Effectiveness of Acidic Solutions as an Antimicrobial Agent on Plain Bread.

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Abstract

Bread spoilage leading to food wastage is detrimental to the environment, economy, and consumers alike. Much of this wastage can be attributed to the spoilage of foodstuffs as it moves from farm to table. The aim of our study is to explore natural preservatives as a means of reducing bread spoilage. To do this, we produced five treatments of various pH solutions and observed their effects on ten samples of 5cm x 5cm bread slices placed indoors in plastic bags for an 18-day period. During this timespan, we recorded biweekly measurements of mold coverage. All treatments inhibited growth by day 4. By day 8, only group 2 (pH = 2.73), group 3, and group 4 (pH 5.51) continued to provide inhibitory effects on mold growth ($p<0.05$). Groups 2 and 3 continued to inhibit growth by day 11, while group 3 was the only group that provided significant growth inhibition after day 15. Lastly, groups 2, 3, and 5 (pH = 5.21) all provided a decrease in mold growth rate which scaled with the treatment's acidity ($p<0.05$). These results are consistent with past literature and suggest that increased acidity facilitates the inhibition of enzymes vital for metabolism, as described in the "weak acid theory". This cytoplasmic acidification increases proportionally to the treatment's acidity, providing both more substantial and longer-lasting inhibition of microbe and fungi growth. These results have important implications for new technology in food sciences, such as lactic acid bacteria, which show promise in the natural preservation of bread products.

Introduction

Bread, a staple food product consumed across the globe, is often spoiled by excess growth of mold [1]. The spoilage of this bread can result in a strain on the entire system supporting it, from the consumers to the environment itself. Increasing the shelf life of bread products is an area of research which could alleviate some of this strain, a strain which has been amplified by the COVID-19 pandemic [2]. With vulnerable consumers looking to limit exposure opportunities in grocery stores, and supply chains limiting bread production and delivery, methods to extend shelf-life that are safe and easily applicable are becoming increasingly important.

One method which can be employed to increase the shelf-life of bread is pH manipulation. Wang and Zhu (2017) have noted that decreasing the pH of bread can lead to improved microbial growth inhibition during storage [3]. More specifically, Debonne et al. (2020) discovered that a significant determinant of sourdough wheat breads' shelf-life is the concentration of undissociated acetic acid [4]. Additionally, it was found by Lee, Jung, & Hwang (2009) that lower pH treatments, even when applied in an aqueous solution after baking in the form of onion juice, reduced the growth of aerobic bacteria and mold on bread, increasing shelf-life [5]. Finally, Gerez, Torino and Valdez (2009) found that organic acids were 2-85 fold more effective at inhibiting mold growth at pH 3.5 compared to 6 [6].

Given this, we sought to create aqueous acidic solutions at multiple pH levels to further test the effect of pH on bread shelf-life. To create solutions of varying acidic pHs, acetic acid in the form of white vinegar and sodium bicarbonate were employed. These two products were selected due to their edibility and availability; anyone can gain access to them. Moreover, the application of these products as an "at home" method of shelf-life extension has not been

investigated, though they both represent great choices for the creation of acidic solutions. In this instance both compounds act as weak acids. Acetic acid dissociates into acetate ion and hydronium while the buffer bicarbonate, acting as an acid in neutral solutions due to its low Kb of 2.25*10^-8, dissociates into carbonate and hydronium. Both of these compounds reach equilibrium in aqueous solutions at acidic pHs.

We investigated the effect of pH on bread mold growth by spraying bread samples with solutions of various pH levels. We anticipated that in accordance with recent literature, samples would see a decreased rate of mold growth in response to decreasing pH.

Methods

We used five different aqueous treatment solutions to test our hypothesis. We chose tap water to be our control treatment, and 2.73, 2.43, 5.51, and 5.21 pH solutions were used as our experimental treatments. First, we prepared 5 cm x 5 cm square bread samples, cut out of Real Canadian Superstore brand harvest grain bread slices, and we used a pre-measured piece of graph paper as a stencil to ensure that all of our samples were the same size. Following this, we created our treatment solutions. For the control treatment (group 1), we collected 250 mL of room temperature tap water. For the 2.73 pH treatment (group 2), we mixed 60.0 mL of white vinegar with 240.0 mL of room temperature tap water. For the 2.43 pH treatment (group 3), we mixed 240.2 mL of white vinegar with 9.8 mL of room temperature tap water. For the 5.51 pH treatment (group 4), we mixed 4.2 g of baking soda with 250.0 mL of room temperature tap water. For the 5.21 pH treatment (group 5), we mixed 16.8 g of baking soda with 250.0 mL of room temperature tap water.

