8	D3-Caproic Acid	8517784	7107086	6689062	6599656

Table 2 Legend. Mice 643, 644, 645, and 646 were tempol treated. Mice 670, 671, and 672 were control mice that were not given tempol. The internal standard used, D3-caproic acid, is highlighted in green. (Units: Peak Area of SCFA)

Table 3: Cecal sample weights (samples with "*" were treated with tempol)

	Sample Number	643*	644*	645*	646*	670	671	672
Cecal sample weight (mg)	-	50.8	54.3	52.8	53.9	51.7	61.2	59.9

Table 4: GC-MS – Mean amounts of SCFAs per cecal sample weights

	Acetic Acid	Propionate	Butyrate
Tempol Mean (units = micromoles/g cecal matter)	44.8	6.2	15.4
Tempol Standard Deviation (units = micromoles/g cecal matter)	4.1	.9	5.4
Control Mean (units = micromoles/g cecal matter)	54.4	9.0	20.0
Control Standard Deviation (units = micromoles/g cecal matter)	8.8	0.2	5.9
P-value (P-value < 0.05 is significant)	0.01	0.01	0.14

Figure 2: Acetic Acid GC-MS Standard Curve

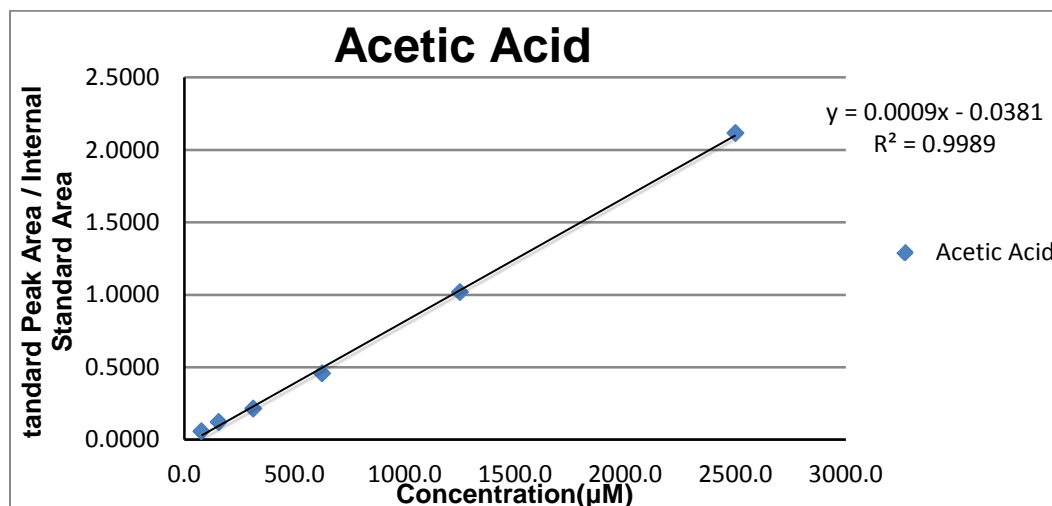


Figure 2 Legend. Standard Curve of acetic acid to determine concentration (units = μM) in each sample. Y-axis plots standard peak area of acetic acid for a specific sample divided by the internal standard (D3-caproic acid) area for same sample. Both of these values are listed in Table 2a-b in the same column for each sample. Once this number, “y,” is calculated, the concentration (in μM) of acetic acid for the sample can be calculated by plugging the y-value into the equation for the best-fit line, “ $y = 0.0009x - 0.0381$ ” and solving for “x.” The R^2 value represents the coefficient of determination and displays how well the data points fit the best-fit line. The closer it is to 1, the better the fit. Hence the data points fit this graph’s best-fit line well because its R^2 value is close to 1.

