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VOLUME 2, ISSUE 4 | APRIL 2020
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## Antibacterial honey
Considering the effects of osmotic stress on honey's antimicrobial properties

## Sugar rush
Investigating factors behind high schoolers' consumption of sugar

## Improving biocatalyst efficiency
A new method of catalase entrapment using "worms"

## QuitPuff: A pre-screening test for oral cancer
A simple saliva-based test to quantify oral cancer risk in smokers

# JOURNAL OF EMERGING INVESTIGATORS

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Article

# Performance of *Panicum virgatum* cultivars in competition with *Bromus inermis* and differing amounts of N fertilizer

**Tyler Ferris and Chelle Gillan**
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## SUMMARY

**With growing demands for ethanol, many researchers are turning to *Panicum virgatum* (switchgrass) as a feedstock of cellulosic ethanol. This study was conducted to examine the germination, biomass, nitrogen, survival, and chlorophyll absorbance of two cultivars of switchgrass grown in competition with *Bromus inermis* (smooth brome) and with two different levels of nitrogen fertilizer. I predicted that these factors would affect the aforementioned variables, as nitrogen promotes plant growth, competition from other plant species detracts from nutrient availability, and these cultivars are physiologically different. The experiment was conducted in a greenhouse as 12 treatments replicated 20 times. Results indicated that the Liberty cultivar had lower germination, but higher survivorship than the Cave-in-Rock cultivar and that treatments with higher levels of competition resulted in lower biomass. Additionally, control treatments of both switchgrass cultivars grown in the absence of smooth brome had the highest levels of chlorophyll absorbance, and competition treatments had overall lower levels of nitrogen than control treatments. Both levels of nitrogen fertilizer decreased chlorophyll absorbance. Furthermore, switchgrass cultivars grown in competition with smooth brome had lower levels of nitrogen compared to control treatments. My results also indicate that during establishment, competition from other species has a greater effect than nitrogen fertilizer. Replicating this experiment for a multi-year randomized block setup would be ideal for extending this study.**

## INTRODUCTION

*Panicum virgatum*, more commonly known as switchgrass, is a native perennial warm-season grass (NPWSG) native to North America. It has been used in hay systems for livestock, as a soil conservation crop, as well as for wildlife cover. Switchgrass is also considered an ideal herbaceous model for biomass energy (1). As a high yielding native prairie grass, switchgrass has the potential to be a major source of bioenergy due to its low input requirements, as well as its broad range of adaptation (2).

I conducted this study to analyze several crucial aspects of switchgrass management as a cellulosic ethanol crop when establishing the crop in competition with *Bromus inermis*, or smooth brome, a non-native cool season perennial grass selected in previous studies as a vigorous competition model (3). This study also addressed the performance of the upland switchgrass cultivar "Cave in Rock", which is typically used for wildlife cover and reestablishing grasslands, and the lowland switchgrass cultivar "Liberty", developed for use as a cellulosic ethanol feedstock for the Great Plains region. Additionally, because nitrogen fertilizer is commonly considered one of the most important inputs for a producer, I varied applied at planting to assess its effects (4-5). Upon the conclusion of the three-month growth period, dry biomass yield, I assessed the number of surviving switchgrass and smooth brome plants, chlorophyll absorbance, plant nitrogen content, and soil nitrate levels.

I hypothesized that treatments receiving urea nitrogen fertilizer would experience increased germination levels, plant survival, nitrogen content, chlorophyll absorbance, and dry biomass yield in both switchgrass cultivars. Conversely, I also hypothesized that smooth brome competition would affect germination levels, plant survival, nitrogen content, chlorophyll absorbance, and dry biomass yield in both switchgrass cultivars, with all of these variables decreasing as the level of competition increased.

## RESULTS

### *Methods Summary*

Cubic foot pots were placed in a heated greenhouse kept at and filled with soil. Each pot received one treatment comprised of one of two switchgrass cultivars (Cave in Rock or Liberty), one of two levels of urea nitrogen fertilizer, and one of three smooth brome competition levels (no competition, 33% competition, and 67% competition). With every combination including 1 variable from each field (switchgrass, fertilizer, and competition level), 12 treatments were created, each of which was replicated 20 times for a total of 240 pots. The location of these pots within the greenhouse was randomized to avoid biased management and to mitigate variable climate conditions in the greenhouse. Pots were planted switchgrass to 30 pure live seed (PLS) per pot, with smooth brome competition percentages (0%, 33%, and 67% making up the remaining seedlot. Plants were allowed to grow normally for three months, during which plants were watered once per week. Two stand counts (one taken shortly after germination, and one taken just before harvest) were performed in which emerged switchgrass plants were counted. After the growth period, a SPAD chlorophyll meter was used to measure chlorophyll absorbance of switchgrass leaves, and plants

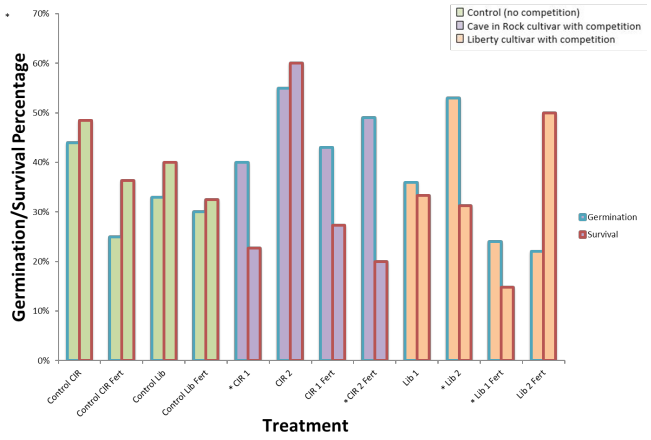


**Figure 1. Germination and Survival.** Germination data (taken 43 days after planting) by treatment compared to survival (taken 38 days after germination recorded) at conclusion of growth period. Single-factor ANOVA and Tukey-Kramer test were run. Asterisks indicate treatment with statistical significance ($p < 0.05$) between treatment and corresponding control treatment conditions according to Tukey Kramer test.

were harvested for a dry biomass measurement and for percent nitrogen, measured by Ward Laboratories.

### *Germination/Survival*

The effect of different levels of nitrogen fertilizer and competition from smooth brome on germination and survival of two cultivars of switchgrass was assessed (**Figure 1**). An ANOVA test performed on germination and survival data showed different levels of nitrogen fertilizer resulted in differences in survival rate ($p = 3.88 \times 10^{-6}$). Individual treatment comparisons revealed that all control treatments, which contained no smooth brome competition (Control CiR, Control CiR Fert, Control Lib, and Control Lib Fert, **Table 1**), resulted in statistically increased survival, but not germination, as compared to those with competition ($p < 0.05$, Tukey-Kramer test) (**Figure 1**). The Cave in Rock treatments,

| Treatment Name | Cultivar | Competition | Fertilizer |
|---|---|---|---|
| Control CiR | Cave in Rock | None | None |
| Control CiR Fert | Cave in Rock | None | 1.1g urea N per rep. |
| Control Lib | Liberty | None | None |
| Control Lib Fert | Liberty | None | 1.1g urea N per rep. |
| CiR 1 | Cave in Rock | 33% smooth brome per rep. | None |
| CiR 2 | Cave in Rock | 67% smooth brome per rep. | None |
| CiR 1 Fert | Cave in Rock | 33% smooth brome per rep. | 1.1g urea N per rep. |
| CiR 2 Fert | Cave in Rock | 67% smooth brome per rep. | 1.1g urea N per rep. |
| Lib 1 | Liberty | 33% smooth brome per rep. | None |
| Lib 2 | Liberty | 67% smooth brome per rep. | None |
| Lib 1 Fert | Liberty | 33% smooth brome per rep. | 1.1g urea N per rep. |
| Lib 2 Fert | Liberty | 67% smooth brome per rep. | 1.1g urea N per rep |

**Table 1. Description of Treatments.**

with the exception of CiR 2 (Cave in Rock cultivar with 67% smooth brome competition and no fertilizer; **Table 1**), all showed relatively low survivorship. The results also indicate that the Liberty cultivar had better survivorship compared to the Cave in Rock cultivar.

### *Biomass*

Dry biomass is depicted as grams of dry aboveground matter taken from four replicates for each treatment (**Figure 2**). Control treatments (**Table 1**) with nitrogen fertilizer resulted in lower germination than their non-fertilized cultivar counterparts (**Figure 2**). With treatments containing the Cave in Rock cultivar and competition from smooth brome, increased competition resulted in lower biomass yields regardless of fertilizer rate. This trend was also observed in the treatments containing the Liberty cultivar and competition from smooth brome, though with less disparity. A correlation test showed a moderate positive correlation between biomass and chlorophyll absorbance (**Figure 5**).

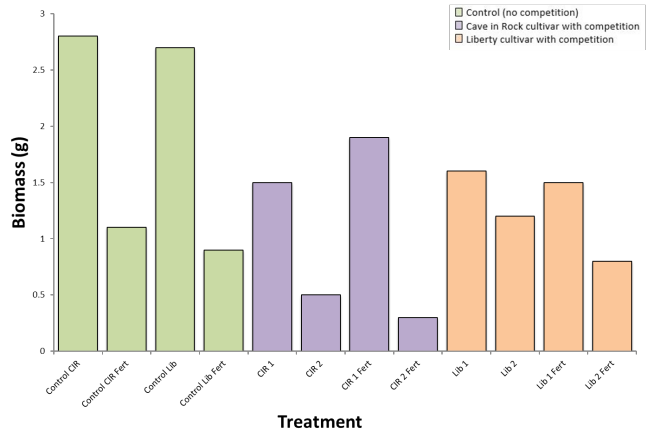


**Figure 2. Dry Biomass.** Dry biomass of four replicate pots combined per treatment.

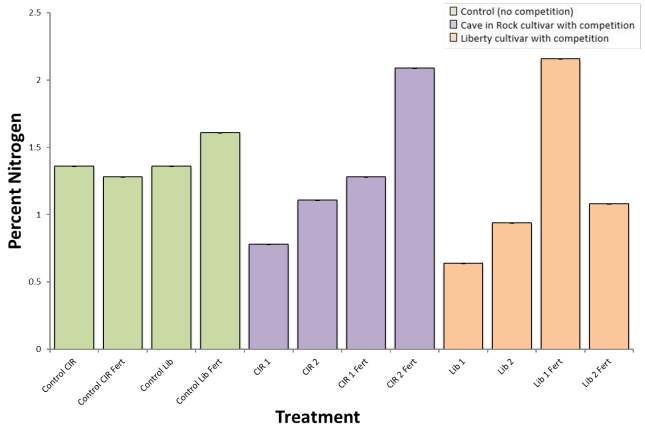


**Figure 3. Percent Nitrogen.** Percent nitrogen of five replicates per treatment of total elements calculated by Ward Labs using Crude Protein Combustion Method.

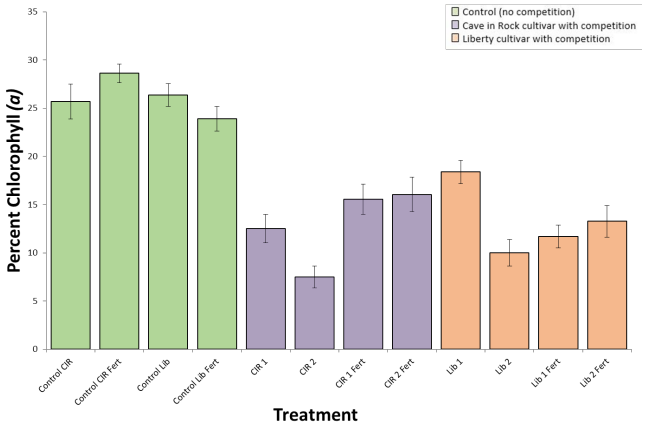


**Figure 4. Percent Chlorophyll Absorption.** Average percent absorbance of chlorophyll per treatment. Single-factor ANOVA and Tukey-Kramer test were run. Single-factor ANOVA yielded *p*-value of $4.7x10^{-41}$. Thin black lines are standard error.

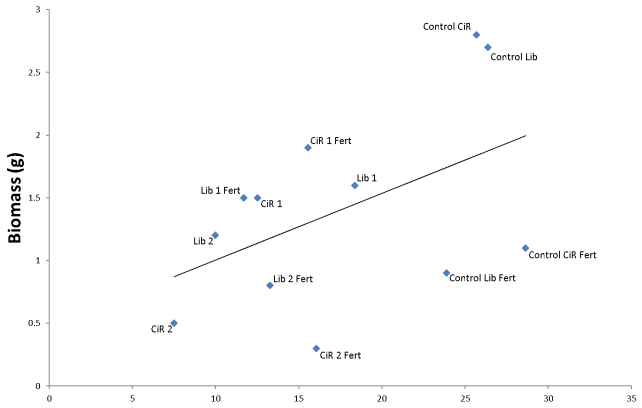


**Figure 5. Correlation Coefficient Test of Dry Biomass and Chlorophyll Absorbance.** Pearson correlation coefficient test indicates moderate positive correlation (R=0.48) between chlorophyll absorbance and dry biomass. Each data point depicts the relationship between chlorophyll and dry biomass per treatment.

### *Nitrogen*

Depicts the nitrogen content refers to five replicates from each treatment (**Figure 3**). These results indicate that all treatments contained similar levels of nitrogen. Control treatments contained higher levels of nitrogen, but all treatments were within two percent of total nitrogen.

### *Chlorophyll*

We next examined the chlorophyll absorbance between the different treatments (**Figure 4**). An ANOVA test performed on chlorophyll data yielded a *p*-value of $4.7x10^{-41}$, which indicated statistical significance. This ANOVA was followed by a Tukey-Kramer test in order to determine statistical significance for individual treatment comparisons. All experimental manipulations (CiR 1, CiR 2, CiR 1 Fert, CiR 2 Fert, Lib 1, Lib 2, Lib 1 Fert, and Lib 2 Fert) all had statistically lower chlorophyll absorbance levels compared to their corresponding control (**Figure 4**). Treatments containing the Cave in Rock cultivar and competition from smooth brome showed a statistical difference between fertilized and unfertilized treatments, with the unfertilized, more-competitive treatment ranking statistically lower than all other treatments containing the Cave in Rock cultivar and smooth brome competition. The chlorophyll absorbance of the treatments containing the Liberty cultivar and competition from smooth brome was statistically equivalent, with the exception of the unfertilized, less-competitive treatment, which showed a statistically greater absorbance in chlorophyll compared to the other three treatments containing the Liberty cultivar and competition from smooth brome. A correlation test showed a moderate positive correlation between chlorophyll and biomass (**Figure 5**).

## DISCUSSION

This study was conducted with the intent of determining the effect of different levels of competition of smooth brome with differing levels of nitrogen fertilizer on two cultivars of switchgrass. Treatments without competition yielded statistically higher survivorship when compared to treatments with competition. Additionally, treatments that contained greater levels of urea nitrogen fertilizer and competition resulted in lower dry biomass yield, while total nitrogen levels were similar for all treatments. It should also be noted that the application of urea nitrogen fertilizer at planting resulted in lower biomass for treatments containing no competition as well as for those treatments which contained competition. Finally, switchgrass grown in the absence of smooth brome competition showed statistically greater levels of chlorophyll absorbance. The data from this study suggests that during stand establishment, the presence of competition has a negative effect on both cultivars of switchgrass. Furthermore, these results suggest that application of urea nitrogen fertilizer at planting did little to affect switchgrass growth, but likely aided the smooth brome competitor and impeded the young switchgrass plants in their ability to produce biomass, which in addition to the quality of major cell wall components such as lignin and cellulose, is a major factor in cellulosic ethanol yield (6). This claim is supported by past studies on switchgrass, which recommend foregoing nitrogen application during switchgrass establishment, as the crop is unable to effectively utilize the nitrogen and outperform competitor species until it has matured for one year (2).

The data collected in this study suggests that that managing competitor species during the establishment period is of the utmost importance to the producer, as nitrogen fertilizer applied once the stand is mature enough to benefit from it is likely to aid and propagate any remaining competitor species like smooth brome. Contrarily, a legume species, when grown in concert with a well-established stand of switchgrass, has been shown to increase switchgrass biomass, as it is able to access nitrogen fixed by the legume species (7). Replicating this experiment in a multi-year random block design study would prove beneficial for gauging the effects of competition

and nitrogen fertilizer application at all times in the life cycle of switchgrass produced for cellulosic ethanol, rather than just the establishment period.

## METHODS

### *Preparation*

Pots were filled with one cubic foot of Lockton series soil (8), saturated with water, assigned a number between 1 and 240, and placed in a location that corresponded with a randomly generated number in a heated greenhouse. Pots were then left overnight to drain. Using damp soil, pots were refilled the following day to create a smooth, level, compact surface for planting. Seed treatments were created using Liberty seed from UNL's USDA Agricultural Research Service, Cave in Rock seed from Stock Seed Farm, urea nitrogen fertilizer sourced from Anderson Farms, and smooth brome seed from Deer Creek Seed Inc.

### *Generating Treatments*

Each treatment was one combination of the aforementioned variables. Each contained one of two switchgrass cultivars (Cave in Rock or Liberty), one of two levels of urea nitrogen fertilizer (0g or 1.1g), and one of three smooth brome competition levels (no competition, 33% competition, and 67% competition). With every combination including 1 variable from each field (switchgrass, fertilizer, and competition level), 12 treatments were created, each of which was replicated 20 times for a total of 240 pots. Treatments were assembled using a sorting knife by counting switchgrass seeds to 30 pure live seeds (PLS) per treatment, with the smooth brome competition percentages (0%, 33%, and 67%) making up the remaining seedlot. This meant that for treatments containing no competition, those pots received either 33 Cave in Rock seeds per pot, or 40 Liberty seeds per pot. Similarly, treatments that contained 33% competition from smooth brome received 22 Cave in Rock seeds per pot or 27 Liberty seeds per pot, with the smooth brome seeds added to 30 seeds total per pot. Finally, treatments that contained 67% competition from smooth brome received 10 Cave in Rock seeds per pot or 16 Liberty seeds per pot (**Table 1**). PLS was only calculated for the switchgrass, not for the smooth brome. Treatments requiring fertilizer received 1.1g of urea nitrogen fertilizer dispersed with the seeds at planting.