When creating each treatment, we used a kitchen scale to measure ingredient mass. We placed a two-cup measuring cup on the scale, and we measured each ingredient directly into the cup. Once all the ingredients were measured and added into the cup, we mixed the solution with a metal spoon until it was thoroughly combined and then poured it into a spray bottle to be applied to the bread slices.

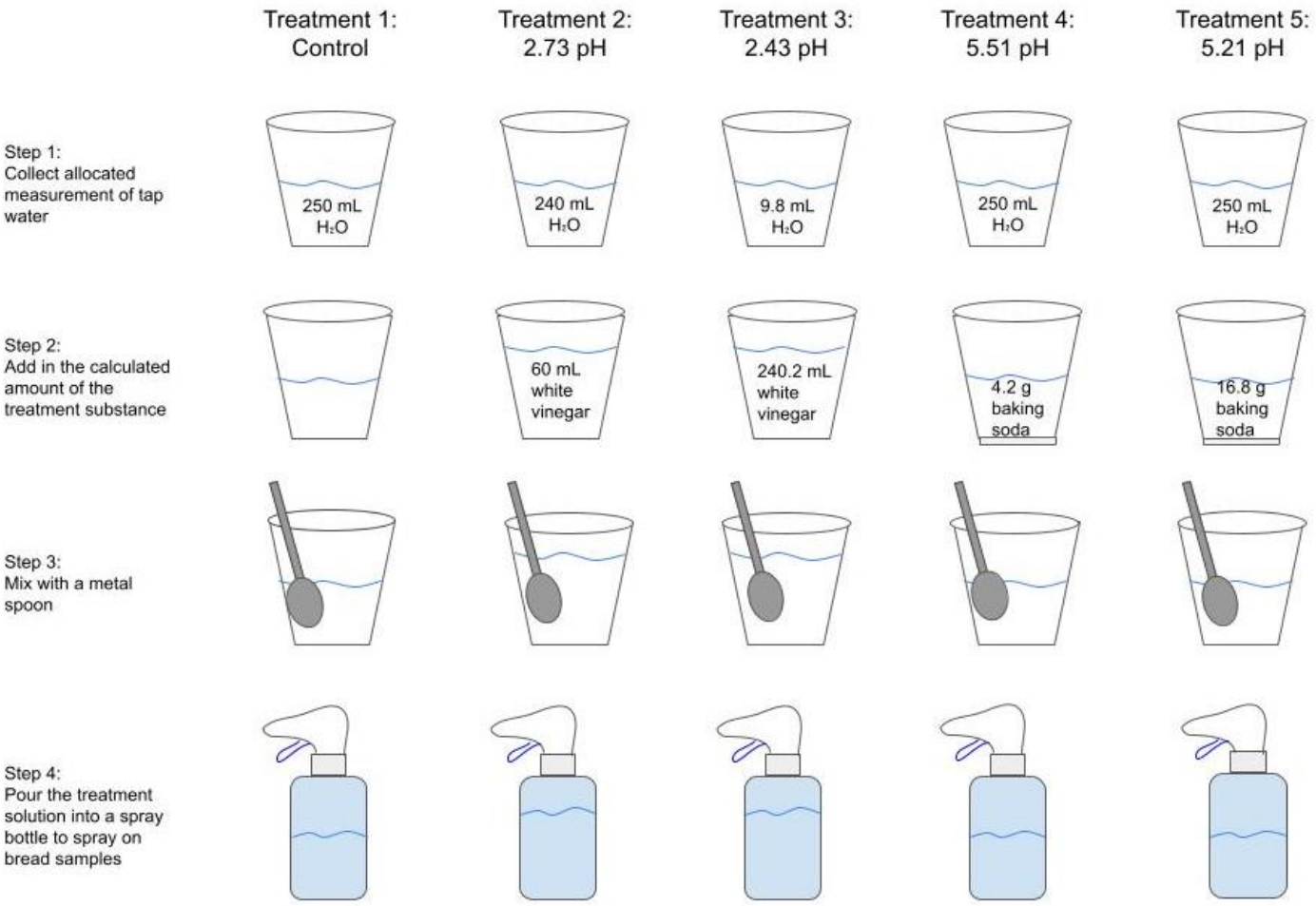

Figure 1: Methodology for creating treatment solutions used to treat bread samples.