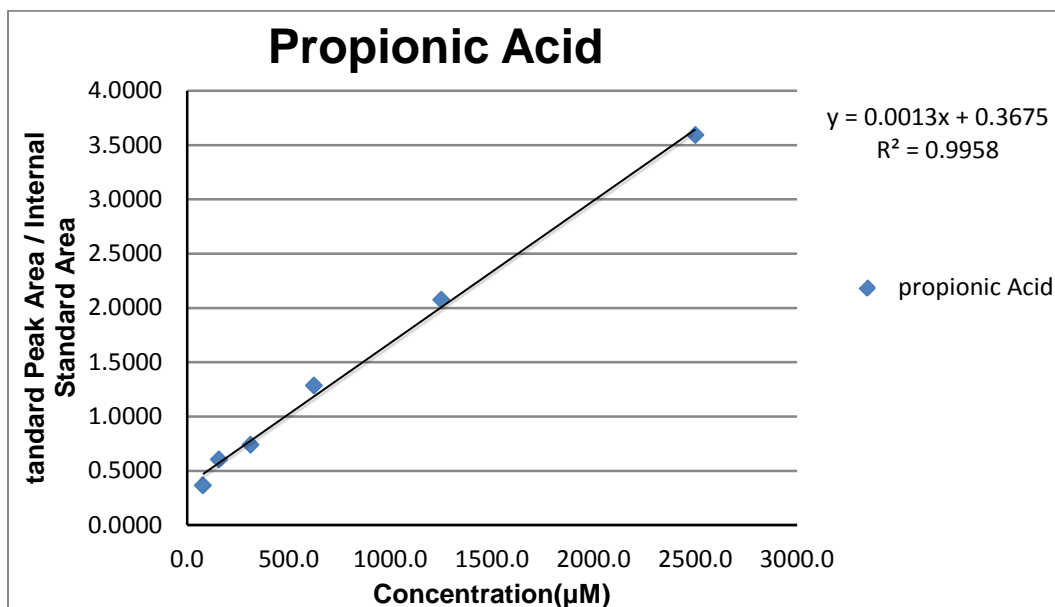
Figure 3: Propionate GC-MS Standard Curve

Figure 3 Legend. Standard Curve of propionic acid/propionate to determine concentration (units = μM) in each sample. Y-axis plots standard peak area of propionic acid for a specific sample divided by the internal standard (D3-caproic acid) area for same sample. Both of these values are listed in Table 2a-b in the same column for each sample. Once this number, “y,” is calculated, the concentration (in μM) of propionic acid for the sample can be calculated by plugging the y-value into the equation for the best-fit line, “ $y = 0.0013x + 0.3675$ ” and solving for “x.” The R^2 value represents the coefficient of determination and displays how well the data points fit the best-fit line. The closer it is to 1, the better the fit. Hence the data points fit this graph’s best-fit line well because its R^2 value is close to 1.