### *Planting and Maintenance*

To avoid bias, each greenhouse location's randomly assigned treatment was recorded onto a master sheet, which was hidden from the researcher until data was evaluated. Pots were planted in a broadcast manner with thin layer of soil applied evenly over the scattered seeds. After the seeds were planted and covered with soil, pots were re-wetted with a seedling wand, taking care not to disturb the seeds. The internal temperature of the greenhouse was kept at 16°C using propane heaters.

### *Data Collection*

Germination data was taken 43 days after planting to ensure easy distinction between brome and switchgrass species. After germination data was taken, pots were watered for three seconds each using an irrigation wand once per week. After the three-month growth period, a SPAD chlorophyll meter manufactured by Konica Minolta was used to measure chlorophyll absorbance, then plants were harvested at soil-level, dried, and weighed for a dry biomass yield. Soil samples were collected from each treatment as well as from soil in which plants were not grown and was dried and packaged for an S1 soil analysis by Ward Laboratories. Additionally, dried harvested plants were sent to Ward Laboratories for nitrogen analysis.

### *Statistical Analysis*

After data was collected, a series of statistical tests were performed using Microsoft Excel 16.0. For germination/survival and chlorophyll results, a single-factor ANOVA was run, with a Tukey-Kramer test performed after that in order to determine statistical significance between individual treatment combinations (e.g. Control CiR (Cave in Rock cultivar) and Control CiR + Fert, Control CiR and Control Lib. (Liberty cultivar), etc.), so every combination was analyzed. The combinations that differed significantly ($p < 0.05$) were then used to draw conclusions. Biomass data was collected by compiling the harvested grass from 4 replicates per treatment into 1 sample, and then weighed on a scale with a linearity of +/- 0.01 which produced 12 individual data points (1 for each treatment). A similar process was used in obtaining percent nitrogen results. 5 replicates per treatment were harvested and compiled into 1 sample (12 data points total) which was measured using Ward Laboratory's Crude Protein Combustion Method which has a nitrogen detection limit of 0.005% and is generally reproducible within +/- 5.0%.

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Article

# Correlates of Sugar Consumption Among High School Students and Faculty

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## SUMMARY

**The majority of Americans are overweight or obese, putting them at increased risk for high blood pressure, stroke, heart disease, type 2 diabetes, cancer, and other adverse outcomes. Multiple factors contribute to this trend, including the increased availability and portion size of Highly Palatable Food (HPF), which is defined by the combination of sugar, fat, and/or salt. Food addiction theory describes a cycle of ingesting sugary food, followed by insulin and dopamine responses that lead to increased cravings for HPF. With repeated exposure to HPF, some individuals become chronic over-consumers with a strong drive for this type of reward-based eating. This study sought to characterize the relationships between Reward-based Eating Drive (RED), consumption of HPF, cravings for sugary beverages, and knowledge of sugar's effects on the brain and body in male and female high school students and faculty. Survey questions were uploaded to Surveymonkey and a link to the survey was sent to the listservs for all individuals with an email account at the high school. 176 anonymous responses were received. The results showed that reward-based eating drive was related to consumption and to cravings. For females, knowledge of sugar's effects was significantly and inversely associated with consumption of sugary food. This finding suggests that public health interventions to increase knowledge of sugar's adverse effects may be an avenue to decreasing consumption of HPF, ultimately decreasing the proportion of overweight and obese individuals, especially in young women.**

## INTRODUCTION

The number of overweight (Body Mass Index, BMI ≥ 25) and obese (BMI ≥ 30) individuals in the United States is approaching epidemic proportions (1). Since 1960, the rate of obesity has more than doubled in adults (from 14.3% to 38%) and more than tripled in children and adolescents (from 5% to 16.9%) (2, 3). A majority of adults (70.7%) are now overweight. Obesity increases risk for many adverse health conditions, including high blood pressure, stroke, heart disease, type 2 diabetes, cancer, and early death (4). A number of factors are believed to have played a role in this upwards trend. Experts point to reductions in physical activity and labor, increases in portion sizes, and increases in added sugar and salt in processed foods (5). Increased access to food and snacks may have led to habitual and mindless overeating (6). Rather than eating three meals a day, approximately 20-35% of Americans consume food hourly or every hour and a half (7).

"Calories in, calories out" (CICO) has been a leading theory of weight balance. According to CICO, the effects of food on weight gain can be entirely explained by calorie count. In other words, what matters is the quantity, and not quality, of the calories consumed (5). However, recent studies have shown that foods vary in their effects on hormones, metabolism, neural activity, calorie absorption, and psychological states (8, 9). Sugary beverages and processed foods are known as Highly Palatable Food (HPF), which is characterized by combinations of fat, salt, and simple carbohydrates. Overconsumption of HPF causes changes in brain pathways involved with rewards and reactions to stress (8). Consuming energy in liquid form may elicit incomplete energy compensation, displacement of more satiating foods, and passive (mindless) caloric overconsumption, thereby promoting weight gain and obesity (10). Consumption of sugar-sweetened beverages (SSB) is associated with weight gain in both children and adults (10).

HPF, especially the combination of sugar and fat, has been shown to create addictive behavior. In behavioral experiments, lab animals chose sweetened water over cocaine (11, 12). HPF also interacts with affective behavior. Humans placed on a high-fat diet for one month report increased anger and hostility when switched to a low-fat diet (13). Normal-weight individuals decrease their food intake when experiencing anxiety and depression, whereas overweight individuals often increase their food intake (9). This suggests that some people consume food to self-medicate negative emotions. The effects of HPF are dynamic and can lead to changes in cravings and learned behavior, resulting in a food addiction (**Figure 1**) (14).

The focus of this paper is hedonic or reward-based food addiction, characterized by a lack of control overeating, a preoccupation with food, and a lack of satiety. The mechanism underlying reward-based addiction to HPF involves both hormones and neurotransmitters (8). One model proposes that sugar intake triggers a glucose spike and leads to the release of dopamine, a neurotransmitter that conveys feelings of pleasure, into the nucleus accumbens of the brain (9). This glucose spike also leads to an insulin reaction, lowering blood-sugar levels by turning glucose into fat. Low levels of glucose are accompanied by fatigue, low energy, poor concentration, and hunger. Repeated exposure


**Figure 1.** Sugar Addiction Cycle. This is a simplified model of one component of sugar addiction, based on the reward aspects of sugar consumption and the pancreatic response of insulin release, which results in lower blood glucose and greater hunger. Adapted with permission from King, 2013.

to sugar or HPF downregulates dopamine receptors in the nucleus accumbens. With fewer dopamine receptors, more food is required to produce the same level of satisfaction (10). Over time, external cues such as the sight, smell, or description of HPF start to signal the opportunity for reward. Strong reward-based temptation can be difficult to resist and can cause cognitive distortions that justify consumption or overeating (13).

HPF, including SSB, are ubiquitous. In 2006, 86% of US high schools had vending machines that sold SSBs (15). An accounting of beverage sales at San Francisco University High School, with a student body of approximately 400, found that the average daily cafeteria sales were 20-30 bottles of water, 70 bottles of soda, and 53 bottles of juice (conducted by the author). This count is likely an underestimate, as it excludes caffeinated beverages with added sugar such as coffee and tea. Two possible inferences from the higher rate of SSB (compared to water) are that students may be unaware of the negative effects of SSB or that they have become dependent on SSB.

High school can be a place where lifelong habits develop; yet, little is known about the factors that relate to sugar consumption in high school students and whether gender plays a role. No prior research has addressed the role of gender, and thus this study hypothesizes on a purely exploratory basis that males and females will differ in their rates of reward-based eating, sugar craving, consumption of sugar, and knowledge of the adverse effects of sugar. Such differences may occur due to differences in hormones and social messaging, and due to the cultural emphasis on slimness in females. This study further hypothesizes that possessing knowledge of the adverse effects of sugar consumption will be negatively associated with actual consumption of sugar.

## RESULTS

This study used survey methodology to evaluate the relationships among Reward-based Eating Drive (RED), Cravings for Sugary Drinks (craving), Sugary Foods Eaten (consumption), and Knowledge of Sugar's Effects on Brain and Body. Correlation and multiple regression were used to describe relationships among the variables, and t-test was used to identify gender differences. The analyses identified gender differences in RED, but not in craving, consumption, or knowledge of sugar's effects. In females, knowledge was inversely associated with consumption.

The correlation coefficients between measures (Pearson's *r*) were modest, but are generally in the expected directions (**Table 1**). Reward-Based Eating Drive was positively correlated with both cravings and consumption of sugar, and these were positively correlated with each other. Knowledge of sugar's adverse effects was negatively correlated with consumption. Next, the joint association of Reward-Based Eating Drive and Craving for Sugary Beverages with sugar consumption (SFEQ) was evaluated using multiple regression. Although both RED and craving were associated with consumption individually (**Table 1**), when evaluated together, only craving was significantly associated with consumption in the regression model ($p<0.001$) (**Table 2**). This means that the association of RED with consumption is mostly accounted for by the association of craving with consumption.

### Effects of Gender

On an exploratory basis, analyses were conducted using sex as a variable. Males and females did not strongly differ on the SFEQ (both genders reported consuming sugary

| Pearson Correlation Coefficients/Probability | | | | |
|---|---|---|---|---|
| | RED-13 | SFEQ | CSBQ | KSEBBQ |
| RED-13 | 1 | $r$=0.163<br>$p$=0.032 | $r$=0.257<br>$p$=0.001 | $r$=-0.098<br>$p$=0.200 |
| SFEQ | | 1 | $r$=0.382<br>$p$<0.001 | $r$=-0.224<br>$p$=0.003 |
| CSBQ | | | 1 | $r$=-0.066<br>$p$=0.393 |
| KSEBBQ | | | | 1 |

**Table 1.** Correlations between sugary food consumption, craving, and reward based eating.

| | *b* | *SE* | *Beta* | *t value* | *p* |
|---|---|---|---|---|---|
| Reward-based Eating (RED-13) | 0.118 | 0.109 | 0.071 | 1.09 | *0.2375* |
| Craving (CSBQ) | 0.3701 | 0.072 | 0.366 | 5.12 | *<0.0001* |

**Table 2.** Predictors of sugary food consumption

foods, on average, between 1–3 times per month and once a week, $p>0.55$). Males and females did not differ on the CSBQ (mean ratings indicated "Slight or mild craving, $p>0.36$) or on the KSEBBQ (mean ratings indicated "a little knowledge", p>0.36). However, significant gender differences were found on the RED scale ($t$=-3.22, $p<0.002$). The means were 1.35 for males (SD=0.65) and 1.73 for females (SD=0.77) (**Figure 2**).

We then conducted analyses within sex, even though there were no interactions found between sex and the associations among other variables (data not shown). Craving was significantly and positively correlated with sugary food consumption for both females and males ($r>0.036$, $p<0.001$). Reward-based eating was positively associated with sugary food consumption for females ($r$=0.24, $p<0.02$), but not males ($p>0.1$). When craving and reward-based eating were put in the statistical models together and ran separately for females and males, only craving remained a significant predictor of sugary food consumption ($p<0.001$).

Males and females also differed in whether their knowledge of sugar's effects on the brain and body was related to their sugary food consumption. For males, knowledge was insignificantly correlated with consumption ($r$=-0.17, $p>0.1$). For females, knowledge of sugar's effects was significantly inversely correlated with consumption of sugary food ($r$=-0.3, $p<0.002$).

## DISCUSSION

Not surprisingly, craving was significantly correlated with consumption in the full sample and for both males and females. This finding could be interpreted to mean that craving has a causal effect on consumption, or that consumption exacerbates cravings. This dynamic would be consistent with the theoretical model of sugar addiction, which states that repeated consumption of sugar creates craving by altering dopamine and insulin.

Females demonstrated a higher reward-based eating drive than males. This may put females at higher risk for sugar addiction. However, craving was a stronger predictor of sugary food consumption than reward-based eating. This finding suggests that potential interventions should focus on strategies to manage cravings for sweets, including warning consumers that stopping consumption of HPF can result in short-term negative emotions and irritability. Females who reported greater knowledge of sugar's effects on the brain and body also reported consuming less sugary food. This is a clear indication it is important to educate people on sugar's effects on the brain and body in order to reduce sugar consumption.

In this study there were too few older subjects to meaningfully address age differences. Future research might be directed toward discovering if there are differences between younger and older populations. In addition, this study looked at data collected at only a single time point. Significant correlations that were found cannot be interpreted as causation. Thus, our interpretations must consider that causation may have occurred from either direction, or may have occurred from the effects of a third variable.

The results here are preliminary but promising in suggesting links between cravings, reward-based eating, sex, and sugar consumption. More research should also be conducted to determine if the gender differences reported here will be found in different populations and in longitudinal designs. If these findings are replicated, they could be used to support targeted educational efforts, such as heavier delivery of internet materials to females. Better dissemination of the known effects of sugar and other HPFs may help to reduce consumption and thus lead to a decrease in the size of overweight and obese populations.

## METHODS

### Study Participants

Our study enrolled 176 participants, including 144 UHS students (approximately 36% of the student body) and 32 faculty/staff (out of 61 faculty and unknown number of staff). 64 participants identified as male, 108 as female, and 4 as other.

### Data Collection

The study collected anonymous data and thus was not subject to human subjects protection. Surveys are a feasible means of gathering large amounts of data anonymously, and are a commonly used method of gathering data on the variables of interest via self-report. For the first three

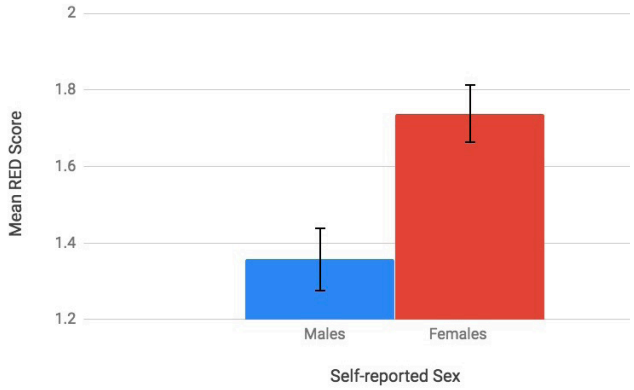


**Figure 2.** Differences in reward-based eating drive between males and females. On average, females had a higher RED score than males ($p<0.05$, $t$-test), implying that females experience greater reward-based eating drive than males.

instruments described below, we adapted a paper self-report form for use with an online administration. A questionnaire that included all study measures was entered into Surveymonkey. A link was sent to all UHS students and faculty/staff, inviting recipients to voluntarily respond to the questionnaire.

### The Reward Based Eating Drive (RED-13) Scale (16).

This 13-item, 5-point scale, self-report measures reward-related eating including food preoccupation, uncontrolled eating, and binge-eating. Response options include: strongly disagree, disagree, neither agree nor disagree, agree, and strongly agree. It is positively correlated with BMI, Type 2 diabetes, and cravings for sweet/savory foods (17).

### Sugary Foods Eaten Questionnaire (SFEQ, 18).

This questionnaire assesses how many sugary foods are being consumed. It is a 3-item self-report with a 7-point scale. Response options to how many times the participant consumes sugary foods include: never, once or twice, 1-3 times per month, once a week, 2-4 times a week, about once a day, and twice a day or more.

### Cravings for Sugary Beverages Questionnaire (CSBQ, 19)

This questionnaire measures how much the respondent is thinking about sugary drinks. It is a 4-item self-report with a ranging 5–7-point scale. Response options range from: almost never to almost all the time (measuring availability of SSB and frequency of craving) and from: no urge to strong urge (measuring intensity of craving).

### Knowledge of Sugar's Effects on Brain and Body Question (KSEBBQ).

This is a single item self-rating with a 4-point scale, developed by the authors, of amount of knowledge of food's effects on the brain and body. Response options include: not knowledgeable at all, a little knowledgeable, fairly knowledgeable, and very knowledgeable.

### Statistics

All data analyses were conducted by a statistical consultant (LJP), using SAS 9.4 (20). First-order relationships among survey measures were evaluated using Pearson correlations (PROC CORR). Concurrent prediction of sugary food consumption was evaluated using multiple regression (PROC REG). Sex differences were evaluated using t-tests. T-test was conducted using PROC GLM (generalized linear model) to allow for unequal variances and n. Analyses were required to use only cases without missing data, and only two cases had data missing from any analysis.

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Article

# Glyphosate Levels in American Food Products Meet Government Safety Levels Yet Exceed Concentrations Associated with Negative Biological Effects

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## SUMMARY

**Glyphosate, the active ingredient in the weed killer Roundup, is the most popular herbicide in the agricultural industry, with 6.1 billion kilograms sprayed worldwide in the past decade (1). Residue findings in breakfast foods and alcoholic beverages have prompted quantitative human and vertebrate studies yielding contradictory results (2). This study tested the hypothesis that glyphosate concentrations in frequently consumed food products exceed the levels legally allowed in Australia, the European Union, and the United States. The enzyme-linked immunosorbent assay (ELISA) method was utilized to measure glyphosate concentrations in cereal, gluten-free white and yellow corn tortillas, regular and whole grain pasta, white and whole wheat bread, and soy chocolate milk. The results showed that mean glyphosate residues were the highest in cereal (1903 parts per billion [ppb]) and significantly higher in whole grain pasta (45 ppb) than regular pasta (19 ppb), in gluten-free yellow corn tortilla (35 ppb) than gluten-free white corn tortilla (none detected), and in whole wheat bread (888 ppb) than white bread (56 ppb). Mean glyphosate concentrations in 25% of samples exceeded the threshold (500 ppb) at which negative effects were observed in previous studies. However, glyphosate concentrations in none of the samples exceeded government-imposed limits. These results raise concerns about whether government-imposed limits are sufficient to protect human health. Future research should quantify glyphosate residues in a wider variety of foods and conduct epidemiological studies to reassess the sufficiency of current safety limits.**

## INTRODUCTION

N-(phosphonomethyl)glycine, more commonly known as glyphosate, is a non-selective herbicide that prevents plant growth by disrupting the shikimic acid pathway, a metabolic pathway responsible for aromatic amino acid synthesis in plants (3). In the early 1970s, American scientist John E. Franz discovered glyphosate's application as a broad-spectrum herbicide while working under the agrochemical company Monsanto, which patented the product as "Roundup®" (4). Glyphosate remains the most frequently used chemical in the agricultural industry, raising concerns about the possible health effects of glyphosate exposure on humans (5).