Next, we wiped 10 samples of the pre-cut bread squares on one side along the kitchen floor to expose them to bacteria and fungi before spraying them with the group 1 treatment solution on the floor-wiped side. We sprayed each sample 25 times, which was equivalent to approximately

5mL of solution, before placing them in separate labeled clear plastic sandwich bags and sealing them. We then placed the samples on the floor of a storage room to be monitored and observed biweekly. We repeated this procedure for each treatment group, and we ensured that each bread sample was wiped on a surface of the kitchen floor that had not been wiped on before in an effort to expose each sample to an approximately equal number of bacteria.

After this, the samples were left on the floor of the storage room for three weeks, and we took measurements of the percent mold cover on the wiped side of the bread every Tuesday and Friday. We took the measurements using a 5cm x 5cm square piece of clear plastic that had 10 x 10 squares drawn onto it, such that each of the 100 squares represented 1% of the sample surface. We then placed the piece of plastic on the surface of the sample, which was still sealed in the clear sandwich bag, and counted each square that contained mold growth, which equated to the percent of the sample surface that was covered in mold growth. These measurements were taken regularly for three weeks. At the time of each measurement the temperature of the floor on which the samples were placed was also measured using a laser thermometer.

We then performed ANOVA analysis on the results to determine if there were any significant trends in mold growth observed on the samples between treatment groups. If the ANOVA detected any significance, we conducted Tukey's post-hoc analysis to determine which treatment groups had significant differences in mold growth compared to the control treatment group.

Results

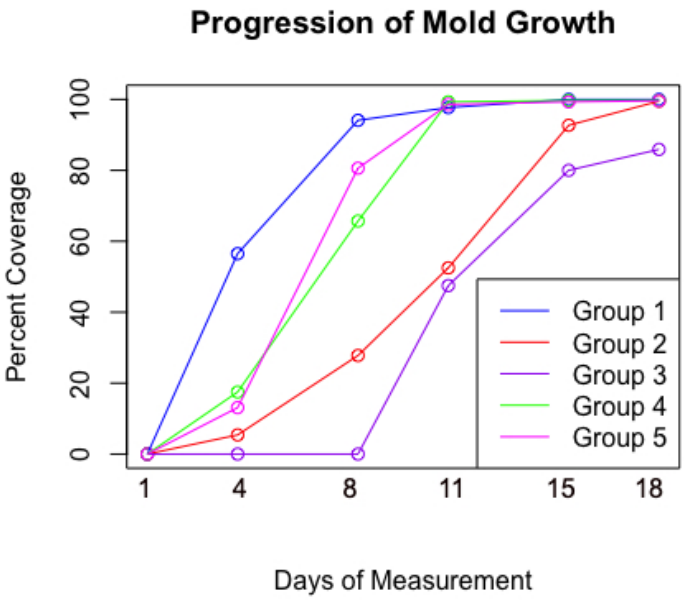

Figure 2: Progression of mold growth displayed with a multiple line graph. Group 1 (pH = 7.0), group 2 (pH = 2.73), group 3 (pH = 2.43), group 4 (pH = 5.51), & group 5 (pH = 5.21). Each data point represents the average percent coverage on the given day. **N** = 50.

Figure 3: Visualization of the mold growth by the end of observation. From top to bottom, group 1 to group 5.

As Figure 2 shows, groups 2 (pH = 2.73) and 3 (pH = 2.43), had the greatest inhibitory effect on the growth of mold. All samples in these groups had minimal to no mold growth for the first few days of observation, but all samples in group 3 experienced no mold growth reported until the 11th day of observation. However, both groups did reach full mold growth by the end of day 18. Unlike groups 4 (pH = 5.51) and 5 (pH = 5.21), which had a minimal inhibitory effect with progression compared to the control group. Using this information, a one-way ANOVA was conducted for each observation day comparing the average for total mold coverage on days (4, 8, 11, 15, and 18).