Figure 4: Butyrate GC-MS Standard Curve

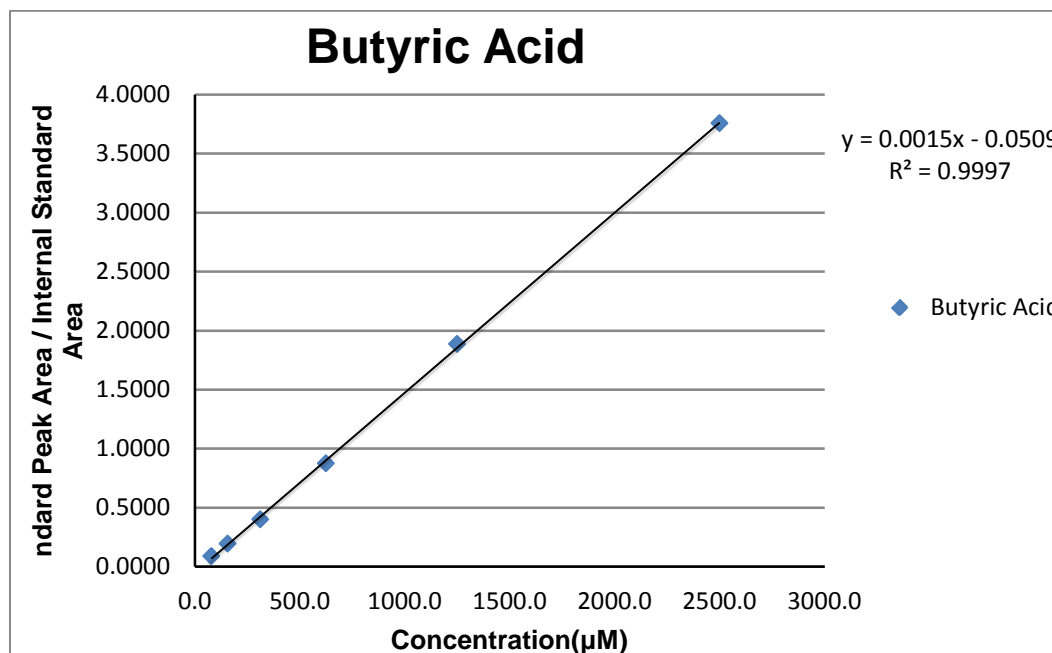


Figure 4 Legend. Standard Curve of butyric acid/butyrate to determine concentration (units = μM) in each sample. Y-axis plots standard peak area of butyric acid for a specific sample divided by the internal standard (D3-caproic acid) area for same sample. Both of these values are listed in Table 2a-b in the same column for each sample. Once this number, “y,” is calculated, the concentration (in μM) of butyric acid for the sample can be calculated by plugging the y-value into the equation for the best-fit line, “ $y = 0.0015x - 0.0509$ ” and solving for “x.” The R^2 value represents the coefficient of determination and displays how well the data points fit the best-fit line. The closer it is to 1, the better the fit. Hence the data points fit this graph’s best-fit line well because its R^2 value is close to 1.

3.3 Results Explanation:

Figure 1 details the results of ^1H NMR Spectroscopy analysis of the samples. The NMR data was used to determine whether there was a significant difference in the relative amounts of the SCFAs butyrate, acetic acid, and propionate as well as oligosaccharides. The figure on the right detailed the coefficient-coded loadings plots for the models from NMR spectra of cecal content extracts. The larger the peak of a substance (i.e., a SCFA, amino acid, or oligosaccharide) and the closer the color is to red, the more significant difference there is between the vehicle and tempol treated mice in the amount of a substance. On the contrast, the bluer a plot is, the less significant a difference there is between the vehicle and tempol treated groups of mice for a substance. The color-coded meter on the far right of the figure ranges from 0.0-1.0, and as the number value increases, the color of the plot becomes redder and less blue. The larger the plot is, the greater the numerical value is, the redder the plot is, and the more significant the difference in the amount of the substance between the tempol treated and vehicle mice. Thus, concentrations of butyrate, acetic acid, and propionate are significantly reduced in the tempol treated mice compared to the vehicle mice because their corresponding plots are close to 1.0 on the color-coded scale of the figure.

The figure on the left of the NMR data was used to determine the model fit and reliability of the ^1H NMR spectroscopy analysis. The four red OPLS-DA dots represented the tempol treated mice and the three black dots represented the vehicle, or control, mice. The two axes of the OPLS-DA graph, $t[1]P$ and $t[2]O$, represent the principle components. The OPLS-DA scores quantify the deviation in the dataset of SCFAs and measure the reliability of the NMR. The R^2X score measured the model fit

and the Q^2 value measured the model's predictive value. The closer each of these values is to 1.00, the better model fit and predictive value. The R^2X value of 0.65 measured the model fit while the Q^2 value of 0.83 represented the model's predictive value. Since both these values were relatively close to 1.00, the NMR data was considered to be significant and reliable.

Both parts of Figure 1 demonstrate that tempol reduces the bacterial fermentation process.

Table 1a-c was used to determine whether or not the differences in the amounts of the three SCFAs mentioned above were significant. The t-tests in the last column of Table 1c ultimately determined if there were significant differences. Significant t-test values (less than 0.05) were highlighted in yellow. With t-tests of 0.02 and 0.01, respectively, acetic acid and propionate had significantly lower levels in the tempol treated mice versus the vehicle mice. Acetic acid and propionate also both had p-values of 0.01 to further support the significance of the difference between their amounts in the tempol treated versus vehicle mice. Contrarily, butyrate had a t-test value of 0.18 and a p-value of 0.14, which both state that there is no significant difference between the tempol treated and vehicle mice.