A previous study which exposed glyphosate-based herbicides (GBH) to human liver cells found androgen receptor endocrine disruption in cells exposed to 500 ppb GBH, transcription inhibition of estrogen receptor genes in cells exposed to 2,000 ppb GBH, and DNA damage in cells exposed to 5,000 ppb GBH (6).

Another study administered 3.8 mg glyphosate/L (Monsanto's recommended maximum dosage of Roundup®) to a pond community and reported the loss of two species and a 70% reduction in total amphibian biodiversity after 13 days (7). It has also been found that African clawed frog (*Xenopus laevis*) embryos injected with 500 pg glyphosate experienced developmental changes in the head and neural crest regions, and chicken embryos injected with 4.4x10-3 µL GBH experienced microcephaly and optic vesicle reduction (8). Brown trout exposed to 10 ppb glyphosate exhibited up-regulation of the tumor-suppressor protein p53 (TP53), which stimulates cell cycle inhibitors that can inhibit cancer, and hypoxia up-regulated protein 1 (HYOU1), which promotes hypoxia-induced cellular perturbation. Brown trout exposed to 50 ppb Roundup® exhibited significant up-regulation of mitochondrion-associated apoptosis-inducing factor (AIFM2) (9-11). Another study on peppered catfish (*Corydoras paleatus*) blood and hepatic cells found increased DNA damage in the treatment group exposed to 3.2 ppb glyphosate for three days (13). Other studies on fish have reported decreased 17β-estradiol in female South American catfish (*Rhamdia quelen*) exposed to 3,600 ppb glyphosate and decreased aggressive behavior and distance traveled in adult zebrafish (*Danio rerio*) exposed to 500 ppb glyphosate (13,14). Increased testosterone levels, estradiol levels, and sperm production in male rats have been observed with 50 mg/kg (50,000 ppb) Roundup® exposure (15).

A population analysis reported that in an Argentinian farming town where crops were grown using GBH, birth defect and miscarriage rates were two and three times greater than the national average, respectively (16). Another study on herbicide and chlorophenol exposure demonstrated an association between glyphosate exposure and non-Hodgkin lymphoma development in adults. Subjects exposed to glyphosate were over two times more likely to have non-Hodgkin lymphoma (17).

Other research, however, has yielded inconsistent results. For example, the Agricultural Health Study (a collaboration between the National Cancer Institute, National Institute of Environmental Health Sciences, Environmental Protection Agency (EPA), and National Institute for Occupational

Safety and Health) on private and commercial licensed glyphosate applicators in Iowa and North Carolina found that the association between glyphosate exposure and cancer was not statistically significant (2). Another study on human umbilical, placental, and embryonic cells reported glyphosate toxicity beginning at a concentration of 1% (10,000,000 ppb) and cell death occurring within 24 hours of exposure to 105 times dilutions (100,00,000 ppb) of Roundup® formulations, levels significantly higher than those found to be detrimental in other studies (18).

Contradictory data have prompted governmental and non-governmental organizations to adopt differing stances on glyphosate safety. The European Food Safety Authority and United States EPA have both determined that glyphosate is unlikely to be carcinogenic (19,20). However, the State of California listed it as a "chemical known to the state of California to cause cancer" under Proposition 65, and the World Health Organization concluded that the herbicide was "probably carcinogenic" after the International Agency for Research on Cancer's Assessment (21,22).

At the time of writing, the EPA limits the amount of glyphosate that should be applied to raw agricultural commodities to 5,000 ppb for corn, 20,000 ppb for soybean seeds, and 30,000 ppb for Group 15 grains, including wheat, oats, barley, and rice (23). Residue limits vary by food because exposure changes with each product's growing environment and processing method. These tolerances are set by evaluating the dietary risk assessments, exposure, and toxicity of contaminants (24). The EPA limits glyphosate's Maximum Contaminant Level (MCL), the maximum concentration of a contaminant permitted in drinking water, to 700 ppb (25). MCLs are determined by the level at which a chemical may be present in drinking water without known risk to health. This level is determined by evaluating the concentration associated with non-carcinogenic effects while accounting for body weight, daily water consumption, and drinking water exposure (26).

The European Union's maximum residue level (MRL) for glyphosate is 1,000 ppb for corn, 20,000 ppb for soybeans, 10,000 ppb for wheat, and 20,000 ppb for oats (27). These MRLs are determined by factors such as application, anticipated residues, and toxicology of each herbicide (28).

Glyphosate's acceptable daily intake (ADI), the recommended amount that can be safely consumed on a daily basis for an extended period of time, in the European Union is 0.5 mg per kg of body weight per day (27). The Australian Government's Pesticides and Veterinary Medicines Authority defines the ADI for glyphosate as 0.3 mg per kg of body weight per day. This level was determined through identifying the concentration at which studies found no observed adverse effects and dividing it by a factor to account for variation in data (29). The limits enforced by the United States, European Union, and Australia are significantly higher than concentrations associated with adverse side effects in previously mentioned studies.

| Food | EPA Glyphosate Tolerance Level (ppb) | European Commission Glyphosate MRL (ppb) |
|---|---|---|
| Oats | 30,000 | 20,000 |
| Corn | 5,000 | 1,000 |
| Wheat | 30,000 | 10,000 |
| Soybean | 20,000 | 20,000 |

**Table 1.** Government enforced glyphosate limits. EPA tolerance levels (ppb) and European Commission MRLs (ppb) for glyphosate and their corresponding foods (23,27).

## RESULTS

Three trials of ELISA were run to measure glyphosate residues in Cheerios Gluten-Free Toasted Whole Grain Oats Cereal, Mission Gluten-Free Yellow Corn Tortilla, Mission Gluten-Free White Corn Tortilla, Kroger Vermicelli Enriched Macaroni Product, Kroger 100% Whole Grain Thin Spaghetti, Van de Kamp's Vegan White Enriched Bread, Van de Kamp's 100% Whole Wheat Bread, and Silk Chocolate Soymilk.

The mean glyphosate concentration was 1903 ± 6.93 ppb in the cereal sample, 888 ± 45.40 ppb in the whole wheat bread sample, 56 ± 4.36 ppb in the white bread sample, 45 ± 6.08 ppb in the whole grain pasta sample, 35 ± 6.08 ppb in the yellow corn tortilla sample, and 19 ± 5.29 ppb in the regular pasta sample. No glyphosate was detected in the gluten-free white corn tortilla or soy chocolate milk samples.

The recorded amount of glyphosate was significantly increased ($p < 0.017$) in whole grain pasta as compared with regular pasta, and in whole wheat bread as compared with white bread. Moreover, the recorded amount of glyphosate was significantly increased ($p < 0.017$) in gluten-free yellow corn tortilla as compared to gluten-free white corn tortilla. The residues of all food samples were significantly ($p < 6.25 \times 10^{-3}$) within permitted government levels deemed safe for human consumption, and European Commission MRLs (**Figure 1**).

## DISCUSSION

Our data did not support our hypothesis that the samples' glyphosate residues were greater than the ADI, MRLs, and tolerance levels outlined by the Australian Pesticides and Veterinary Medicines Authority, European Commission, and the EPA (**Table 1**). Lower-tailed, Bonferroni corrected t-tests indicated statistically significant evidence at the $\alpha = 6.25 \times 10^{-3}$ level that the glyphosate concentrations of the foods tested were lower than their corresponding EPA tolerance levels and European Commission MRLs (**Figure 1**). However, glyphosate levels measured in the cereal and whole wheat bread samples were significantly higher than the threshold at which negative effects were observed in previous studies (500 ppb) by as much as three times. The whole grain pasta and whole wheat bread samples may have had higher glyphosate concentrations than the regular pasta and white bread samples, respectively, because unlike refined grain products, whole grain products contain both bran and germ.

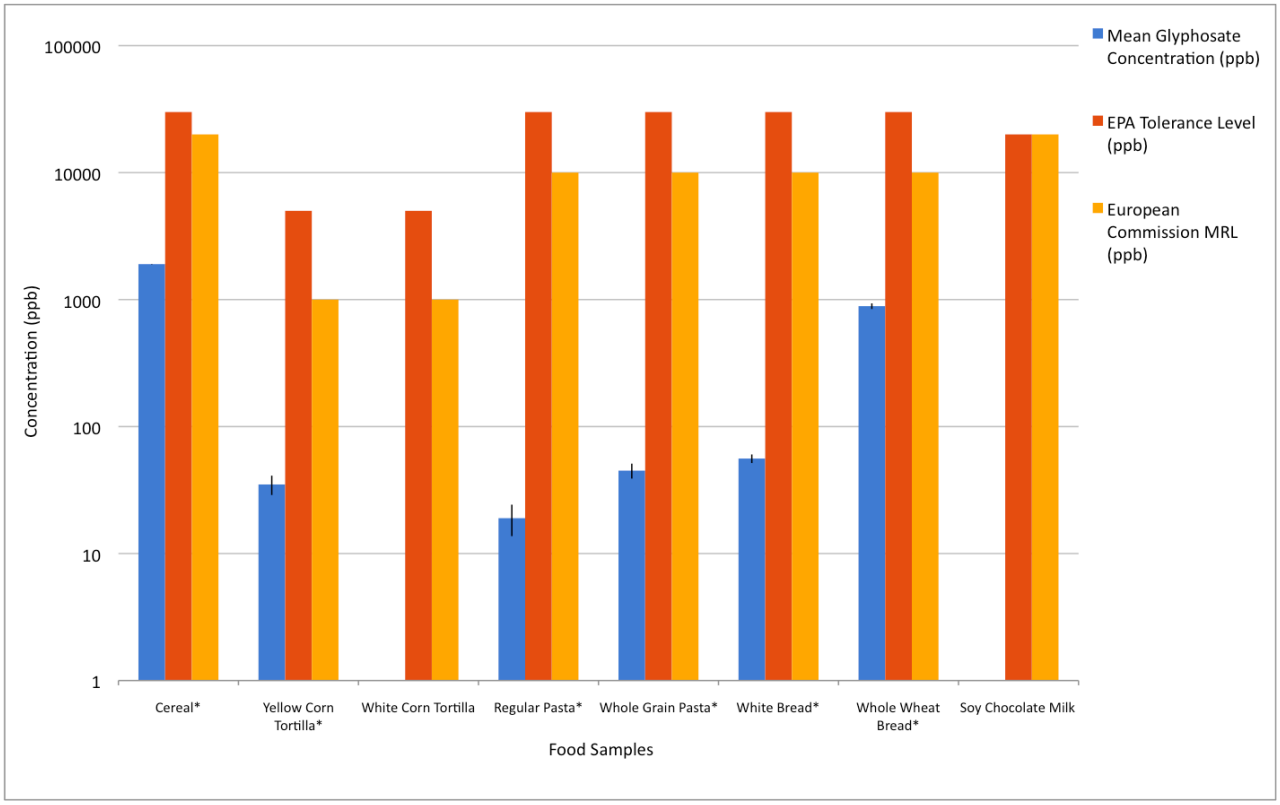


**Figure 1.** ELISA results reveal glyphosate levels in common household foods are within EPA regulations but may still exceed safe ingestion limits. Three independent trials of competitive ELISA were run to measure glyphosate concentrations in each food. Bars represent the average glyphosate concentrations (ppb) of the three independent trials, error bars represent the standard deviation, * indicates $p < 6.25 \times 10^{-3}$ by *t*-test. The Bonferroni correction, a conservative test that protects from Type I error, was applied to *t*-tests by dividing the initial critical *p*-value ($\alpha = 0.05$) by the number of foods test (n = 8)

The bran, the protective outer layer, and germ are removed from the kernel during processing.

In order to exceed daily regulatory guidelines placed by government agencies, a person would need to consume an overwhelming amount of these foods. To surpass the Australian ADI of 0.3 mg per kg of body weight per day, the average American adult male would have to consume approximately 110 servings of cereal, 2,995 servings of yellow corn tortilla, 5,518 servings of regular pasta, 2,330 servings of whole grain pasta, 3,744 servings of white bread, or 116 servings of whole wheat bread a day. If the average American 2-year-old, 10-year-old, adult male, or adult female fulfilled their USDA grain serving recommendation by only consuming cereal, the food with the greatest glyphosate concentration, their daily glyphosate consumption would still not exceed the Australian or European ADI (30-32).

This research studied a small number of food products and their glyphosate residues. More research is needed to test a wider variety of food products for glyphosate. These experiments were performed under controlled laboratory settings, and three trials were conducted to verify results.

It is interesting to note that while gluten-free and non-GMO labels often prompt consumers to assume certain products have increased health benefits across the board, this is not always the case. For instance, the cereal tested in this experiment was labeled as non-GMO, but it contained the greatest glyphosate concentration among the eight foods tested with an average glyphosate concentration of 1903 ppb (33). United States Department of Agriculture Organic Certification labels are not adequate for consumer awareness concerning pesticide contamination, because glyphosate residues may come from adjacent crops grown with Roundup® or other GBH. If food products containing glyphosate concentrations similar to these samples are consumed on a regular basis, individuals may unknowingly ingest unsafe levels of glyphosate due to lack of awareness and insufficient governmental regulation.

Despite the fact that glyphosate residues in all foods tested were lower than government limits, two were still significantly higher than concentrations associated with negative effects in humans and fish. The United States Food and Drug Administration, along with other governmental organizations, should consider lowering glyphosate tolerance levels to reflect this threshold.

## METHODS

### Sample Preparation

Cereal, gluten-free yellow corn tortilla, gluten-free white corn tortilla, regular pasta, whole grain pasta, white bread, and wheat bread samples: According to the protocol included

in the Glyphosate Microtiter Plate ELISA Kit (PN500086) purchased from Abraxis Inc., Warminster PA, USA, a 0.5 g aliquot of sample was transferred to a beaker containing 10.0 mL of boiling deionized water. Samples were vortexed, put on a shaker, and allowed to rest. A 2.0 mL aliquot of supernatant was centrifuged. An 800 µL aliquot of glyphosate sample diluent from the ELISA Kit and a 200 µL aliquot of the supernatant were added to a beaker and vortexed.

Soy chocolate milk sample: According to Abraxis procedure, a 0.5 g aliquot of sample was added to 0.5 mL of 1.0N hydrochloric acid and vortexed in a microcentrifuge tube. 40.0 µL of this solution was vortexed in a glass vial containing 3.96 mL of glyphosate diluent.

### Sample Derivatization

According to Abraxis procedure, a 1.0 mL aliquot of glyphosate assay buffer was dispensed into test tubes containing 250 µL aliquots of the control, standards 0-5 (containing 0, 0.075, 0.20, 0.5, 1.0, 4.0 ppb glyphosate), and each sample, and vortexed. The derivatization reagent was diluted with a 3.5 mL aliquot of derivatization diluent and 100 µL of this solution was dispensed into each test tube and vortexed. Each test tube was incubated for 10 minutes before ELISA analysis.

### Plate Procedure and ELISA Analysis

According to Abraxis procedure, the 100 mL of 5X wash buffer was diluted with 400 mL of deionized water to make a 1X solution. 50.0 µL of the derivatized standard solutions, control, and samples were transferred into the wells containing goat anti-rabbit antibody. New pipette tips were used for every sample to avoid possible contamination. Each pipette tip was conditioned by aspirating and discharging the sample into and from the tip before drawing the correct volume. A 50.0 µL aliquot of the anti-glyphosate antibody solution was added into the wells. The wells were covered with parafilm, mixed, and incubated for 30 minutes at room temperature. A 50.0 µL aliquot of enzyme conjugate was added to the wells that were mixed and incubated for 60 minutes at room temperature. Glyphosate residues in samples competed with enzyme-labeled glyphosate, glyphosate enzyme conjugate horseradish peroxidase, for antigen-binding sites in the microtiter wells. The covering of the wells was removed, and contents washed three times using the 1X wash solution. A 150 µL aliquot of "color solution", composed of hydrogen peroxide and 3,3'5,5'-tetramethylbenzidine, was added to each well and allowed the enzyme-labeled glyphosate in antigen-binding sites to catalyze the production of color, which was inversely proportional to the samples' glyphosate residue concentrations. The wells were covered, and the strips were incubated for 30 minutes at room temperature without light exposure. A 100 µL aliquot of "stop solution", or diluted acid, was added to the wells to terminate the chemical reaction.

### Evaluation

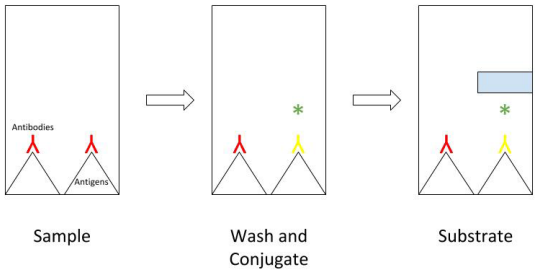


**Figure 2.** Competitive ELISA process. Competitive ELISA was utilized to determine the foods' glyphosate concentrations. Sample antibodies (red) were added to microtiter plate wells coated with antigens and competed with enzyme-labeled antibodies (yellow) for antigen-binding sites; the introduction of enzyme substrate (green) allowed the sample remaining after wash to catalyze the production of color (blue), which was inversely proportional to the concentration of sample.

Spectrophotometric analysis was used to quantify the color with a microplate ELISA reader measuring absorbance at 450 nm. A commercially available ELISA data solver (Abraxis Inc.) determined the foods' glyphosate concentrations in ppb using the standard curve run with the ELISA. The glyphosate concentration of each food sample was determined in ppb using the standard curve run with the ELISA and was inversely proportional to the resulting color (**Figure 2**). Samples with lower absorbances than a standard had greater glyphosate concentrations than the standard. Sample handling and preparation, such as the addition of boiling water, was taken into account when calculating glyphosate concentrations in ppb.

### Statistical Analysis

Lower-tailed *t*-tests were conducted to verify that the foods' glyphosate concentrations were significantly lower than their corresponding government tolerance levels. Two p-values were calculated for each food, one with the null (Ho) and alternative hypotheses (Ha) based on the corresponding EPA tolerance level, and one with Ho and Ha based on the corresponding European Commission MRL.