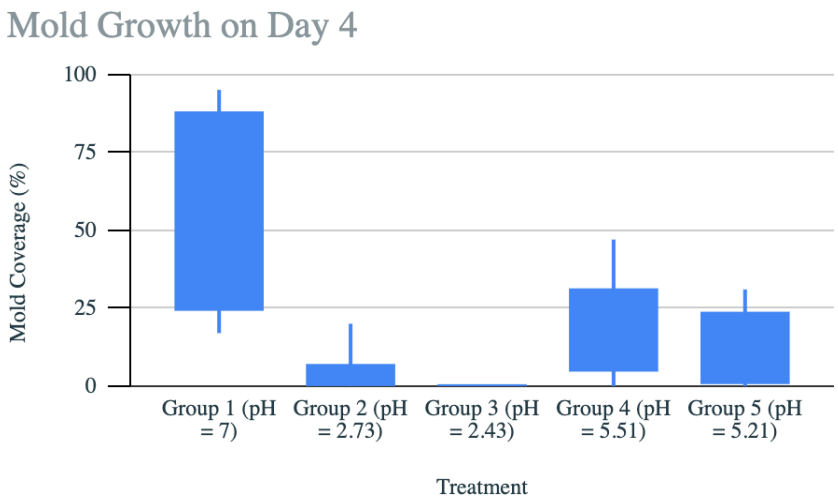

Figure 4: Boxplot for the distribution of percent mold coverage on Day 4 for treatment groups 1 (pH = 7), 2 (pH = 2.73), 3 (pH = 2.43), 4 (pH = 5.51), and 5 (pH = 5.21). Each data point represents the percentage of area covered by mold for a sample. Lower end is the minimum value, and the upper end is the maximum value. **N** = 50. **α** = 0.05. **P-value (One-way ANOVA)** < 0.00001. ***σ* (1 - 5)** = 32.353, 7.618, 0, 17.148, 12.449. **Q1 (1 - 5)** = 24.75, 0, 0, 5.25, 1.25. **Q3 (1 - 5)** = 87.5, 6.5, 0, 30.75, 23.25. 3

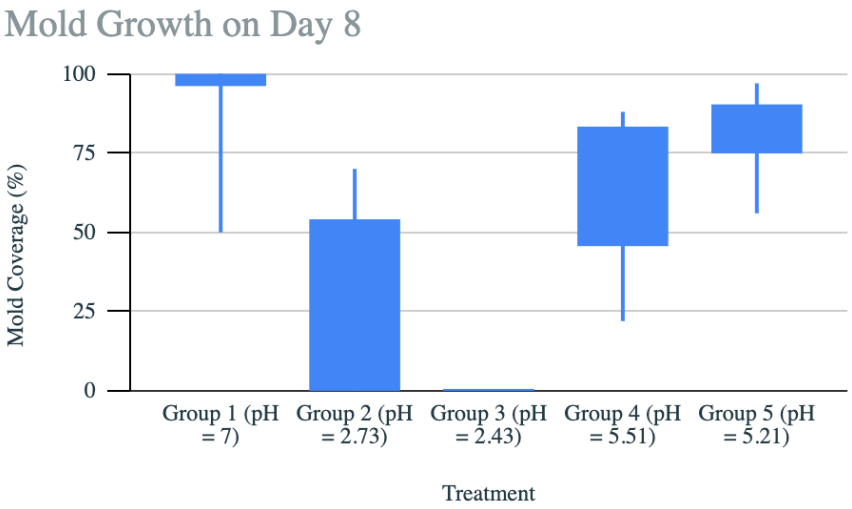

Figure 5: Boxplot for the distribution of percent mold coverage on Day 8 for treatment groups 1 (pH = 7), 2 (pH = 2.73), 3 (pH = 2.43), 4 (pH = 5.51), and 5 (pH = 5.21). Each data point represents the percentage of area covered by mold for a sample. Lower end is the minimum value, and the upper end is the maximum value. **N** = 50. **α** = 0.05. **P-value (One-way ANOVA)** < 0.00001. ***σ* (1 - 5)** = 15.581, 29.272, 0, 23.209, 13.672. **Q1 (1 - 5)** = 96.75, 0, 0, 46.25, 75.5. **Q3 (1 - 5)** = 100, 53.5, 0, 82.75, 89.75.