Standard curves (Figures 2-4) were included in the GC-MS analysis of the dataset in order to determine the relative concentrations of each short-chain fatty acid was found in the samples. On the y-axis of each of these figures was the standard peak area of the short-chain fatty acid divided by the internal standard area. Table 2a-b helped determine these values by dividing the area of the short-chain fatty acid of a particular sample by the area of the internal standard (D3-caproic acid) for the same sample. This y-value was

then plugged into the figure's linear equation to determine the x-value, or the concentration of the SCFA in micromoles for the sample. Then, this concentration was divided by the sample's weight in Table 3 to provide a concentration to sample weight ratio. These ratios for a given SCFA was then averaged for all the tempol treated samples (samples 643-646) and compared to the average for the control samples (samples 670-672). These comparisons for all the SCFAs and their respective t-tests were listed in Table 4 for further analysis.

These experiments were repeated twice in two sets of mice and similar results were obtained.

Chapter 4: Discussion

4.1 NMR Results Discussion:

The results of the NMR analysis, detailed by Figure 1, confirmed the hypothesis that tempol supplementation of a normal diet would significantly affect the gut microbial fermentation of SCFAs. Particularly, the NMR peaks from 0.8-2.25 ppm in Figure 1 demonstrated how the three SCFAs butyrate, acetic acid, and propionate were significantly reduced in the tempol treated mice relative to the control mice. These results were consistent with the hypothesis because the less SCFAs that are present in the digestive tract of mice (particularly the cecum in the case of this experiment), the less likely the mice are to become obese and experience accompanying complications (Boden 2007). For example, higher levels of free fatty acids (FFA) are associated with greater risk for insulin resistance and gastrointestinal tract inflammation, which can lead to a number of cardiovascular disease developments like type 2 diabetes, hypertension, and atherosclerosis (Boden 2007).

It is important to note that Figure 1 also showed that the oligosaccharides found on the NMR from about 3.0-4.0 ppm were significantly increased in the tempol treated group relative to the control group. This makes sense because oligosaccharides are the substrate material for short-chain fatty acids, like the three of interest. This was demonstrated in a 2009 study that showed prebiotic administration of oligosaccharides increased concentrations of SCFAs in the cecal contents of mice (Pan et al 2009). Thus if

the SCFA fermentation is reduced, the substrate material (oligosaccharides) should be increased (Pan et al 2009).

The plot on the far left of Figure 1 represents the principle components, t[1]P and t[2]O. The purpose of this graph was to quantify the deviation in the dataset of short-chain fatty acids and measure the reliability of the NMR. The R^2X value of 0.65 demonstrated an accurate model fit. The Q^2 value of 0.83 demonstrated the model's highly accurate predictive value. Since both these values were around 1.00, the NMR data was considered to be significant and reliable so conclusions could be reasonably drawn from the results of NMR analysis.

4.2 GC-MS Results Discussion:

The results of GC-MS analysis of the dataset partially supported the hypothesis that tempol supplementation of a normal diet would significantly affect the gut microbial fermentation of short-chain fatty acids. It only partially supports the hypothesis because the GC-MS results only confirm that the fermentation of acetic acid and propionate, but not butyrate, were affected by tempol administration.

Table 4 sums up the results of GC-MS analysis of the samples. Although the average concentrations per body weight for all three SCFAs were reduced in the tempol mice compared to the control mice, only the acetic acid and propionate results had significant differences, given by their p-values. Since acetic acid and propionic acid/propionate both had p-values of .01, it can be concluded that the microbial fermentation of these two short-chain fatty acids was significantly affected by tempol administration.

4.3 Main Conclusions:

The introduction discussed many of the important functions of tempol including its identity as a nitroxide, scavenger of reactive oxygen species, and its relationship with the microbial environment in the gut. Several studies were even cited to demonstrate the ability of this antioxidant to prevent and treat complications and diseases associated with obesity and cardiovascular disease. As our nation collectively struggles with curbing obesity and its associated chronic illnesses, the need to explore anti-obesity compounds like tempol has never been more imperative.