The critical *p*-values were calculated using the Bonferroni correction, which divided the initial critical p-value ($\alpha = 0.05$) by the number of foods tested ($n = 8$) for comparisons to corresponding government tolerance levels, and by the number of ELISA trials ($n = 3$) for comparisons to other foods. The Bonferroni correction is a conservative test that was applied to protect from Type I error by constricting the critical *p*-value.

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Article

# QuitPuff: A Simple Method using Lipid Peroxidative Changes in Saliva to Assess the Risk of Oral Pre-cancerous Lesions and Oral Squamous Cell Carcinoma in Chronic Smokers

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## SUMMARY

**Smoking carries a risk of oral cancer. Smoking generates free radicals, which are responsible for the high levels of cellular lipid peroxidation along the oral mucosa. Free-radical-induced lipid peroxidation has been implicated in the pathogenesis of oral cancer. Malondialdehyde (MDA) is the end-product of lipid peroxidation and can serve as a marker of the degree of lipid peroxidation. This study aimed to determine the salivary MDA levels in smokers and to use salivary MDA levels to assess the risk of developing oral pre-cancer and cancer. We hypothesized that heavier smokers would exhibit greater levels of salivary lipid peroxidation which in turn would correlate with a greater risk of oral pre-cancer and cancer. To test our hypothesis, we devised QuitPuff, a simple, home-based test consisting of a diagnostic reagent that reacts with MDA and produces a color change in a sample of saliva. We measured the MDA level in each sample by matching the color change with a colorimetric Lipid Peroxidation Index (LPI) chart. This method was tested on 125 people and the results were validated by UV Spectroscopy. The test detected the degree of salivary lipid peroxidation with 96% accuracy. We found that heavier smokers exhibited greater levels of salivary MDA. QuitPuff, a simple and inexpensive home-based test, can serve as an early, safe, non-invasive test for smokers to measure their degree of salivary lipid peroxidation and thereby assess their risk of developing oral pre-cancer and cancer.**

## INTRODUCTION

### The high mortality rate associated with oral cancer is attributed mainly to late diagnosis

Oral cancer, also known as mouth cancer, is any cancerous tissue growth located in the oral cavity (1). It most commonly involves the tongue, floor of the mouth, cheek lining, gums, lips, or roof of the mouth. More than 90% of all oral cancers are squamous cell carcinoma (2).

By country, the incidence of oral cancer is the highest in India, which accounts for almost one-third of cases found in the world (1). Over five people in India die every hour because of oral cancer (3). Public health centers and private hospitals have recognized oral cancer as a grave problem and efforts towards early detection and prevention can help reduce this burden.

The high prevalence of oral cancer in India is mainly due to the influence of tobacco and betel quid chewing (4). Greater than 90% of patients with oral cancer report using tobacco products (5). The incidence of oral cancer in patients who smoke and chew tobacco is 8.4 times greater than that of patients who do not (6).

Globally, the 5-year mortality rate of oral cancer is approximately 50% and has not improved despite advances in diagnostic techniques and improvements in treatment modalities (4). The high mortality rate in oral cancer is attributed to late diagnosis, which is either due to lack of knowledge or access to medical care (1,2,7). Most patients seek help only in later stages when symptoms like pain, ulceration, or a neck mass appear (1).

Detection of an oral cancer at stage I carries a prognosis of 80% survival, while the same lesion at stage III carries a prognosis of 20% survival (8). This difference could affect not only the quality of life for the patients, but also the cost of the medical treatment. Thus, there is a need for improvement in early risk detection of oral carcinomas, because in the initial and pre-cancerous stages, treatment is more effective and morbidity is minimal.

### Link between salivary malondialdehyde and oral pre-cancer and cancer

Free-radical-induced lipid peroxidation has been implicated in the pathogenesis of oral cancer (9-11, 14, 15). Smoking generates free radicals and reactive oxygen species (ROS). ROS-induced cell damage causes lipid peroxidation. It most commonly affects polyunsaturated fatty acids, which causes alteration in the structure and function of cell membranes and also initiates and promotes the multistep process of carcinogenesis (10).

Malondialdehyde (MDA) is the end-product of lipid peroxidation and can be used as a marker for assessing the extent of lipid peroxidation (9-11, 14, 15). MDA is mutagenic and genotoxic, as it readily reacts with deoxynucleosides to produce adducts that cause DNA damage (11). An increase in MDA concentrations in saliva have been widely reported in various oral pre-cancers and cancers in the early stages (9-11, 14, 15). Additionally, previous studies have shown that salivary MDA could serve as a potential diagnostic marker

| Group Name | N | Mean LPI by Colorimetric method | Mean LPI by UV Spectroscopy method |
|---|---|---|---|
| Non-smokers | 25 | 0.24 | 0.2 |
| Smokers (<10 cig/day) | 25 | 3.56 | 3.68 |
| Smokers (10-20 cig/day) | 25 | 3.64 | 3.72 |
| Smokers (>20 cig/day) | 25 | 4.44 | 4.48 |
| Smokers with oral pre-cancer & cancer | 25 | 4.48 | 4.52 |

**Table 1.** Comparison of mean LPIs (Lipid Peroxidation Index) between the Colorimetric and UV Spectroscopy methods with standard deviation.

and that by measuring the salivary MDA level we can measure the extent of lipid peroxidation and thereby assess the risk of a smoker towards developing oral pre-cancer and cancer (9, 10, 12-15).

With this background, we were motivated to investigate the relationship between smoking, lipid peroxidation and oral cancer and hence we carried out our study. We hypothesized that the heavier the smoker, the greater the level of salivary lipid peroxidation and the higher the risk of developing oral pre-cancer and cancer. To test our hypothesis we devised a simple, quick, colorimetric, home-based test named QuitPuff to determine the salivary malondialdehyde levels in smokers.

## RESULTS

The mean lipid peroxidation index (LPI) of non-smokers was 0.24. In the smokers group, the mean LPI of smokers who smoked less than 10 cigarettes a day was 3.56, that of smokers who smoked 10 to 20 cigarettes a day was 3.64 and that of smokers who smoked more than 20 cigarettes a day was 4.44. The mean LPI of smokers with oral pre-cancer and oral squamous cell carcinoma was 4.48 (**Table 1**).

The mean LPI was consistently and significantly elevated ($p$<0.001) in smokers with oral pre-cancer and cancer and smokers who smoked more than 20 cigarettes a day, 10-20 cigarettes a day and less than 10 cigarettes a day as compared to non-smokers.

The mean lipid peroxidation index (LPI) obtained by QuitPuff colorimetric method was compared with the mean LPI obtained by the validation method of UV Spectroscopy. The mean LPIs from both methods were in agreement (**Table 1**). Variations in LPI were observed in 20 of 125 samples, but only 4 out of 125 (3.2%) resulted in misclassification error when compared to the UV Spectroscopy results. Thus, the diagnostic test was able to detect the degree of lipid peroxidation in the saliva of smokers, patients with pre-cancerous mouth lesions, and Oral Squamous Cell Carcinoma (OSCC) patients with 97% accuracy.

Among smokers, 24 of 25 smokers with oral pre-cancer and cancer were found to have high degree of lipid peroxidation, an increase compared to that of otherwise healthy smokers (22/25 subjects who smoked more than 20 cigarettes a day, 18/25 who smoked 10-20 cigarettes a day, and 16/25 who smoked less than 10 cigarettes a day). In contrast in the non-smokers group, 20 of 25 subjects (80%) had no detectable lipid peroxidation, and none showed a high degree of lipid peroxidation (**Table 2**).

The color change in each sample was matched with the colorimetric lipid peroxidation index (LPI) chart (**Figure 1**). It was found, the higher the number of cigarettes smoked per day, greater the levels of salivary MDA and deeper the color produced. In the absence of salivary MDA, there was no color change found. The color change results correlated with the readings performed by UV Visible Spectroscopy, performed to validate the results. Results of the spearman correlation indicated there was a significant positive association between the color changes and the readings of the UV Spectroscopy (r=0.86, $p$<0.001)

## DISCUSSION

Cancer is caused by the accumulation of multiple lesions occurring in a single cell. It can be described by three stages: initiation, promotion, and progression. Previous reports indicate that ROS not only initiate, but also promote multistep carcinogenesis (10). ROS-induced lipid peroxidation is implicated in the pathogenesis of oral cancer (9-11, 14, 15). Malondialdehyde (MDA) is the end-product and the most widely studied product of lipid peroxidation (9-11, 14, 15). By measuring the level of salivary MDA we can determine the degree of salivary lipid peroxidation and thereby assess the risk of a smoker developing oral pre-cancer and oral cancer.

In our study, smokers with oral pre-cancer and oral squamous cell carcinoma were found to have a higher degree of salivary lipid peroxidation as compared to non-smokers. Within the smoker's category, the heavier the smoker, the greater the degree of salivary lipid peroxidation. This is in line with other studies (9-11, 14, 15) on oral pre-cancer and cancer, which have reported similar findings, validating the relationship between free radical activity, lipid peroxidation, and cancer.

We have also observed in our study that saliva can be used as a suitable diagnostic medium, as its collection is

| Group Name | N | Degree of Lipid Peroxidation | | | |
|---|---|---|---|---|---|
| | | Zero | Low | Moderate | High |
| Non-smokers | 25 | 20 | 4 | 1 | 0 |
| Smokers (<10 cigarettes/day) | 25 | 0 | 0 | 9 | 16 |
| Smokers (10-20 cigarettes/day) | 25 | 1 | 0 | 6 | 18 |
| Smokers (>20 cigarettes/day) | 25 | 0 | 0 | 3 | 22 |
| Smokers with oral pre-cancer & cancer | 25 | 0 | 0 | 1 | 24 |

**Table 2.** Degree of Lipid Peroxidation in Study Groups

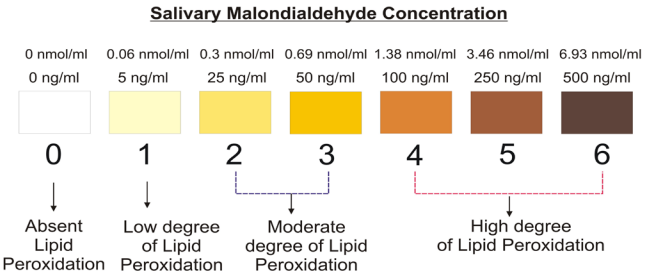



**Figure 1. The Colorimetric Lipid Peroxidation Index Chart:** The numbers from 0 to 6 denote the Lipid Peroxidation Index (LPI). These were interpreted as LPI 0 representing no risk, LPI 1 denoting low risk, an LPI between 2 and 3 corresponding with moderate risk, while an LPI of 4-6 respresenting high risk.

easy, non-invasive, not time-consuming, and inexpensive. Our findings are thus in accordance with studies that have shown that salivary MDA could serve as a potential diagnostic marker in potentially malignant disease and OSCC (9, 10, 12-15). Our diagnostic test was able to detect the salivary MDA level as an indicator of the degree of lipid peroxidation in the saliva of smokers, pre-cancerous mouth lesions, and OSCC patients with 97% accuracy, and therefore could serve as an early, safe, non-invasive test for smokers to assess their risk of developing oral pre-cancer and cancer.

The simplicity of the procedure does not rule out scope for systematic errors. Inappropriate saliva collection, contamination, or possible degradation of saliva after collection, as well as errors in measurement of saliva or reagent and inconsistent temperatures of the water bath, could lead to false positive results. One way to overcome these errors would be to repeat the test on different occasions. Furthermore, as the test is colorimetric, interpretation of results is largely subjective. In application, QuitPuff salivary diagnostic test does not aim to substitute conventional diagnostic tests, but is meant to act as a qualitative, self-diagnostic test for flagging early risk, and to be followed by laboratory tests for confirmation of diagnosis.

There is ample awareness that cigarette smoking can lead to cancer. For years, cigarette packets have been portraying images of cancer lesions, but this has not discouraged people from smoking. The number of smokers in India has risen from 79 million in 1998 to 108 million in 2015 (16). As the oral cavity is more accessible to complete examination, it could be used in early detection of oral precancerous and cancerous lesions. The disease however gets detected in the later stages, due to reasons such as lack of knowledge and inaccessibility of medical care (7). Careful annual examination of the oral cavity in persons above the age of 40 years can result in significant improvement in the rate of early detection of oral cancer with all the therapeutic advantages. However, a great proportion of those at risk of oral cancer do not attend annual healthcare check-ups (4).

Our aim was to devise a test that could detect the early risk of oral pre-cancer and cancer in smokers. We propose that if such a simple, do-it-yourself, home-based test could be provided with every cigarette pack, then more people might be willing to check their risk and make behavioral changes prior to the development of oral cancer. More people taking these tests would mean more people finding out their risks in the early stages. Early detection could enable better treatment outcome and improvement in the quality of life. It could reduce healthcare costs and the economic burden of treating oral cancer. The test kit requires no elaborate storage conditions and could be easily transported to remote locations and stored in small pan-beedi shops (tobacco selling shops & kiosks) in rural areas. At per sample cost of Rs. 38.15 (approximately 50 cents), it could offer an inexpensive and affordable option especially to the lower income populations, where such a test is often most needed. On account of its simplicity, non-invasive nature, low-cost and easy accessibility, QuitPuff may have great potential as a point-of-care test for oral pre-cancer and oral cancer. The test could be useful as a mass screening tool not only for routine clinics, but also for rural areas and remote locations with limited laboratory facilities or minimally trained health workers.

## METHODS

In this study, a simple home-based diagnostic method named QuitPuff was developed for the determination of lipid peroxidation in saliva by simple means i.e. through a visible color change which can be observed by naked eye.

2-Thiobarbituric Acid (TBA) 98% was purchased from Sigma Aldrich; Malondialdehyde Tetrabutylammonium salt (MDA) 96% pure was purchased from Sigma Aldrich. Extra pure distilled water was used. Trichloro Acetic Acid 1% and Ortho Phosphoric Acid 85% pure were purchased from Emplura.

The study was conducted on patients from the Bangalore Medical College and Research Institute, Victoria Hospital and Dr. Health Clinic. Informed consent was obtained from all groups. The Ethics Approval was granted by the Institutional Ethics Committee, Gurushree Hi-Tech Multi-Speciality Hospital. The sample analysis and experiments were conducted at Indian Institute of Science, Bengaluru.

### Preparation of MDA standards in saliva of healthy non-smokers

As our diagnostic medium was saliva, the MDA standards were also prepared in saliva. 10 healthy subjects were selected in the age group of 30 to 45 years, with no prior history of smoking or tobacco chewing. To rule out any pre-existing MDA, their saliva samples were sent to Indian Institute of Science, Bengaluru for MDA detection through Liquid Chromatography Mass Spectroscopy (LCMS). The LCMS report confirmed the absence of MDA in all 10 samples collected from the healthy people.

10 mL of unstimulated saliva was collected from each of the ten healthy subjects. MDA standards using saliva of each of the 10 healthy subjects were prepared in the concentrations of 500 ng/mL, 250 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL

and 5 ng/mL.

### Preparation of the diagnostic QuitPuff reagent

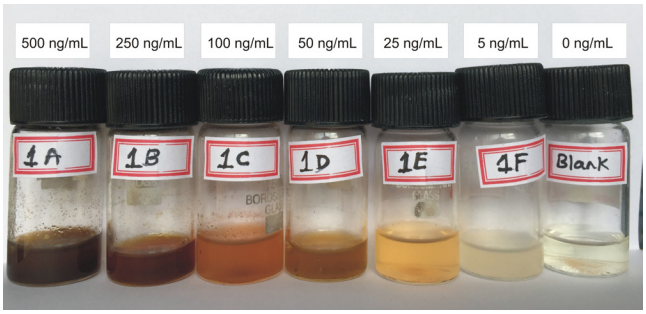
One molecule of MDA reacts with two molecules of TBA under high temperature and acidic conditions to produce a colored MDA-TBA adduct. This is the basic principle of TBARS assay which is the most widely employed assay used to determine lipid peroxidation. Although being accurate, these tests are not routinely done in laboratories. They are expensive, require technical skills, laboratories and instrumentation and sometimes need complex methods like High Performance Liquid Chromatography (HPLC) or Liquid Chromatography Mass Spectrometry (LCMS). Thus, they are fairly out of reach of ordinary people.

As the aim of our study was to develop a simple, home-based test, we explored the possibility of converting a complex laboratory based TBARS assay into a simple colorimetric test that could be self-conducted by the user and the results could be interpreted by simply matching the color change in a sample of saliva to a color chart.

To formulate the most sensitive TBA reagent that would detect MDA concentrations as low as 5 ng/mL, by producing a visible color change, MDA standards using saliva of 10 healthy subjects were prepared in the concentrations of 500 ng/mL, 250 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL and 5 ng/mL and TBA reaction was performed using various TBA formulations (**Table 3**).

As seen in **Table 3**, the formulation used in method 8 proved to be most sensitive, detecting MDA levels as low as 5 ng/mL by producing a visible color change. Thus, the QuitPuff TBA Reagent was prepared by dissolving 0.375 g of TBA in 85% Ortho-Phosphoric acid (1 ml) and 1% Trichloro Acetic Acid (1 ml).

**A**




**B**

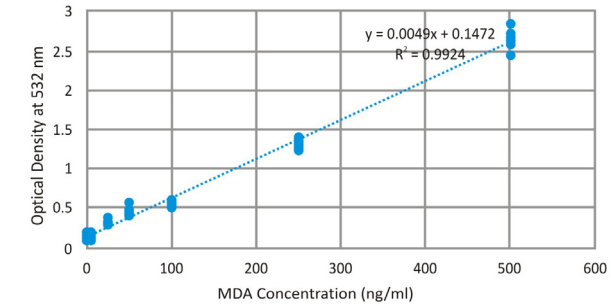



**Figure 2. TBA reaction on MDA saliva standards of Healthy Non-Smokers:** (**A**) The color change is observed in a gradient with A being the darkest and F being the lightest. No color change is seen in the control. (**B**) A standard curve was plotted based on optical density and MDA concentration.