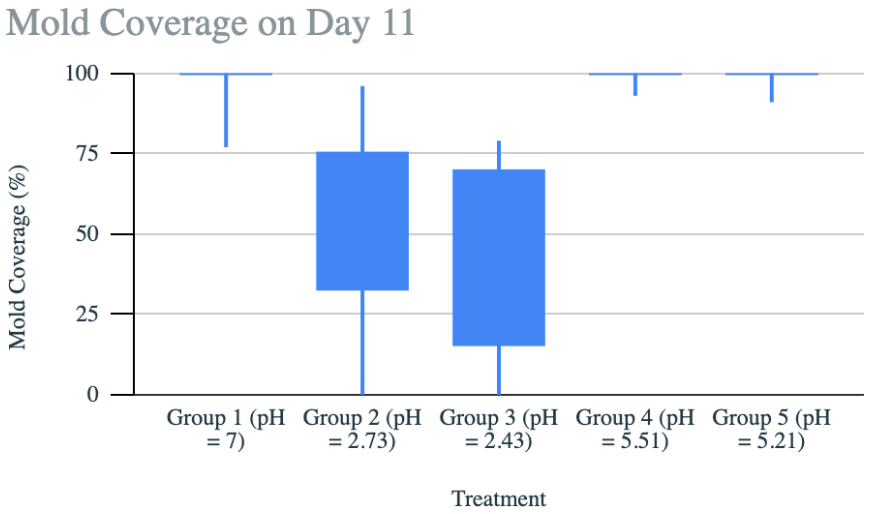

Figure 6: Boxplot for the distribution of percent mold coverage on Day 11 for treatment groups 1 (pH = 7), 2 (pH = 2.73), 3 (pH = 2.43), 4 (pH = 5.51), and 5 (pH = 5.21). Each data point represents the percentage of area covered by mold for a sample. Lower end is the minimum value, and the upper end is the maximum value. **N** = 50. **α** = 0.05. **P-value (One-way ANOVA)** < 0.00001. ***σ* (1 - 5)** = 7.273, 33.046, 31.384, 2.213, 3.098. **Q1 (1 - 5)** = 100, 33, 15.75, 100, 100. **Q3 (1 - 5)** = 100, 75, 69.5, 100, 100.

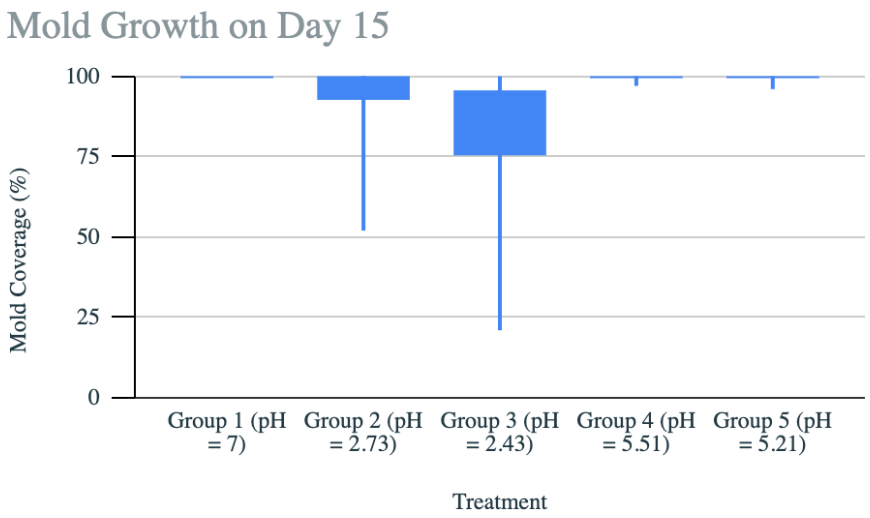

Figure 7: Boxplot for the distribution of percent mold coverage on Day 15 for treatment groups 1 (pH = 7), 2 (pH = 2.73), 3 (pH = 2.43), 4 (pH = 5.51), and 5 (pH = 5.21). Each data point represents the percentage of area covered by mold for a sample. Lower end is the minimum value, and the upper end is the maximum value. **N** = 50. **α** = 0.05. **P-value (One-way ANOVA)** = 0.002635. ***σ* (1 - 5)** = 0, 15.305, 22.997, 0.948, 1.494. **Q1 (1 - 5)** = 100, 93.25, 76, 100, 100. **Q3 (1 - 5)** = 100, 100, 95, 100, 100.