For the most part, this experiment contributed valuable information about the way in which tempol affects the gastrointestinal environment and health of mice. NMR and GC-MS analysis results showed that tempol administration significantly reduced the amount of two out of the three SCFAs analyzed. By reducing the amount of SCFAs present in the cecal contents of the mice, tempol effectively reduced the mice's risk for obesity, inflammation, and several cardiovascular diseases. Although levels of the SCFA butyrate were not significantly reduced in the tempol treated mice, this is consistent with data mentioned in the introduction that type-II diabetes patients were associated with a decrease in bacteria that produce butyrate, the ideal energy source for colonic epithelial cells and an important proponent of the immune system (Qin et al 2012). Thus, it was not surprising that of the three SCFAs of interest in the study, butyrate was the one that was not significantly reduced in the tempol treated group. All of this indicates that tempol supplementation may be able to combat obesity and cardiovascular disease in humans under the proper dosage.

4.4 Future Considerations:

One of the most difficult parts of this experiment was perfecting the derivatization process. It took several attempts to finally arrive on a method that provided acceptable results for data analysis. With this knowledge, future projects' derivatization process should be performed with much less hassle.

Future projects involving tempol administration should focus on the dosage of the antioxidant in greater detail. Although this project was able to produce significant results, only one dosage was used (100 mg/kg body weight). Future studies would be benefited by using multiple doses to determine the lowest dosage of tempol that still produces the same benefits.

Lastly, future studies would be benefited by analyzing serum and urine samples in addition to cecum samples. This study only looked at the cecum contents of the mice, but analysis of the two other types of samples could provide a more complete picture of how tempol affected the gut microbial fermentation of SCFAs.

Appendix A

List of Abbreviations:

FFA – Free Fatty Acids

FID – Free Induction Decays

GC-MS - Gas Chromatography - Mass Spectrum

HR - Heart Rate

MAP - Mean Arterial Pressure

NMR - Nuclear Magnetic Resonance

NO – Nitroxide

OPLS – Orthogonal Projection to Latent Structures with Discriminant Analysis

PCA – Principle Component Analysis

PVAT - Perivascular Adipose Tissue

ROS - Reactive Oxygen Species

RSNSA - Renal Sympathetic Nerve Activity

SCFA – Short-Chain Fatty Acid

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ACADEMIC VITA

Ryan D. Lynch
1021 Edmonds Ave, Drexel Hill, PA 19026/rdl5102@psu.edu

Education:

The Pennsylvania State University, University Park, PA

Schreyer Honors College

Major: Science (B.S.), Health Professions Option and Honors in Veterinary and Biomedical Sciences

Honors and Awards:

- Dean's List Scholar Fall 2010 - present (every semester)
- Schreyer Honors College Gateway Scholar Summer 2012 - present
- James F. Modliszewski Integrity Award Winner Fall 2013

Science and Medical Experience:

- Honors Thesis in Veterinary and Biomedical Sciences Spring 2014
- *Hospital of the University of Pennsylvania*, Premed Volunteer Summer 2013
- Undergraduate Teaching Assistant Fall 2013 – Spring 2014
 - Department of Biobehavioral Health (TA for BBH 432)
 - Department of Sociology (TA for SOC 003)
- Undergraduate Research Assistant Fall 2012 - Spring 2013
 - *Pennsylvania State University's Center for Molecular Toxicology and Carcinogenesis*
 - *Penn State University's General Clinical Research Center*
 - *Noll Physiological Research Center*
- Independent Study in Kinesiology & Exercise Science Spring 2012
- Clinical Externship in Ophthalmology, *Johns Hopkins Hospital* Summer 2011

Collegiate Leadership Experiences:

- President of Alpha Gamma Rho Fraternity Fall 2012 – Fall 2013
- Vice President of Alpha Gamma Rho Fraternity Fall 2011- Fall 2012
- Coordinator of Penn State University's Bone Marrow Drive Spring 2011