### TBA-Reaction on known standards using QuitPuff

Using the QuitPuff diagnostic TBA reagent, TBA reaction was carried out on the known standards of MDA to generate a visibly colored MDA-TBA adduct. 2 mL of TBA reagent was added to 1 mL of each of the six MDA standards as well as a saliva control. The mixtures were then heated in a boiling water bath for 15 minutes and the color change was observed. The color change was observed in a gradient, with A being the darkest and F being the lightest. The color of control in the saliva control remained unchanged (**Figure 2A**).

The colored samples were analyzed through UV Visible Spectroscopy at Indian Institute of Science (IISc), Bengaluru and the absorbances were measured at 532 nm. Based on the readings a standard curve was prepared (**Figure 2B**). The optical density was plotted along the Y-axis and the MDA concentration in ng/mL was plotted along the X-axis. The following linear equation was derived: [y = 0.0049x + 0.1472]

### Preparation of colorimetric chart or the Lipid Peroxidation Index

Based on the color change and UV spectroscopy readings, a colorimetric chart was prepared (**Figure 1**). The colors are numbered from zero to six and denote the Lipid Peroxidation Index (LPI). The results can be interpreted by noting the LPI, which indicates the degree of lipid peroxidation, and thereby the risk status.

### Testing the method on 125 subjects

The QuitPuff saliva kit was tested on 125 subjects (aged 30-60 years) from Bangalore Medical College and Research Institute, Victoria Hospital and Dr Health clinic. These subjects were divided into 5 groups. Each group consisted of 25 subjects. Informed consent was obtained from all groups.

*Inclusion Criteria*: The etiology of oral cancer in India, is dominated mainly by tobacco use, alcohol consumption and smoking (3-7). As the aim of our study was to investigate the relationship between smoking and oral cancer, subjects with significant alcohol consumption and tobacco chewing habits were excluded.

*Non-smokers (n=25):* No history of alcohol consumption or tobacco chewing habit, absence of long-term systemic diseases, and no oral lesions.

*Smokers who smoked less than 10 cigarettes per day for a minimum of 5 years (n=25):* no significant alcohol consumption or tobacco chewing habit, absence of long-term systemic diseases, and no oral lesions.

*Smokers who smoked 10-20 cigarettes per day since for a minimum of 5 years (n=25):* no significant alcohol consumption or tobacco chewing habit, absence of long-term systemic diseases, and no oral lesions.

| Method No | TBA Formulation | TBA Reaction | Detection limit of color change |
|---|---|---|---|
| Method 1 | Formulation adapted from Ohkawa et al. (17), 0.2mL of 8.1% sodium dodecyl sulphate (SDS), 1.5mL 20% acetic acid at pH3.5, 1.5mL of 0.8% of Thiobarbituric Acid (TBA). | 0.2 mL TBA reagent was added to 0.2 ml of each of the MDA standards as well as a saliva control and boiled in water bath for 1 hour at 95 degree Celsius. | 100 ng/mL |
| Method 2 | Formulation adapted from Buege J.A and S.D. Aust et al. (18), 0.5% Thiobarbituric Acid, 20% Trichloro Acetic acid, 2.5N Hydrochloric acid. | 0.5 mL TBA reagent was added to 0.5 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 20 minutes. | 100 ng/mL |
| Method 3 | Formulation adapted from Esterbauer H. et al. (19),1 mL 0.37% Ethylenediaminetetraacetic-acid (EDTA), 1 mL 2% Butylated hydroxytoluene (BHT), 2 mL 10% Trichloro Acetic Acid, 1 ml 0.67% Thiobarbituric Acid. | 5 ml TBA reagent was added to 1ml of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 10 minutes. | 100 ng/mL |
| Method 4 | Formulation adapted from Uchiyama M and Midori Mihara M. (21), 3 mL 1% Phosohoric acid, 1 mL 0.6% Thiobarbituric acid aqueous solution. | TBA reagent was added to 0.5 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 45 minutes. | 25 ng/mL |
| Method 5 | Formulation adapted from Asakawa T. and Matsushita S. (20), 2 mL of 20% TCA containing 20 umol Ferrous Sulphate, 1 mL 0.67% TBA. | TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 30 minutes. | 25 ng/mL |
| Method 6 | Formulation adapted from experimentation, 0.1875 g of TBA in 0.4562 mL of Acetic Acid, added distilled water to bring the volume to 50 mL. | 2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes. | 5 ng/mL, color change observed in control. |
| Method 7 | Formulation adapted from experimentation,1 mL 0.375 g of TBA in 1 mL 85% Ortho-Phosphoric Acid. | 2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes. | 25 ng/mL |
| Method 8 | Formulation adapted from experimentation, 0.375 g of TBA in 1 mL 85% Ortho-Phosphoric Acid, 1 mL 1% Trichloro Acetic Acid. | 2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes. | 5 ng/mL |
| Method 9 | Formulation adapted from experimentation, 0.375 g of TBA in 1 mL 1% Ascorbic Acid, 1 mL 1% Trichloro Acetic Acid. | 2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes. | 5 ng/mL, Color change observed in control. |
| Method 10 | Formulation adapted from experimentation, 0.3 g of TBA dissolved in 3 mL of Dimethyl Sulfoxide (DMSO). | 2 mL TBA reagent was added to 1 mL of each of the MDA standards as well as a saliva control and heated in a boiling water bath for 15 minutes. | 25 ng/mL |

**Table 3:** Detection limit of color change seen in MDA standards with various TBA formulations

*Smokers who smoked above 20 cigarettes per day since for a minimum of 5 years (n=25)*: no significant alcohol consumption or tobacco chewing habit, absence of any long-term systemic diseases and having no oral lesions.

*Smokers who smoked 10-20 cigarettes per day since for a minimum of 5 years with recently diagnosed pre-cancerous mouth lesions and oral cancer stage 1-2 (n=25):* yet to start on treatment (oral erosive lichen planus, n=12; oral leukoplakia [raised, indurated, white lesion in the oral mucosa more than 5mm in diameter with dysplastic changes in the epithelium], n=6; sub-mucous fibrosis, n=2; and oral squamous cell carcinoma [OSCC], n=5). Of the five subjects with OSCC, four were diagnosed with Stage 1 OSCC and one was diagnosed with Stage 2 OSCC.

Patients with chronic alcohol addiction, tobacco chewing habits and patients with advanced stages of oral squamous cell carcinoma and/or already on treatment or operated upon were excluded from the study.

The tests were performed on fresh samples of saliva. Subjects were asked not to eat, drink, smoke or chew tobacco an hour before the collection. Subjects rinsed their mouths with 10 mL of water and thereafter saliva was collected in sterile bottles. The QuitPuff TBA reaction was performed as previously described. The colour change was matched with the colorimetric chart (**Figure 2**) and the Lipid Peroxidation Index (LPI) was noted.

### Validation by UV Visible Spectroscopy

For further validation, all 125 samples were sent to Indian Institute of Science for MDA determination through UV Spectroscopy (Supporting Information). The readings of UV Visible Spectroscopy were then plotted on the standard curve and the linear equation [y = 0.0049x + 0.1472] was used to determine the MDA concentration values. A spearman correlation analysis was performed to understand the correlation between the color change in the samples and the readings of of UV Visible Spectroscopy. On the basis of the MDA concentration, the LPI was again derived and noted. A detailed report of these 125 samples, including photos, UV Spectroscopy results, MDA concentrations, and Lipid Peroxidation Index classification is available in this article's Supporting Information.

## ACKNOWLEDGEMENTS

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Article

# Developing a portable, reusable, and inexpensive magnesium-air fuel cell

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## SUMMARY

**Global warming is becoming an increasingly bigger problem, and it is important that we work to find alternative sources of electricity. The goal of this project was to determine what metal is best for use in a portable and reusable metal-air fuel cell. The three metals tested for use in a metal-air fuel cell were aluminum, zinc, and magnesium. These metals were selected due to their high theoretical voltage, faradic capacity, and widespread usage as sacrificial anodes in the naval shipbuilding industry. We created a design for a portable and reusable fuel cell frame in Google Sketchup and then 3D-printed. For each of the three types of metal, we tested the fuel cell three times over a total span of six hours. Every hour, we recorded the voltage and current produced by the fuel cell. After the three trials, we averaged the data at every measurement point. Our data shows that magnesium produced the greatest voltage and current. On average, magnesium produced 197% more voltage and 740% more current than zinc. Additionally, magnesium produced a voltage 280% greater and a current 1593% greater than aluminum during the six hours of testing. This project indicates that magnesium produces the greatest voltage and current in a metal-air fuel cell, as well as the viability of magnesium-air fuel cells. Due to their long shelf life and self-sufficiency, they can be used by emergency response teams and in developing areas.**

## INTRODUCTION

A metal-air fuel cell generates power through a redox reaction. Like all fuel cells, a metal-air fuel cell possesses a positive electrode, the anode, a negative electrode, called the cathode, and an electrolytic substance separating the two electrodes (1). The redox reaction oxidizes the metal, and reduces oxygen and water vapor in the atmosphere. The freed electrons from the metal then generate an electrical current and can be used to power an appliance. The metal is the anode, while the surrounding air acts as the cathode (5). In this project, carbon electrode fabric is used to enable the diffusion of oxygen and water vapor into the fuel cell (6). The reduced hydroxides and metal then react to form a metal oxide byproduct. The voltage produced by a metal-air fuel cell is primarily dependent on its reactivity. More reactive metals tend to produce greater voltages.

The metals most commonly used in metal-air batteries are aluminum and zinc. These metals are popular because they are relatively reactive yet remain stable when not in use (5). Magnesium is another metal that displays these qualities. Further research shows that magnesium possesses a variety of desirable traits that make it suitable for use in a metal-air fuel cell, such as a high theoretical voltage and faradic capacity (**Table 1**). Magnesium is the least dense of the three metals and also produces the greatest theoretical voltage. The faradic capacity of magnesium comes close to that of aluminum; however; aluminum's low voltage makes it less viable to use compared to magnesium. The primary barrier that prevents magnesium's use as an anode material in batteries is a difficulty in identifying a long-lasting electrolytic substance to use in conjunction with it. However, in a fuel cell, which is replenishable, magnesium becomes a viable option because both the electrolyte and metal can be replaced.

Additionally, the naval shipbuilding industry uses sacrificial anodes to protect metal parts that are submerged underwater. Sacrificial anodes function by being more reactive than the metal that they are protecting. When the part is submerged, the saltwater acts as an electrolyte, and the more reactive sacrificial anode is corroded away, protecting the part itself. The three most commonly used metals for sacrificial anodes are aluminum, zinc, and magnesium. This is due to their relatively high reactivity compared to the metal they are protecting (**Figure 1, Table 1**) (4). As a result, we expected these metals would produce the greatest voltage in a metal-air fuel cell.

The goal of this project, therefore, was to determine which type of metal produces the greatest voltage and current in a metal-air fuel cell. Based on the theoretical voltage and highest reactivity of magnesium out of the three metals, we hypothesized that magnesium would produce the greatest voltage and current in a metal-air fuel cell.

## RESULTS

Given that the goal of this project was to determine which metal is the most viable for use in a metal-air fuel cell, we first tested the voltage and current the fuel cell generated for each candidate metal. The metal-air fuel cell consisted of a fuel cell frame, a saltwater electrolyte, a carbon fabric cathode and a metal anode. All three metals were tested over a period of six hours, with three trials per metal. All three trials were conducted in the same environment with the same setup. We

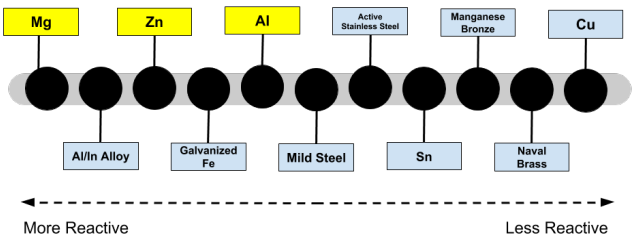


**Figure 1:** Reactivity series of various metals.

| | Mg | Al | Zn |
|---|---|---|---|
| Density (g/cm$^3$) | 1.74 | 2.70 | 7.14 |
| Faradic capacity (Ah/g) | 2.21 | 2.98 | 0.82 |
| Theoretical voltage (V) | 2.93 | 1.20 | 1.65 |

**Table 1:** Comparison between the density, faradic capacity, and theoretical voltage of magnesium, aluminum, and zinc (5).

visualized the recorded data with a line graph for each trial (**Figure 2A-C, 3A-C**). Then, we averaged the voltage and current output at each measurement for each metal, and we plotted each average on another line graph (**Figures 2D, 3D**).

The results of the experiment show that magnesium consistently produced a far greater voltage and current than both zinc and aluminum. In terms of voltage, magnesium produced an average of 4.04 volts after 6 hours, compared to 2.16 volts for zinc and 1.5 volts for aluminum (**Figure 2D**). This equates to a 197% difference in voltage between zinc and magnesium, and a 280% difference between aluminum and magnesium. Magnesium also produced far more current than zinc and aluminum, averaging 646.67 mA at the 6-hour mark, compared to 105.37 mA for zinc and 53.03 mA for aluminum (**Figure 3D**). Magnesium consistently outperformed both zinc and aluminum by a wide margin.

The average standard deviation of the voltage produced by magnesium was 0.05, which is lower than that of both zinc and aluminum (0.15 and 0.19, respectively) (**Table 2**). This result shows that magnesium is also able to produce a more consistent voltage. The average standard deviation of the current produced by the magnesium was 24.89, compared with 1.94 and 1.83 for zinc and aluminum, respectively (**Table 2**).

We also tested the magnesium-air fuel cell's ability to charge a smartphone using an Adafruit PowerBoost. We connected the fuel cell to the PowerBoost using alligator wires, then plugged in a standard USB charging cable into the PowerBoost and a phone. We found that we were successfully able to charge the smartphone.

## DISCUSSION

The results of this experiment show that magnesium is the best metal for use in a metal-air fuel cell. Averaging 4.04 V and 646.67 mA over the six hours of testing across three trials, not only can magnesium-air fuel cells produce electricity, they produce enough to be a viable energy source. The difference in the average current standard deviation of magnesium can be attributed to the fact that magnesium produced far more current than the other two test metals. If the current produced by the zinc and aluminum were to increase by using more metal, we predict that the standard deviation would also scale up to a value comparable to that of magnesium. In our testing, the magnesium-air fuel cell produced enough electricity to charge a smartphone as well. Additionally, metal-air fuel cells possess a very long shelf life and simply require saltwater

| | Mg | Al | Zn |
|---|---|---|---|
| Avg voltage at 6 hours (V) | 4.04 | 1.50 | 2.16 |
| Avg current at 6 hours (mA) | 646.67 | 53.03 | 105.37 |
| Avg voltage std deviation (V) | 0.05 | 0.19 | 0.15 |
| Avg current std deviation (mA) | 24.89 | 1.94 | 1.83 |

**Table 2:** Average voltage and current produced over six hours for all three metals. Values are reflective of three trials.

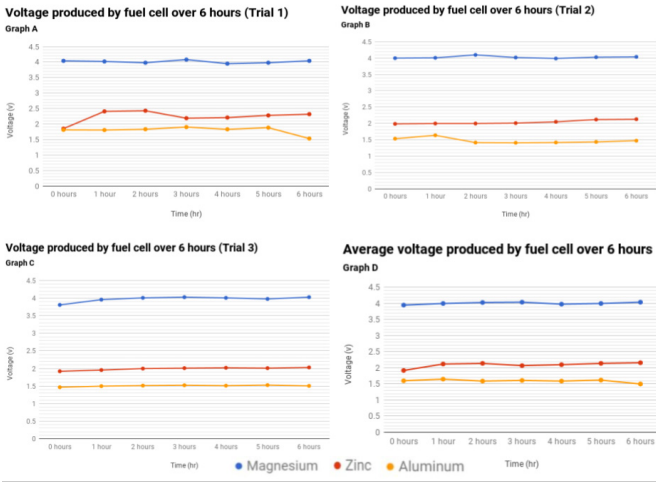


**Figure 2:** Voltage produced by the three metals over the first 6 hours of the first (A), second (B), and third (C) trials, as well as the average voltage produced by each metal at each measurement (D).

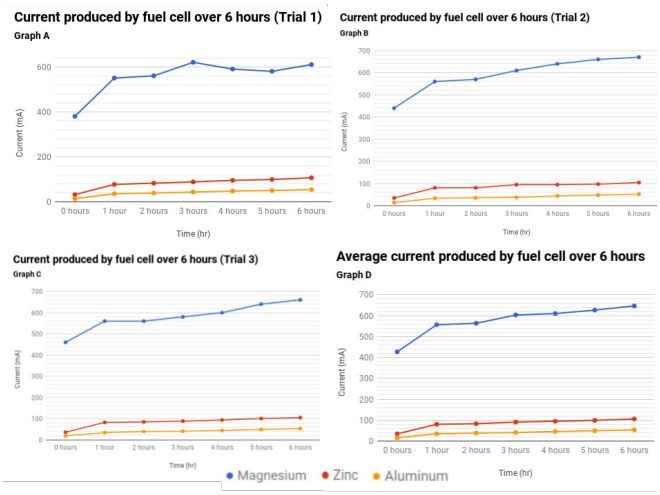


**Figure 3:** Current produced by the three metals over the first 6 hours of the first (A), second (B), and third (C) trials, as well as the average current produced by each metal at each measurement (D).