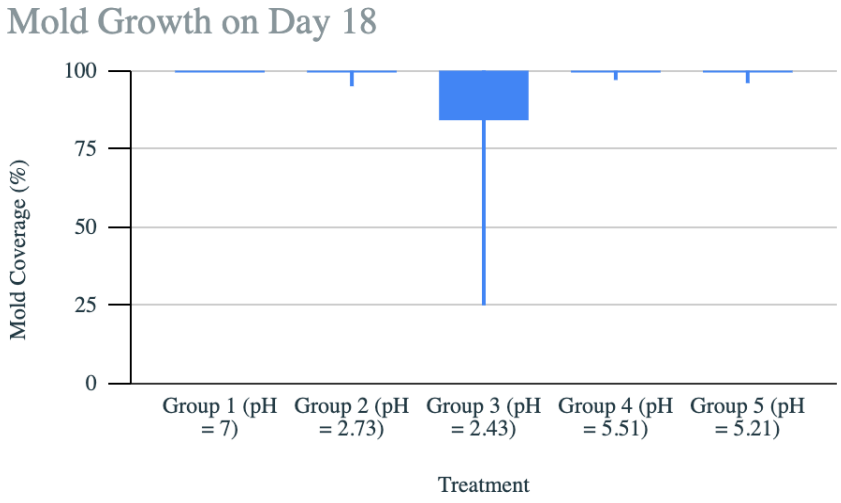

Figure 8: Boxplot for the distribution of percent mold coverage on Day 18 for treatment groups 1 (pH = 7), 2 (pH = 2.73), 3 (pH = 2.43), 4 (pH = 5.51), and 5 (pH = 5.21). Each data point represents the percentage of area covered by mold for a sample. Lower end is the minimum value, and the upper end is the maximum value. **N** = 50. **α** = 0.05. **P-value (One-way ANOVA)** = 0.013411. ***σ* (1 - 5)** = 0, 1.581, 23.072, 0.948, 1.265. **Q1 (1 - 5)** = 100, 100, 84.75, 100, 100. **Q3 (1 - 5)** = 100, 100, 100, 100, 100.

Using $\alpha = 0.05$ for the following ANOVA analyses, a p-value of < 0.00001 was found for day 4 of mold growth. A posthoc test had findings that all treatments had significant results compared to the control. For day 8, p-value = < 0.00001 and posthoc testing found treatments 2, 3, and 4 had significance compared to the control. Next, day 11 had a p-value of < 0.00001 but further testing discovered only treatments 2 and 3 were signifcant. For day 15, p-value = 0.00263 with treatment 3 having significant results. Finally, on the last day of observation, the p-value was 0.0134 and similarly, only treatment 3 had significant results.

To compare the complete mold growth of all treatments, an ANOVA was done to compare the average total mold coverage on the last day of observation. Analysis showed that the results were significant, having a p-value of 0.0134 with $\alpha = 0.05$ and group 3 had significance compared to the control group. In comparison, group 3 had the lowest mean mold coverage percentage for the ten samples at 85.9% by the end of observation. In addition, Figure 2 shows that group 3 (pH = 2.43) had the greatest distribution of total mold coverage for the duration of the study.

Finally, to compare the relative progression of growth for each treatment for the entire duration of the experiment, an ANOVA was done to compare the mean growth rates for all treatments. An application of a logarithmic function was applied to the slopes of each sample to illustrate a linear relationship, which was then used as the growth rates for the statistical test. Using $\alpha = 0.05$, we found significant results at a p-value of < 0.00001. Following Tukey's posthoc analysis, groups 2, 3, & 5 were significant compared to the control.

Discussion

Our study aimed to explore preservatives as a means of reducing bread spoilage and wastage. In particular, our study focuses on waste reduction using natural preservatives, which have gained popularity in recent years due to the negative shift in public opinion on artificial additives [8; 9; 10]. Our results clearly support our hypothesis that acidic solutions influence total mold coverage (Figure 2; 4; 5; 6; 7; 8). These results are supported by previous research, which illustrated a correlation between antimicrobial and antifungal ability with increased treatment acidity [11; 12; 13; 14; 15].