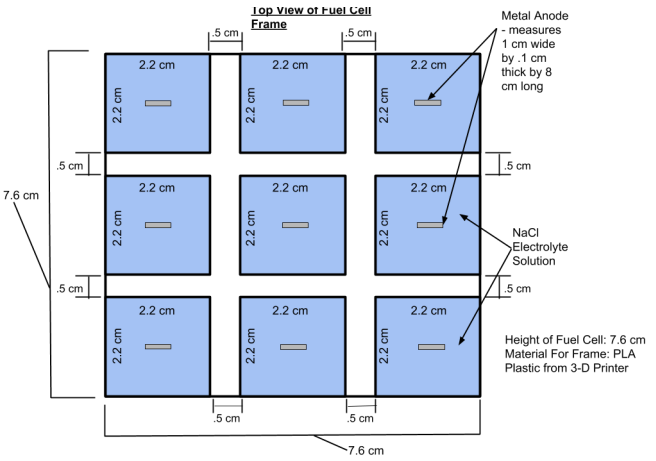


**Figure 4:** Top view of the design of the metal-air fuel cell frame used in the experiment.

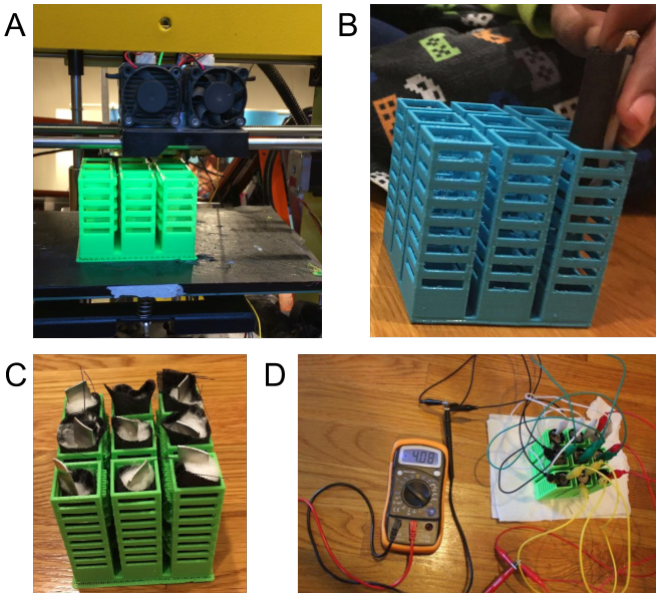


**Figure 5:** Assembly and testing of a reusable fuel cell. A: A fuel cell frame being 3D-printed. B: A completed set (magnesium anode wrapped with cotton and carbon fabric) being inserted into a subcell of a fuel cell frame. C: A fuel cell frame with all subcells filled but not yet wired. D: A running fuel cell whose voltage output is being measured. The fuel cell is fully wired, and the alligator clips at the end of each "row" of subcells are connected to the multimeter's leads.

| | Mg | Al | Zn |
|---|---|---|---|
| 9 anode metal strips | $3.15 | $1.16 | $3.41 |
| 9 carbon cathode pieces | $34.47 | $34.47 | $34.47 |
| 3D-printed fuel cell frame | $10.00 | $10.00 | $10.00 |
| **Total** | **$47.62** | **$45.63** | **$47.88** |

**Table 3:** Component cost of the magnesium, aluminum, and zinc fuel cells. The total cost is the sum of each individual part of the fuel cell – the anode, cathode, and frame. Cost of water and salt was marginal and is therefore not represented in the table.

to begin the reaction to produce electricity. We attribute the results of this experiment to magnesium's highest reactivity and theoretical voltage out of the three metals tested (**Figure 1, Table 1**).

A breakdown of the costs of the fuel cell used in this project shows that a complete fuel cell was constructed for under $50 (**Table 3**). We calculated the cost of each material in the fuel cell based on the amount of the material that was actually used in the fuel cell. However, since the materials were purchased in small amounts for the purpose of experimentation, costs can be reduced if they are purchased in greater quantity. If cost-cutting factors are taken into account, we estimate that these fuel cells can be constructed for less than $15, with anodes able to be replaced for less than $2 every time.

The applications of a portable, reusable, and inexpensive fuel cell such as the one tested in this experiment are far-reaching. For example, remote areas that are not connected to a power grid could power essential appliances using these fuel cells. Emergency responses teams who require quick access to electricity can also use this fuel cell to generate power. Magnesium-air and other metal-air fuel cells can even be implemented in suburban and urban environments as large units for powering individual houses or apartment complexes. For future research, this project could be further advanced from either an engineering or scientific standpoint. The fuel cell frame currently uses bulky alligator wires for the purpose of testing. In the future, we can design a more streamlined frame with a lid that encases all wiring, such that closing the lid over the fuel cell frame will automatically close the circuit. Additionally, companies such as MagPower are developing magnesium-air fuel cell technology with hydrogen inhibitors added to their fuel cells (2). In magnesium-air fuel cells, a variation of the normal reaction causes hydrogen evolution, which leads to the faster corrosion of the anode. By determining effective additives that inhibit hydrogen evolution, the longevity of the fuel cell can be increased.

## MATERIALS AND METHODS

### Design and construction of a reusable fuel cell frame

In order to be portable, the fuel cell was designed as a cube with side lengths of 7.6 centimeters. Within this cube were 9 subcells, each containing its own anode, cathode, and electrolyte (**Figure 4**). A mockup of the frame was created in Google Sketchup and then 3D-printed (**Figure 5A**).

In order to construct the fuel cells, we cut ELAT carbon electrode fabric (NuVant Systems) into 7.5 centimeter square swatches (nine per fuel cell frame, one per subcell). Then, we cut zinc, aluminum, and magnesium sheet metal into thirty 8 cm by 1 cm pieces. We wrapped one standard cotton ball (CVS brand) around each piece of metal, leaving about 1 cm of room at the top for wiring. We then tightly wrapped the carbon fabric swatch around the cotton and metal piece, forming the internals of one full subcell. We then inserted that set into one of the subcells in the fuel cell frame (Figure 5B). The same was done for all nine subcells (Figure 5C). We then

mixed iodized salt (Morton Salt, Inc.) and distilled, bottled water at room temperature until the salt crystals would no longer dissolve in order to create a fully saturated saltwater solution. Using a pipette, we pipetted 15 mL of the saltwater solution onto the cotton of each of the subcells, which then acted as an electrolyte (2, 3). Next, we used alligator clips to wire the fuel cell. We wired three subcells in series, forming three rows of three series-wired subcells. We then wired these rows in parallel to form the fully constructed fuel cell. At this point, the oxidation reaction began, enabling the fuel cell to produce electricity. We took an initial measurement of voltage and current using a multimeter (Figure 5D).

### Voltage and current testing

We tested the voltage and current output of the fuel cell using a multimeter for six hours once every hour, at which point the fuel cell was disassembled and set up once again for the next metal. After determining that magnesium was the best metal to use in a metal-air fuel cell, further experimentation was conducted with a PowerBoost (Adafruit) to test whether the fuel cell could produce enough electricity to charge a smartphone. The alligator clips from the fuel cell were wired to the PowerBoost, directly supplying power to the USB port on the PowerBoost unit. A smartphone was attached to the USB port to test whether the fuel cell supplied enough electricity to successfully charge it.

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Article

# Determining the Contribution of Osmotic Stress to the Antibacterial Properties of Honey

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## SUMMARY

**Researchers have repeatedly shown that honey possesses distinctive antimicrobial properties; however, there is uncertainty over which compounds in honey are responsible for these properties. In this research study, we sought to quantify the role of osmotic stress in honey and determine the efficacy of two types of honey: Manuka and raw pasture honey. Bacteria were sequentially cultured in sublethal concentrations of Manuka and raw pasture honey for five days. The role of osmotic stress as a contributor to the antibacterial properties of Manuka and raw pasture honey was quantified in the first culture and over five serial cultures. The growth levels of bacteria in honey were compared to growth levels in glucose, an osmotic control, to quantify the role of osmotic stress in the two types of honey. The results of this study indicate that in the first culture, the antibacterial impacts of Manuka and raw pasture honey were primarily attributable to osmotic stress. However, over five days of sequential transfers, both raw pasture honey and Manuka honey showed significant antibacterial properties beyond osmotic strength. It was established that the antibacterial properties of honey cannot be investigated based solely on the first culture. Serial transfers over several days should be employed to investigate the efficacy of honey as an antibacterial substance.**

## INTRODUCTION

Over the past century, antibiotics have saved countless lives; however, the recent rise of antibiotic resistance has established a critical need for the use of novel antibacterial agents. Moreover, few new antibiotics are being developed by pharmaceutical companies, which further increases this need (1). This quandary has instigated a re-examination of several ancient plant-based remedies, such as honey, to assess their potential to be used therapeutically (2).

Honey has been used as a traditional remedy for centuries (3), yet its use in contemporary medicine has been limited due to a lack of scientific support (4). Recently, however, researchers have been re-evaluating honey as a potential therapeutic treatment, and several studies have reported its antibacterial properties (1, 3, 5, 6). Honey has been shown to possess antibacterial effects against microorganisms, such as *Salmonella*, *Shigella*, *Escherichia coli*, and *Helicobacter pylori*, according to several laboratory and clinical investigations (3).

Honey has been reported to contain about 200 different substances. Sugar and water are the two main constituents, and fructo-oligosaccharides, minerals, enzymes, and various amino acids are some of the other components of honey (3). Honey is also hygroscopic, meaning it absorbs moisture from its environment, which partially contributes to its antimicrobial effects (2). In addition to the poor environment honey creates for bacteria, many phytochemical factors have been found in honey, which contribute to its antibacterial effects (3).

Certain types of honey are more effective than others. Commercially available honeys differ in their antibacterial activity compared to newly identified medical-grade honey, which may be used therapeutically in the future (7).

Manuka honey (*Leptospermum scoparium*), a monofloral honey from New Zealand, is distinctive for its potent and broad-spectrum antibacterial properties (8). This honey has been proven to be effective against pathogenic bacteria such as *S. aureus* and *H. pylori*, making it a possible effective treatment for wounds or stomach ulcers (2). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *S. aureus* that is resistant to most of the modern-day antibiotics, rendering it a pertinent threat to worldwide health, often leading to high treatment costs and several deaths over the past several decades (9). In conjunction with oxacillin, a penicillin antibiotic, Manuka honey has been shown to restore the susceptibility of MRSA to oxacillin (10).

While the antibacterial effects of several honeys are solely due to the production of hydrogen peroxide (8), Manuka honey has been shown to exhibit other antibacterial effects. Methylglyoxal (11) and leptosin (12) have already been identified as some of the compounds responsible for its unique antibacterial effect. Despite the lack of notable antibacterial compounds, raw pasture honey may still be beneficial as it is more accessible than medical-grade honeys in local communities.

Since sugar is the most abundant component of honey, it is expected that some of the antibacterial effects of honey are due to the high osmotic stress (3). However, it is unclear what the contribution of osmotic stress is to the antibacterial properties of Manuka or other commercial types of honey. The purpose of this study was to investigate the antibacterial effects of Manuka honey and raw pasture honey. By quantifying

the impact of osmotic stress on the antibacterial properties of Manuka and raw pasture honey, the other antibacterial effects of honey beyond osmotic stress can be discerned. A native strain of *S. aureus*, a gram-positive bacterium, was used. *S. aureus* has a thick cell membrane with a large amount of peptidoglycan, which is a mesh layer that preserves the shape of the bacteria and allows it to better endure intracellular pressure (13). We hypothesized that both Manuka and raw pasture honey would exhibit unique antibacterial properties beyond osmotic stress. Instead of just a single culture, this study employed multiple serial transfers to assess the efficacy of different concentrations of honey. Bacteria were serially transferred into sublethal concentrations of Manuka and raw pasture honey for a total of five cultures to evaluate the efficacy of a range of honey concentrations.

## RESULTS

### Determining Treatment Concentrations to Use in Serial Transfers

In a brief pilot study, bacteria were first grown in Manuka honey concentrations of 50, 60, 70, 80, 90, 100, and 110 mg/mL for 24 hours. The growth levels of these cultures are shown in **Figure 1**. Concentrations of Manuka honey of 90 mg/mL and greater were lethal in the first culture, and thus, they were not used in serial transfers. Sublethal concentrations of Manuka honey between 50 and 80 mg/mL were utilized in serial transfers.

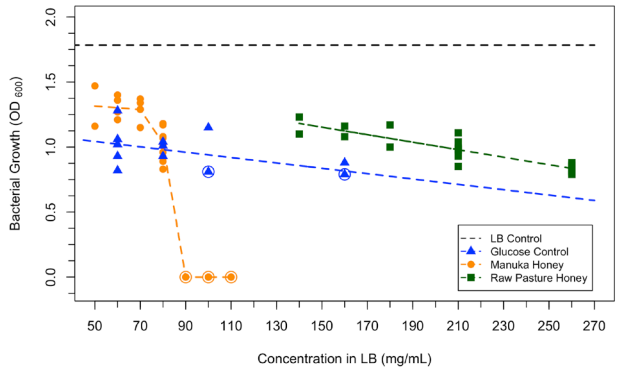


**Figure 1. Dose-response curves for Manuka honey and raw pasture honey.** Manuka honey concentrations from 50–80 mg/mL and raw pasture honey concentrations from 140–260 mg/mL were sublethal upon first exposure. Multiple Manuka honey culture losses are indicated by outer circles around points at an optical density of 0. The dotted black line represents the average growth level of bacteria grown in LB under no stress in the first culture. The dotted blue line represents growth levels in glucose concentrations in the first culture with circles around points representing multiple points with the same value.

Bacteria were also grown in raw pasture honey concentrations of 140, 160, 180, 210, and 260 mg/mL. All concentrations of raw pasture honey tested were sublethal upon initial exposure as shown in **Figure 1**, and therefore, all tested concentrations of raw pasture honey were utilized in serial transfers. Note that the range of effective concentrations used for raw pasture honey was drastically higher than the range of effective concentrations used for Manuka honey.

Effective honey concentrations used in this study and concentrations of glucose, ranging from 0–200 mg/mL, were measured with an osmometer. Glucose concentrations measured were 0, 60, 80, 100, 120, 150, and 200 mg/mL; **Figure 2** is a plot for glucose concentrations between 0 and 200 mg/mL with a regression line for osmolality on concentration included. As an osmotic control, concentrations of glucose were chosen to mimic the osmolality of the highest concentrations of each honey that permitted growth in the first culture. A 60 mg/mL glucose concentration was found to have a similar osmolality to Manuka honey at 80 mg/mL (765 mOsm/kg) and was chosen as a glucose concentration for the experiment. A 160 mg/mL glucose concentration had a similar osmolality to raw pasture honey at 260 mg/mL (1577 mOsm/kg); thus, it was chosen as another glucose concentration for the experiment.

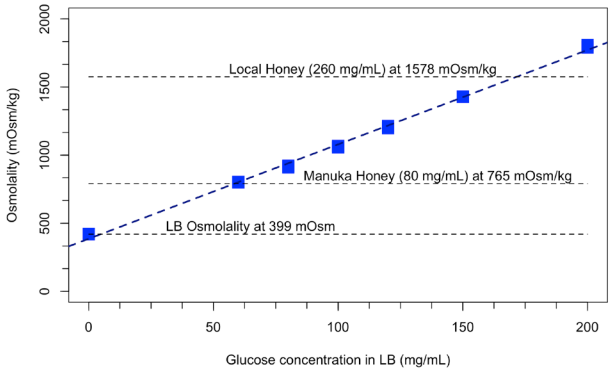


**Figure 2. Osmolalities of seven different concentrations of glucose in LB, shown as a linear relationship.** Marked on the graph are the osmolalities of Manuka honey (80 mg/mL), raw pasture honey (260 mg/mL), and LB (0 mg/mL glucose). The dotted line is the regression line of osmolality on concentration of glucose. Regression line equation is $\hat{O} = 385.98 + 6.93C$, where $\hat{O}$ is predicted osmolality on the line and $C$ is glucose concentration (in mg/mL).

Bacteria were serially transferred into 60 and 160 mg/mL glucose growth media for five days, similar to honey. Osmotic stress was clearly effective in lowering bacterial growth, as both glucose cultures grew to nearly half the growth level of bacteria grown in sterile LB. Both concentrations showed stable growth throughout the five culture days. Although the 160 mg/mL glucose concentration was slightly more effective in inhibiting bacterial growth than the 60 mg/mL glucose, on average, the difference between the growth levels of bacteria cultured in these two media was relatively small ($OD_{600}$ = 0.1887 ± 0.0335, $p < 0.001$).

### Serial Transfers are Essential in Evaluating the Efficacy of Honey

A majority of the concentrations which were sublethal in the first culture did not permit growth upon later transfers

into fresh honey-containing media. Concentrations that did not permit detectable growth upon further transfers were tested several times to ensure that this loss of growth was not coincidental. It was confirmed that Manuka honey (70 and 80 mg/mL) and raw pasture honey (180, 210, and 260 mg/mL), which were sublethal in the first culture, were in fact lethal when bacteria were retransferred into them (**Figure 3**).

Lethal concentrations of Manuka honey (70 and 80 mg/mL) showed no detectable growth after the first transfer, and were thus, discontinued from further serial transfers. Sublethal concentrations of Manuka honey (50 and 60 mg/mL) maintained stable growth for all five culture days. Raw pasture honey concentrations of 210 and 260 mg/mL did not permit growth in the second culture, while 140 and 160 mg/mL raw pasture honey concentrations showed lower levels of growth. After the second sequential transfer, 140 and 160 mg/mL raw pasture honey strains returned to the growth levels of day one and sustained stable growth for the remaining culture days.

## Osmotic Stress Fully Accounts for Antibacterial Effects of Sublethal Concentrations of Honey in the First Culture

In model equation [1], treatment-concentration factor $(TC)_i$ was leveled to have the LB control as the reference level to verify that each bacterial culture was placed under significant stress. Relative to the LB control, all treatment-concentrations showed significantly lower growth levels upon initial exposure (**Table 1**). This verified that Manuka honey, raw pasture honey, and glucose exhibited significant antibacterial effects.

**Table 1** clearly shows that both Manuka and raw pasture honey cultures grew at a significantly lower level than the LB control bacteria, and the following results quantified how much of this inhibitory effect was due to osmotic stress. The efficacy of Manuka and raw pasture honey was evaluated by comparing the growth of bacteria in honey versus glucose, an osmotic control.

The osmolalities of honey and glucose were then used as grounds for comparing osmotic strength. The osmolality of glucose and honey concentrations were measured three times with an osmometer, and their average osmolalities are given in **Figure 2** and **Table 2**, respectively.

The effect of osmotic stress on the growth levels of bacteria in the first culture was quantified using statistical model [2], verifying several things about sublethal honey concentrations in the first culture, which are summarized in **Table 3**.

The effect of osmolality on bacterial growth had a significant negative slope, verifying that osmotic stress effectively depressed the growth of bacteria. In addition, treatment effects, $T_i$, were accounted for in the model to detect other antibacterial properties beyond osmotic stress in each treatment. Both Manuka and raw pasture honey had nonsignificant positive estimates relative to glucose (Raw pasture honey $OD_{600}$ = 0.0304 ± 0.0719, $p$ = 0.6738; Manuka honey $OD_{600}$ = 0.0164 ± 0.0650, $p$ = 0.8025), indicating that when osmotic stress was accounted for, the antibacterial properties of Manuka and raw pasture honey in the first culture did not differ significantly from glucose. In the first culture of bacteria into sublethal concentrations of honey, neither Manuka nor raw pasture honey exhibited substantial antibacterial properties beyond osmotic stress; therefore, the inhibitory effects of honey in the first culture seemed to be primarily attributable to osmotic impact.

While the unique antibacterial properties of sublethal honey concentrations beyond osmotic impact are not detected in the first culture, further serial transfers will reveal

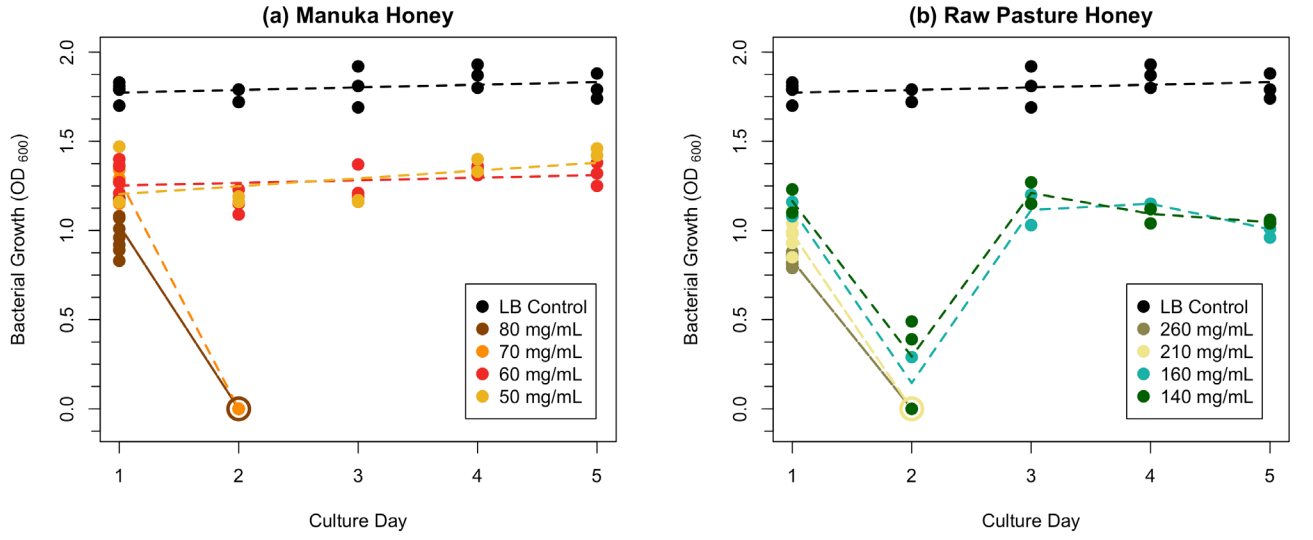


**Figure 3. (a) Growth levels of bacterial strains grown in concentrations of Manuka honey ranging from 50 to 80 mg/mL and LB control.** Bacteria grown in 70 and 80 mg/mL Manuka honey did not show visible growth upon second exposure. **(b) Growth levels of bacterial strains grown in concentrations of raw pasture honey ranging from 140 to 260 mg/mL and LB control.** Bacteria grown in 210 and 260 mg/mL raw pasture honey did not show visible growth upon second exposure. Multiple culture losses are indicated by outer circles around points at $OD_{600}$ = 0.

| Treatment | Concentration (mg/mL) | $OD_{600}$ Estimate | SE[b] | *p* | Significance[c] |
|---|---|---|---|---|---|
| Overall mean ($\hat{\mu}$)[a] | --- | 1.8233 | 0.0417 | < 0.001 | *** |
| Day ($\hat{\alpha}$)[a] | --- | -0.0393 | 0.0139 | 0.0071 | ** |
| Glucose | 160 | -0.9247 | 0.0657 | < 0.001 | *** |
| | 60 | -0.6680 | 0.0657 | < 0.001 | *** |
| Raw pasture honey | 140 | -0.3045 | 0.1334 | 0.0274 | * |
| | 160 | -0.3888 | 0.1221 | 0.0027 | ** |
| | 180 | -0.3845 | 0.1334 | 0.0061 | ** |
| | 210 | -0.5742 | 0.0944 | < 0.001 | *** |
| | 260 | -0.8930 | 0.0525 | < 0.001 | *** |
| Manuka honey | 50 | -0.2331 | 0.1113 | 0.0420 | * |
| | 60 | -0.2461 | 0.1003 | 0.0182 | * |
| | 70 | -0.3388 | 0.0787 | < 0.001 | *** |
| | 80 | -0.7157 | 0.0502 | < 0.001 | *** |

a. Note: $\hat{\mu}$ and $\hat{\alpha}$ are estimates for overall mean and regression coefficient of OD on day.
b. Note: SE are standard errors for the OD estimates.
c. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

**Table 1.** Day-adjusted estimates of first-day cultures studied relative to an LB control strain grown with no antibacterial additives. All strains studied were put under stress as shown by their significantly lower growth levels, relative to the LB control strain. LB was the first level of the treatment factor, hence, estimates of all other levels were obtained relative to LB.

a more comprehensive picture about the efficacy of these honey concentrations.

The antibacterial effects of Manuka and raw pasture honey in the first culture were verified using an alternative method of analysis involving contrasts of statistical model [1]. The growth levels of bacteria in certain concentrations of honey were compared against growth in solutions of glucose with similar osmolality measures.

First, the growth levels of bacteria in 80 mg/mL Manuka honey were compared to the growth levels of bacteria in 60 mg/mL glucose. A 60 mg/mL glucose solution and 80 mg/mL Manuka honey have similar osmolality measures (**Figure 2**). Bacterial growth in 80 mg/mL Manuka honey and 60 mg/mL glucose was not significantly different in the first culture

| Treatment | Concentration (mg/mL) | Osmolality (mOsm/kg) | Standard Deviation |
|---|---|---|---|
| LB | 0 | 398.7 | 2.52 |
| Raw pasture honey | 140 | 992.67 | 8.74 |
| | 160 | 1090.00 | 9.64 |
| | 180 | 1167.33 | 6.66 |
| | 210 | 1323.33 | 5.13 |
| | 260 | 1577.67 | 10.69 |
| Manuka honey | 50 | 605.00 | 7.21 |
| | 60 | 671.00 | 7.94 |
| | 70 | 699.67 | 10.97 |
| | 80 | 765.33 | 1.53 |

**Table 2.** Average osmolality of effective sublethal concentrations of Manuka and raw pasture honey used in this study along with standard deviation.

| Effect | $OD_{600}$ Estimate | SE[b] | *p* | Significance[c] |
|---|---|---|---|---|
| Overall mean ($\hat{\mu}$)[a] | 1.3623 | 0.1444 | < 0.001 | *** |
| Day ($\hat{\alpha}$)[a] | 0.0196 | 0.0082 | 0.0214 | * |
| Raw pasture honey | 0.0264 | 0.0703 | 0.7085 | |
| Manuka honey | 0.0117 | 0.0650 | 0.8577 | |
| Osmolality ($\hat{\beta}$)[a] | -0.0004 | 0.0001 | < 0.001 | *** |

a. Note: $\hat{\mu}$, $\hat{\alpha}$, and $\hat{\beta}$ are estimates for $\mu$, $\alpha$, and $\beta$, respectively, in statistical model [2].
b. Note: SE are standard errors for the OD estimates.
c. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

**Table 3.** The effect of treatment and osmolality on growth level of bacteria in the first culture, relative to glucose. Manuka and raw pasture honey exhibited nonsignificant antibacterial effects beyond the contribution of osmotic stress in the first culture. Therefore, osmotic impact was the primary antibacterial effect of honey in the first culture.

($OD_{600}$ = -0.0477 ± 0.0579, $p = 0.4149$). This affirms the results in **Table 3**: the impact of sublethal concentrations of honey in the first culture was not significantly different from glucose solutions with equivalent osmolalities.

However, the antibacterial effects of Manuka honey, as an overall treatment in the first culture, were not the same as those of glucose. It is important to emphasize that concentrations of Manuka honey above 80 mg/mL were completely lethal, but concentrations of glucose greater than 60 mg/mL (i.e. 160 mg/mL glucose) were not, thus nullifying the impression that Manuka honey does not differ significantly from glucose as an overall treatment in the first culture.

Next, to verify whether raw pasture honey has antibacterial properties other than osmotic stress in the first culture, another contrast was used for statistical model [1], with 160 mg/mL glucose as a reference. A 160 mg/mL glucose solution and 260 mg/mL raw pasture honey had similar osmolalities (**Figure 2**), and their growth levels were not significantly different from each other in the first culture ($OD_{600}$ = 0.0317 ± 0.0583, $p = 0.5900$). These results are consistent with **Table 3**: the inhibitory effects of sublethal concentrations of honey in the first culture did not differ significantly from glucose concentrations with similar osmolalities.

The first culture of bacteria into honey did not provide a comprehensive picture of the efficacy of either raw pasture honey or Manuka honey. To truly evaluate the efficacy of honey, the role of osmotic stress was quantified over five subsequent cultures.

## Osmotic Stress of Honey has Nonsignificant Contribution on Bacterial Growth Beyond the First Culture

Similar procedures to the analysis for first culture were utilized to assess the role of osmotic strength in each treatment for five cultures. The same treatment-concentrations used in the first culture analysis were also used in the five-culture-day analysis.

Statistical model [1] was once again used with the data over all five culture days to verify that each treatment-concentration used in this experiment was in fact inhibitory. By

comparing the growth level of bacteria in honey and glucose to growth levels in the LB control, it was affirmed that each treatment-concentration studied was inhibitory. All bacterial cultures in this study were placed under stress, as indicated by their significantly lower growth levels, relative to the LB control (**Table 4**).

The role of osmotic strength in honey and glucose over five cultures was then quantified. Due to the change in the behavior of bacteria in response to further transfers into fresh honey-containing media, as shown in **Figure 3**, it was necessary to evaluate the efficacy of honey over several serial transfers. The role of osmotic stress in Manuka and raw pasture honey was quantified over five bacterial cultures using statistical model [2] with data now spanning five cultures. The results of this analysis, with glucose as the reference level, are summarized in **Table 5**.

The effect of osmolality had a nonsignificant impact on the growth levels of bacteria in honey over five culture days (slope of $OD_{600}$ on osmolality = -0.0003 ± 0.0002, $p = 0.1259$). Both Manuka and raw pasture honey had significant negative estimates relative to glucose (Raw pasture honey $OD_{600}$ = -0.4512 ± 0.1147, $p < 0.001$; Manuka honey $OD_{600}$ = -0.3391 ± 0.1180, $p = 0.0047$), indicating that when osmotic stress was accounted for, the growth levels of bacteria in sublethal concentrations of both Manuka and raw pasture honey over five cultures were significantly lower than in glucose. Thus, Manuka and raw pasture honey exhibited antibacterial properties beyond osmotic stress when bacteria were serially transferred over five cultures.

The results of statistical model [2] with bacterial growth over five cultures were different from those of the first-culture. The first culture gave an insufficient representation of how bacteria responded to honey; concentrations which were sublethal in the first culture were actually lethal in later cultures. Due to the incomplete depiction of the efficacy of honey in the first culture, it is imperative to evaluate the antibacterial activity of honey over several serial transfers, as this study has done.

| Treatment | Concentration (mg/mL) | $OD_{600}$ Estimate | SE[b] | *p* | Significance[c] |
|---|---|---|---|---|---|
| Overall mean ($\hat{\mu}$)[a] | --- | 1.8287 | 0.1008 | < 0.001 | *** |
| Day ($\hat{\alpha}$)[a] | --- | -0.0165 | 0.0124 | 0.1839 | |
| Glucose | 160 | -0.8566 | 0.1150 | < 0.001 | *** |
| | 60 | -0.6679 | 0.1150 | < 0.001 | *** |
| Raw pasture honey | 140 | -0.7174 | 0.1285 | < 0.001 | *** |
| | 160 | -0.7440 | 0.1311 | < 0.001 | *** |
| | 180 | -1.1101 | 0.1161 | < 0.001 | *** |
| | 210 | -1.3631 | 0.1096 | < 0.001 | *** |
| | 260 | -0.3879 | 0.1317 | 0.0037 | ** |
| Manuka honey | 50 | -0.4062 | 0.1082 | < 0.001 | *** |
| | 60 | -1.0938 | 0.1295 | < 0.001 | *** |
| | 70 | -1.2729 | 0.1123 | < 0.001 | *** |
| | 80 | -0.7157 | 0.0502 | < 0.001 | *** |

a. Note: $\hat{\mu}$, $\hat{\alpha}$ are estimates for μ and α, respectively, in statistical model [1].
b. Note: SE are standard errors for the OD estimates.
c. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

**Table 4.** Estimates of cultures studied throughout the five cultures relative to an LB control strain grown under no stress. All strains studied were put under stress as shown by their significantly lower growth levels, relative to the LB control strain.

| Effect | $OD_{600}$ Estimate | SE[b] | *p* | Significance[c] |
|---|---|---|---|---|
| Overall mean ($\hat{\mu}$)[a] | 1.1094 | 0.2631 | < 0.001 | *** |
| Day ($\hat{\alpha}$)[a] | 0.0553 | 0.0136 | < 0.001 | *** |
| Raw pasture honey | -0.4594 | 0.1126 | < 0.001 | *** |
| Manuka honey | -0.3414 | 0.1195 | 0.0049 | ** |
| Osmolality ($\hat{\beta}$)[a] | -0.0003 | 0.0002 | 0.1310 | |

a. Note: $\hat{\mu}$, $\hat{\alpha}$, and $\hat{\beta}$ are estimates for μ, α, and β, respectively, in statistical model [2].
b. Note: SE are standard errors for the OD estimates.
c. Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

**Table 5.** The effect of treatment and osmolality on growth level of bacteria over five cultures, relative to glucose. Manuka and raw pasture honey exhibited significant antibacterial effects beyond osmotic stress over five cultures.

The antibacterial properties of Manuka honey over five cultures were confirmed by comparing the growth of bacteria in 80 mg/mL Manuka honey with bacterial growth in a glucose concentration of similar osmolality, 60 mg/mL glucose. While bacteria grown in 60 mg/mL glucose maintained stable growth throughout the five culture days, bacteria grown in 80 mg/mL Manuka honey did not show detectable growth after the first culture. This loss of viability in bacteria clearly suggests that Manuka honey exhibits antibacterial properties other than just osmotic stress. Although both 80 mg/mL Manuka honey and 60 mg/mL glucose had similar osmolalities, bacteria were only able to sustain stable growth in glucose, which confirms that sublethal concentrations of Manuka honey had antibacterial properties beyond osmotic stress (**Table 5**).

The antibacterial properties of Manuka honey were detected by using serial transfers. The first culture provided evidence to suggest that Manuka honey was only effective due to its high osmotic impact; however, further cultures revealed the antibacterial effects of Manuka honey. This further demonstrates the importance of using serial transfers when evaluating honey.

The antibacterial properties of raw pasture honey were verified next. The growth of bacteria in 260 mg/mL raw pasture honey was compared against glucose solution with a similar osmolality, 160 mg/mL glucose. Raw pasture honey at 260 mg/mL did not permit detectable growth beyond the first culture, whereas 160 mg/mL glucose permitted bacterial growth throughout all five culture days. Although both 260 mg/mL raw pasture honey and 160 mg/mL glucose had the same osmotic impact on the growth of bacteria, bacteria behaved substantially poorer in 260 mg/mL raw pasture honey, which suggests that raw pasture honey exhibited antibacterial properties other than causing osmotic stress.

## DISCUSSION

The difference in the role of osmotic strength in honey in the first culture versus over five cultures suggests that the efficacy of honey cannot be evaluated without the use of several serial transfers. Bacteria behaved drastically differently upon further exposures into honey; thus, the first culture provides a deficient depiction of how bacteria behave in honey. For example, if the efficacy of the sublethal Manuka honey concentrations studied here had only been evaluated in the first culture, there would be flawed evidence to suggest that Manuka honey is only effective because of the high osmotic environment, which contradicts the literature (2, 3, 5, 8, 11, 12, 14).

Although several concentrations of Manuka and raw pasture honey were sublethal in the first culture of bacteria and seemed appropriate to use throughout the study, further serial transfers revealed a more comprehensive picture about the behavior of bacteria in response to honey. The loss of bacteria grown in concentrations which were sublethal in the first culture emphasizes the importance of utilizing serial transfers not only when selecting honey concentrations, but also when measuring the effectiveness of honey. Data from the initial 24-hour incubation of bacteria in a certain honey concentration provided insufficient information about the behavior of bacteria in that medium.

The results of this study indicate that honey has unique antibacterial properties beyond the osmotic impact in the first culture. Even when honey is administered at low concentrations that have no impact beyond causing osmotic stress in the first culture, bacterial populations seem to be transformed in future generations to make them more vulnerable upon further treatments with honey. The unique effects of sublethal concentrations of honey only became noticeable upon further transfers into honey, which further necessitates the use of serial transfers when evaluating the efficacy of honey.

It is important to emphasize that the analysis did not include lethal concentrations of honey (those that did not permit growth in the first cultures). Because only sublethal concentrations of honey were included, results cannot be generalized about the effectiveness of honey as an overall treatment in the first culture. The antimicrobial effects of lethal honey concentrations are attributable to factors beyond osmotic impact starting right in the first culture, e.g., Manuka concentrations of 90, 100, and 110 mg/mL (**Figure 1**).