More specifically, our results clearly indicate that group 3 (pH 2.43) significantly inhibited total mold coverage by day 18 (Figure 2). This observation correlates with past research and can be explained according to the "weak acid model" [11; 12; 13; 14; 15; 16; 17]. Under this model, the partial dissociation of weak acids facilitates cytoplasmic acidification, which ultimately leads to a decrease in metabolism and growth [18; 19; 20]. Considering this, we conclude that the group 3 solution was sufficient to drive a degree of cytoplasmic acidification which stunted microbe growth even 18 days following application. The opposite is true for treatments not showing a significant difference. Previous studies have shown a curvilinear relationship between maximum microbial-specific growth rate (μ_{max}) and pH [15]. This relationship explains why the less acidic treatments were unable to significantly inhibit mold growth following 18 days of treatment: the solutions did not sufficiently displace environmental pH to a degree that it significantly affected μ_{max} [15].

Our study also demonstrated that certain treatments exhibited significant growth inhibition in a temporally transient nature (Figure 2; 4; 5; 6; 7; 8). These findings are consistent

with the results expected under the “weak acid model”, as the effect of cytoplasmic acidification only provides temporary metabolic inhibition [11; 16; 17]. As expected, the more acidic the treatment, the longer the duration of growth inhibition, which explains why only group 3 displayed significant inhibition 18-days post-treatment (Figure 2; 8). These results are of particular significance: they illustrate that advances in natural preservative technology will have to consider the strength of acid in relation to how it affects the duration of preservation and palatability, as there is an implicit tradeoff between the two. Future studies should be conducted to explore the relationship between using acidic, natural preservatives in the pre- or post-baking process which could positively affect the continual preservation of baked goods. Additionally, studies should be conducted into the possible relationship which underlies increasing the volume of acidic preservatives and increased strength of mold inhibition.

During observations of the treatments, all samples excluding group 3 grew the same-coloured mold (Figure 3). This suggests that the acidity of group 3 hinders the growth of a subset of microorganisms that are known to commonly grow on grain products [21]. Future research in identifying the specific microorganisms present after acidic treatments would be beneficial in developing new preservative strategies.

Lastly, our results clearly demonstrate that pH influences mold growth rate (Figure 2). Our statistical analysis determined that every treatment, other than group 4 (pH = 5.51), differed significantly in their growth rates. As expected, all affected treatment groups displayed a decreased growth rate compared to the control group. Additionally, the growth rate decreased linearly with a reduction in treatment pH (Figure 2). This was consistent with the results described in previous studies which illustrated an inversely proportional relationship between increased acidity and growth rate of microbes or fungi [11; 13; 14]. Interestingly, the fact that

group 4 did not have a significant effect on growth rate suggests that the pH of this solution was not adequate to significantly disrupt the μ_{max} to inhibit the growth of microbes [15].

Ultimately, we rejected our null hypothesis in addition to confirming previous trends concerning the relationship between acidity and mold growth inhibition. We demonstrated a possible temporal pattern between the magnitude of acidity and growth inhibition (Figure 2; 4; 5; 6; 7; 8). Future research would be well-served by exploring the relationship between this transient inhibition and how it is affected by acidic treatments. This study provides the groundwork upon which future studies into natural preservatives, such as lactic acid bacteria, can be built [22].

Conclusion

Our study was aimed at determining whether aqueous solutions of different acidic pH levels would be successful at hindering the growth of mold on bread, and our results indicate that pH levels of solutions have a significant effect on mold growth. The results of our study show that a solution with a pH of 2.43 had a statistically significant negative effect on mold growth over the 18-day observation period, and the level of growth inhibition increased with acidity levels. Our study therefore indicates that acidic solutions have a significant impact on mold growth, and that increasing acidity can affect both the magnitude and duration of mold growth inhibition.

Acknowledgements

We would like to thank Professor Celeste Leander for providing us with instruction on how to formulate an experimental study, as well as on how to write a scientific paper. We would also like to thank the xʷməθkʷəy̓əm (Musqueam) people for allowing us to learn on their traditional land. Finally, we would like to thank UBC for providing us with the opportunity to take this course.

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