Several authors have described the antibacterial properties of honey based only upon the first culture of bacteria. Osato, Reddy, and Graham sought to evaluate the efficacy of U.S.-produced honey (14). Similar to this study, the authors compared the growth of bacteria in honey to growth in a sugar solution and found no significant antibacterial properties in their U.S.-produced honey. This is consistent with the results of this study during the first culture. Since only the first culture of bacteria into honey was used, accurate conclusions regarding the antibacterial properties of honey cannot be made.

There are several other methods of evaluating the efficacy of honey which are employed in the literature; however, these methods did not account for the behavior of honey beyond the first culture. Perhaps the most common methods of evaluating honey involve determining the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentration (MBC) of honey. To determine the MIC, as described by Mohapatra, Thakur, and Brar, bacteria are cultured using the broth dilution method into several concentrations of honey growth medium; the lowest concentration showing no observable growth after the incubation period is considered the MIC (6). To determine the MBC, microorganisms from the test tubes that did not permit visible growth are streaked onto a sterile nutrient agar plate. After incubation of the plates, the lowest concentration that shows no growth is considered the MBC (6). A plethora of studies have used the MIC or MBC to determine the efficacy of different types of honey (2, 6, 15, 16). However, they did not consider the fact that bacterial response to honey varies over several serial exposures.

It is important to emphasize that the results of this study do not necessarily apply to all concentrations of Manuka and raw pasture honey. Only concentrations of honey which permitted growth in the first culture were utilized in data analysis. Lethal concentrations of honey, which did not permit growth in the first culture, were not included in the analysis. This study quantified the role of osmotic stress in the antibacterial properties of honey; it also emphasized the importance of serial transfers in evaluating honey as an antibacterial agent.

## METHODS

The effect of osmotic stress in honey was statistically separated from the effect of other antibacterial compounds in honey. Osmotic strength was quantified in the first culture of bacteria into honey as well as over five serial cultures in sublethal honey concentrations.

### Bacteria

The role of osmotic strength in honey-containing media on the growth level of bacteria was quantified twice throughout this experiment. First, the osmotic strength of honey was quantified in the first culture of Manuka and raw pasture honey. An amount of 100 µL of *S. aureus* bacterial broth was cultured into 5 mL of growth media containing sublethal concentrations of Manuka honey, raw pasture honey, and glucose, as well as an LB control. Three to five replicates of each treatment-concentration were prepared. The test tubes with inoculated growth media were placed in a VWR Incubating Orbital Shaker at 37°C and a speed of 200 RPM for 24 hours. To measure the growth levels in each strain and evaluate the efficacy of each treatment, a Varian Cary 50 Ultraviolet–Visible spectrophotometer (Agilent Technologies,

Santa Clara, CA) was utilized to measure the optical density of each culture at 600 nm, which is the standard wavelength used to measure bacterial cultures. The extent to which osmotic strength contributes to the inhibitory properties of Manuka and raw pasture honey in the first culture was quantified.

### Treatments

Manuka honey, raw pasture honey, and glucose were utilized as the three treatments in this experiment. First, Manuka was Kiva certified with Unique Manuka Factor (UMF) of 20+. UMF rating is a measure of the valuable contents in Manuka honey which guarantees its quality and purity (17). This specific honey is medical-grade with a methylglyoxal (MGO) of 825+. Second, raw pasture honey was polyfloral honey harvested in Northeast Wisconsin. Neither of the honeys were purified nor heated so as to avoid damaging or deactivating their antibacterial properties. Heating honey has been shown to reduce its antibacterial properties (18). Third, glucose was utilized as an osmotic control. Glucose inhibits bacterial growth by creating a poor environment for bacteria (19, 20). Finally, bacteria were grown in a sterile LB growth medium with no antibacterial additives. This culture served as an LB-only negative control and represented maximum growth for each day of the experiment.

Concentrations of Manuka honey from 50–80 mg/mL, raw pasture honey from 140–260 mg/mL, and glucose from 0–400 mg/mL were prepared. Osmolality values for all concentrations were measured by a Wescor vapor pressure Osmometer.

### Serial Transfers

Serial transfers of bacteria into honey and glucose took place over a total of six days. Viable bacterial strains from the first culture were transferred into fresh medium with the same treatment-concentration the following day. 100 µL of the previous day's growth were transferred into the same concentration of growth medium in a new, sterile test tube. This procedure was performed for a total of five sequential cultures. After each bacterial strain was incubated for 24 hours each day, the growth level was measured with the spectrophotometer. Throughout the experiment, concentrations which did not permit visible growth were discontinued and excluded from further serial transfers to the next day. After all growth measurements were taken, the impact of osmotic stress on the antibacterial properties of Manuka and raw pasture honey over five subsequent culture was quantified.

### Statistical Analysis

The impact of osmotic stress on the antibacterial properties of honey in the first culture was quantified by comparing the growth levels of bacteria in honey with growth levels in glucose, an osmotic control. Concentrations of honey which were lethal at the first culture were excluded from data analysis; only concentrations of honey which showed growth in the first culture were used. Growth levels from Manuka honey concentrations of 50, 60, 70, and 80 mg/mL and raw pasture honey concentrations of 140, 160, 180, 210, and 260 mg/mL in the first culture were utilized to quantify the role of osmotic strength in honey. R statistical software (21) was used for data analysis.

Model [1] was fit to verify that each bacterial culture was placed under significant stress relative to the LB control. Model [1] equation is:

$$OD_{ij} = \mu + (TC)_i + \alpha D + e_{ij} \qquad [1]$$

where $OD_{ij}$ is the $j^{th}$ replicate of the optical density measurement associated with treatment-concentration $i$; $\mu$ is an overall mean for optical density across all treatment concentrations, $(TC)_i$ is the $i^{th}$ treatment-concentration, which is one of LB or several Manuka honey, raw pasture honey, or glucose concentrations; $D$ is the day on which the optical density measure was taken, $\alpha$ is the regression coefficient of $OD_{ij}$ on $D$, and $e_{ij}$ is the random residual component of the model. In this experiment, not all cultures of bacteria were grown on the same day; therefore, day was considered as a covariate in model [1] to adjust for random day differences.

To quantify the contribution of osmotic stress on inhibiting bacterial growth and to separate it from other antibacterial effects of honey, the osmolality of all honey concentrations was accounted for as a covariate in model [2]. The following model fits optical density against treatment, day, and osmolality:

$$OD_{ij} = \mu + T_i + \alpha D + \beta O + e_{ij} \qquad [2]$$

where $OD_{ij}$ is the $j^{th}$ replicate of the optical density measurement associated with treatment $i$; $\mu$ is an overall mean for optical densities across treatments; $T_i$ is the $i^{th}$ treatment which is one of glucose, Manuka honey, and raw pasture honey; $D$ is the day on which the $OD_{ij}$ measure was taken; $O$ is the osmolality associated with $T_i$; $\alpha$ and $\beta$ are regression coefficients of $OD_{ij}$ on $D$ and $O$, respectively; and $e_{ij}$ is the random residual component of the model.

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Article

# Covalently entrapping catalase into calcium alginate worm pieces using EDC carbodiimide as a crosslinker

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## SUMMARY

**Catalase is a biocatalyst used to break down toxic hydrogen peroxide into water and oxygen in industries such as cheese and textiles. Improving the efficiency of catalase would help us to make some industrial products, such as cheese, less expensively. The best way to maintain catalase's conformation, and thus enhance its activity, is to immobilize it. The primary goal of this study was to find a new way of immobilizing catalase. There are many ways to immobilize an enzyme, one of the foremost being entrapment in calcium alginate. In past literature, researchers used calcium alginate as a bead, and an enzyme was physically entrapped into it. This form of immobilization has been proven inefficient because the enzyme is loosely attached to the material. To address this issue, we used calcium alginate as worm pieces with a carbodiimide as a crosslinker. Worm pieces here refer to small uniform pieces of cylindrical calcium alginate made by cutting long strings (worms) into small pieces. This research set out to understand whether or not EDC(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) can increase the immobilization of catalase into the calcium alginate. We found that EDC is able to triple the amount of catalase immobilized in calcium alginate compared to only calcium alginate entrapment. This research devised a new type of covalent entrapment that can be used to immobilize many other enzymes that also contain lysine residues. The result suggests that catalase can be better covalently entrapped into the calcium alginate worm pieces using EDC.**

## INTRODUCTION

Catalase (EC 1.11.1.6) is an enzyme that is found in nearly all living organisms. The main activity of catalase is to break down toxic hydrogen peroxide into simple water and oxygen. This activity of catalase helps the cell to main its homeostasis by reducing the number of reactive oxygen species (ROS). Catalase also has a variety of potential uses in the medical industry, food industry, bioremediation, and much more (1). One of the popular uses of catalase is to remove hydrogen peroxide ($H_2O_2$) from milk to produce cheese, because $H_2O_2$ is toxic to the bacteria that help in cheese production (1). Bioremediation is a process of treating pollutants from the environment using biological specimens. Catalase can also be used to bioremediate $H_2O_2$ from bleached textile effluents that are not treated well (1).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase must be immobilized into some material if it needs to be used in an industrial setting because this enhances its activity. There are many ways to immobilize catalase, including inorganic supports, natural polymers, and synthetic polymers (2). The primary goal of immobilization is to increase the efficiency of the enzyme. This is important because it helps to promote the enzyme's activity so the industrial company can successfully use it to increase the speed of a chemical reaction.

Calcium alginate is one of the natural polymers popularly used for both cell immobilization and enzyme immobilization (3). Alginate is found in algae, and through an industrial process it is commercially purified and sold (3). When sodium alginate (NaAlg) is placed in a divalent solution (Group 2 elements), the carboxylate groups in the alginate get crosslinked with the divalent ions, allowing the catalase to be entrapped into the spaces (2). The sodium in the alginate gets displaced by the calcium ions once it is placed in calcium chloride solution (3). The final material calcium alginate is a hard structure that is easy to handle.

To immobilize catalase using alginate, carbodiimide chemistry can be used. In this chemical reaction, EDC(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride), a carbodiimide that can crosslink carboxylates to primary amines, is used to crosslink proteins with the immobilizing material (4). Catalase and most other enzymes have lysine residues in their amino acid composition. Since the lysine group (NH2) holds a positive charge, it is found mainly on the surface of a protein. This property allows for easy crosslinking without denaturing the enzyme (4). Sodium alginate is composed of multiple carboxylates which can be used for crosslinking. EDC is first allowed to activate these carboxylate groups in an aqueous solution and then the protein containing lysine residues is added to the solution for effective crosslinking between the carboxylates and the protein's lysine residues (4). After the crosslinking, isourea is released as a byproduct (4). We employed this carbodiimide chemistry in this study between alginate and catalase.

Traditionally, calcium alginate has been used as a bead to immobilize enzymes and cells (5). In contrast, this study uses calcium alginate worm pieces. Worm pieces are made by cutting the calcium alginate strings (worms) into small pieces. Worm pieces are effective because they have a better surface area to volume ratio than beads. We hypothesized

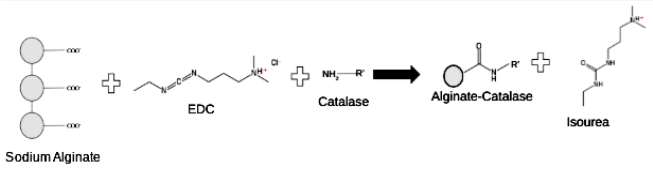


**Figure 1: Mechanism of crosslinking using EDC.** The carboxylate group of alginate is first activated by EDC (not shown in the figure). Once the carboxylate is activated, the catalase with a lysine residue is added to crosslink with the carboxylate. After the reaction, isourea is released as a byproduct. Isourea is removed from the mixture when it is placed in a calcium chloride solution. The structures were designed using PubChem Sketcher V2.4.

that catalase activity would increase drastically when the calcium alginate worm pieces are crosslinked to catalase with EDC carbodiimide because EDC has the ability to form a covalent bond between the carboxylate group in the alginate and lysine residue group in the catalase. Due to this, the enzyme can firmly attach to the material.

## RESULTS

The following observations were made by immobilizing catalase in calcium alginate under various conditions. In order to first determine the molarity of the 3% $H_2O_2$, we first measured the amount of $H_2O_2$ present in a control sample containing 3% $H_2O_2$ by titrating with an oxidizing agent and monitoring the color change. The molarity of the solution was found to be 0.9 M. In order to understand the baseline activity of catalase, free catalase was added to an $H_2O_2$sample solution. In order to measure the catalase's activity, we measured the amount of $H_2O_2$ remaining after a specific period of time in the 0.9 M sample by titrating it with KMnO4. This showed an activity of 1182 IU. We then prepared catalase immobilized in calcium alginate worm pieces, either with or without EDC (**Figure 1**). Sodium alginate showed the lowest activity after immersion in calcium chloride solution for 30 minutes and showed the highest activity after immersion for 15 minutes (**Table 1**).

The next step was to check whether EDC improved the activity. Adding EDC to give a final concentration of 10 mM increased the activity of catalase three-fold compared to calcium alginate alone (**Table 2**). When the calcium alginate worm pieces with and without EDC were compared, the calcium alginate with EDC showed the highest activity (**Figure 2**). This result supports the hypothesis that calcium alginate with EDC carbodiimide is a better candidate for catalase immobilization.

## DISCUSSION

The main goal of this study was to effectively immobilize catalase into calcium alginate worm pieces by using EDC carbodiimide. When EDC was used to activate the carboxylate group in NaAlg, the catalyst's activity was higher than in calcium alginate without EDC. We observed the highest activity when EDC was dissolved to a final concentration of 10 mM. This might be due to the fact that there was more EDC to activate carboxylate groups in 10 mM EDC than in 4 mM EDC.

| | |
|---|---|
| $H_2O_2$ (control) | 0 IU |
| Catalase-$H_2O_2$ | 1182 IU |
| Calcium Alginate 2%, 15 min | 251 IU |
| Calcium Alginate 2%, 30 min | 130 IU |

**Table 1:** Catalase activity in each sample with 20 µL undiluted catalase solution

| Calcium Alginate 2%, 30 min | IU Immobilized |
|---|---|
| 4 mM EDC | 282 IU |
| 10 mM EDC | 399 IU |

**Table 2:** The activity of catalase immobilized in calcium alginate using EDC

The catalase was able to be successfully immobilized both by entrapment and by covalent linkage in calcium alginate. The reason why immobilized catalase had less activity than free catalase might be due to enzyme activity loss during immobilization.

This study developed a new way of immobilizing an enzyme that is more efficient and easier to handle than regular calcium alginate beads. This result may lead to further research on enzyme immobilization. In the future, research can be done to find out whether the same technology can be used to immobilize many other enzymes that also contain lysine residues in their nonactive sites.

There are many applications of this technology in the manufacturing sector, including the cheese industry. It is widely accepted that hydrogen peroxide halts the growth of the bacteria in the milk that is essential for cheese production. We can use the immobilized catalase to break down this toxic hydrogen peroxide. The same immobilized catalase can also be used in the textile industry to remove peroxides from fabric. The long-term outcome of this study would be to develop a universal, cost-effective way to immobilize enzymes.

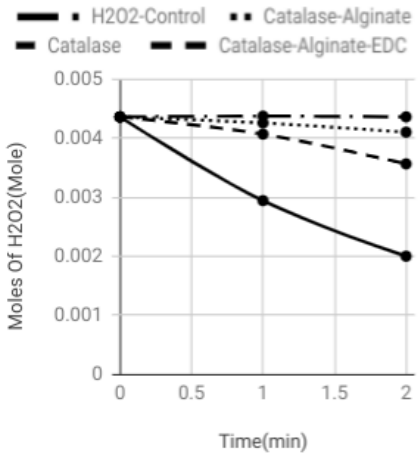


**Figure 2: The activity of catalase for 2% NaAlg- 30 minutes.** The graph shows the moles of H2O2 present in the sample after a t=0,1 and 2 minutes. The graph compares the moles of H2O2 remaining in a 5.0mL sample with no catalase, free catalase, immobilized catalase without EDC and immobilized catalase with EDC.

## MATERIALS AND METHODS

### Sodium Alginate And Calcium Chloride Solution

The 2% sodium alginate solution was made by dissolving 2.00 g NaAlg in 100.0 mL phosphate buffer (0.1M, pH 7.0) overnight. The 0.5 M calcium chloride solution was made by dissolving 27.745 g in 500.0 mL distilled water. All these solutions were stored at room temperature for future use.

### Generation of CaAlg and catalase-containing worms without EDC

In a beaker, 20.0 mL of 2% NaAlg was added and then 5720 IU of catalase was added to it. 5.0 mL was loaded into a 10.0 mL syringe and made into string-like structures called worms by dispensing into the 0.5 M calcium chloride solution. The sodium alginate with catalase was incubated in calcium chloride solution separately in two different time periods-15 and 30 minutes. The sodium in alginate is displaced by calcium and gives it a hard structure. After the time, the CaAlg was made into worm pieces with a diameter of about 2-4 mm and length of about 1-2 cm. The activity was determined by placing the pieces in 5.0 mL $H_2O_2$ solution and titrating after 1 and 2 minutes.

### Generation of CaAlg and catalase-containing worms with EDC

To the 20.0 mL 2% NaAlg, EDC was added to give final concentrations of 4 mM and 10 mM. It was allowed to react for 15 minutes, after which 5720 IU of catalase were added. Then 5.0 mL of this solution was made like worms in a 0.5 M calcium chloride solution. It was in the calcium chloride solution for 30 minutes. The activity was measured using the same protocol as described for calcium alginate without EDC.

### Determination of H2O2 concentration and measurement of catalase activity

$H_2O_2$ concentration was determined by titrating the sample with potassium permanganate. For ~5.0 mL $H_2O_2$, 12.0-13.0 mL sulfuric acid was added to give a source of hydrogen ions. 2% $KMnO_4$ was loaded into a 50.0 mL burette, then the sample $H_2O_2$ was titrated until the color of the sample H2O2 solution changed to light pink. The light pink marked the endpoint of titration for $H_2O_2$. The volume of $KMnO_4$ consumed was used to determine the moles of $H_2O_2$ using the following equation:

$$5H_2O_2 + 2KMnO_4 + 3H_2SO_4 \rightarrow$$
$$5O_2 + 2MnSO_4 + K_2SO_4 + 8H_2O$$

The catalase activity was determined indirectly by measuring the amount of $H_2O_2$ decomposed over time.

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