Failure of colony growth in probiotic *Lactobacillus casei* Shirota as result of preservative sorbic acid

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SUMMARY

Preservatives have been a part of the food industry for years. Even before the days of bacterial pesticides and other antimicrobial technology, people used items like salt to prevent bacteria from growing. However, questionable claims on the negative effects of preservatives have been made, starting a new wave of "clean eating." In this study, we tested the proficiency of different concentrations of the antimicrobial sorbic acid to inhibit the probiotic *Lactobacillus casei* Shirota. We hypothesized that sorbic acid's use as a bacterial deterrent would also target this bacterial strain of *Lactobacillus*. We inoculated petri dishes in trials of 0%, 0.03%, and 0.1% sorbic acid concentrations and examined them over the 36-hour study. The results supported our hypothesis, with the colony count of *L. casei* Shirota having significant decreases at all concentrations of sorbic acid. These results additionally suggest that even under the FDA sorbic acid restrictions of 0.03% concentration, damaging effects could be seen in *L. casei* Shirota.

INTRODUCTION

Preservatives have been used since prehistoric times, and continually play a part in human survival against microbes. In more recent years, the use of both artificial and naturally created preservatives has become more common (1). As globalization of the human food chain has continued, preservatives have become important in ensuring that no mold or other substances grow during transport. One common compound used as a preservative is sorbic acid, which along with its salts are claimed to be some of the most used preservatives (2, 3). The antimicrobial properties of sorbic acid were first discovered in the late 1930s and early 1940s, and since then, its application in foods has expanded greatly (4). Common examples of sorbic acid uses include cakes, jams and jellies, cheese, and notably, fermented vegetables. This is of interest as fermented products often culture high concentrations of bacteria, something sorbic acid wards against. Such wide use of sorbic acid is partially due to its resilience and efficacy in weakly acidic pH levels and its unnoticeable taste (2). These weak acid preservatives characteristically inhibit growth of cells rather than killing them (5). Along with sorbic acid, its salts, like potassium salt, are utilized as preservatives in a comparable way. This also means the bactericidal mechanisms of sorbic acid and its salts are the same: penetrating through the cell membrane,

accumulating, and affecting the internal pH, disrupting the transport functions and metabolic activity of the microbe, creating proton flux into the cell, or a mixture of these (6). To add, sorbic acid has better antimicrobial efficacy at more acidic pH levels (7). This information influenced the choice of culture media, as sorbic acid is generally regarded as ineffective above 6.5 pH, and yeast malt agar has a pH level of 4.7 ± 0.2.

There are still many questions about the use of artificial preservatives. Some argue that it increases the risk of certain cancers and hyperactivity disorders (3). Another common argument stems from probiotics. Probiotics (commonly referred to as "good gut bacteria") are a group of bacteria that balance the microbiome of the gut (8). Understanding that preservatives are designed to target microbes puts forth the possibility that preservatives could affect the viability of beneficial microbes.

The particular bacterial genus and probiotic examined in this study is *Lactobacillus*. *Lactobacillus* is a Gram-positive, non-spore-forming bacterium (9). It is often found in the digestive system and urinary tracts of humans and other animals, helping the body break down food, absorb nutrients, and kill strains of "bad" bacteria (9-11). *Lactobacillus*, along with other probiotic genera like *Bifidobacterium*, are commonly available in pill capsules and over the counter at pharmacies; some literature considers these pills among the most popular dietary supplements (12). However, these probiotics are not just limited to pills, with certain foods such as kimchi and fermented yogurt being natural sources of these "good" bacteria (13). Some *Lactobacillus* species are commonly commodified, including *L. acidophilus*, *L. casei*, and *L. fermentum* (14).

In this study, we tested the *Lactobacillus casei* strain Shirota, which is known for being in the yogurt drink Yakult®. This strain was chosen because of its wide availability and commercialization. *L. casei* Shirota is also claimed to have the same positive intestinal effects as other *Lactobacillus* species and probiotics (15).

Although *L. casei* Shirota and the *Lactobacillus* genus can benefit the human gut, they still have bacterial functions that may be susceptible to preservatives. The antibacterial properties of sorbic acid attack most microbes, as they all have membranes or systems that can be permeated (i.e., how sorbic acid disrupts bacterial metabolism). Due to this, we hypothesized that sorbic acid would negatively affect the growth of the *Lactobacillus* colonies resulting in a decreased growth rate (5). In this experiment, yeast malt agar was altered

by adding sorbic acid at two concentrations: .03%, the highest amount allowed by FDA regulations, and .1% (16). These plates were then inoculated with *L. casei* Shirota in three trials per concentration. Over a 36-hour period, these plates were kept in dark boxes at 33.5°C until results were recorded. After analyzing the collected data through a one-way ANOVA, it was found to be significant. This data shows that sorbic acid negatively affects the growth of *L. casei* Shirota.

RESULTS

We first tested how sorbic acid could have applications in damaging the human gut microbiome by analyzing the effects of sorbic acid on *L. casei* Shirota in-vitro. Three sets of three trials of yeast malt agar plates were created with 0%, 0.03% and 0.1% concentrations of sorbic acid. Experimentation was done to determine how much to dilute the Yakult® for prime colony viewing. This value was found to be 10^{-7} mL of Yakult per 1mL of saline solution, which was pipetted via the pour plate method. All plates were then placed in an insulated cooler at 33.5°C. Petri dishes were monitored in 12-hour periods until colonies were visible on hour 36.

The control group of *L. casei* Shirota (no sorbic acid) had an average colony count of 78 ± 18.7. This range of colonies was the widest of all results. To the naked eye, colonies were small, and were close together. Despite this, they were still distinguishable from each other. Some colonies varied in size while others developed small translucent circumferences (**Figure 1**).

L. casei Shirota grown with .03% sorbic acid resulted in an average colony count of 21.67 ± 5.6. The range of colonies was much smaller than the no sorbic acid group. Colonies on these plates were less crowded. These colonies appeared visually similar to the ones that appeared in the agar plates without sorbic acid (**Figure 1**).

L. casei Shirota grown with .1% sorbic acid had a consistent colony count of 0. This was consistent across every single plate, and there were no colonies visible (**Figure 1**). After running a one-way ANOVA test, all decreases in colony count were determined to be statistically significant (**Figure 2**).

DISCUSSION

From this data, *L. casei* Shirota shows a statistically significant decrease in the number of colonies when grown with both .03% and .1% concentrations of sorbic acid. The collected data supports the hypothesis that sorbic acid reduces colony growth in *L. casei* Shirota. This aligns with recent literature that shows similar preservatives impacting the diversity of the gut microbiota in mouse models (17). Another recent paper shows that sulfite preservatives can negatively impact the human mouth's microbiome (19). It is promising that the results of our experiment seem to follow these current studies.

Utilizing what was available under environmental constraints, there were many different techniques used to compensate for not having certain equipment. As previously mentioned, a standard 1100W microwave was used to reheat the nutrient agar liquid instead of an autoclave. Additionally, neither an actual sterile air box nor a Bunsen burner was
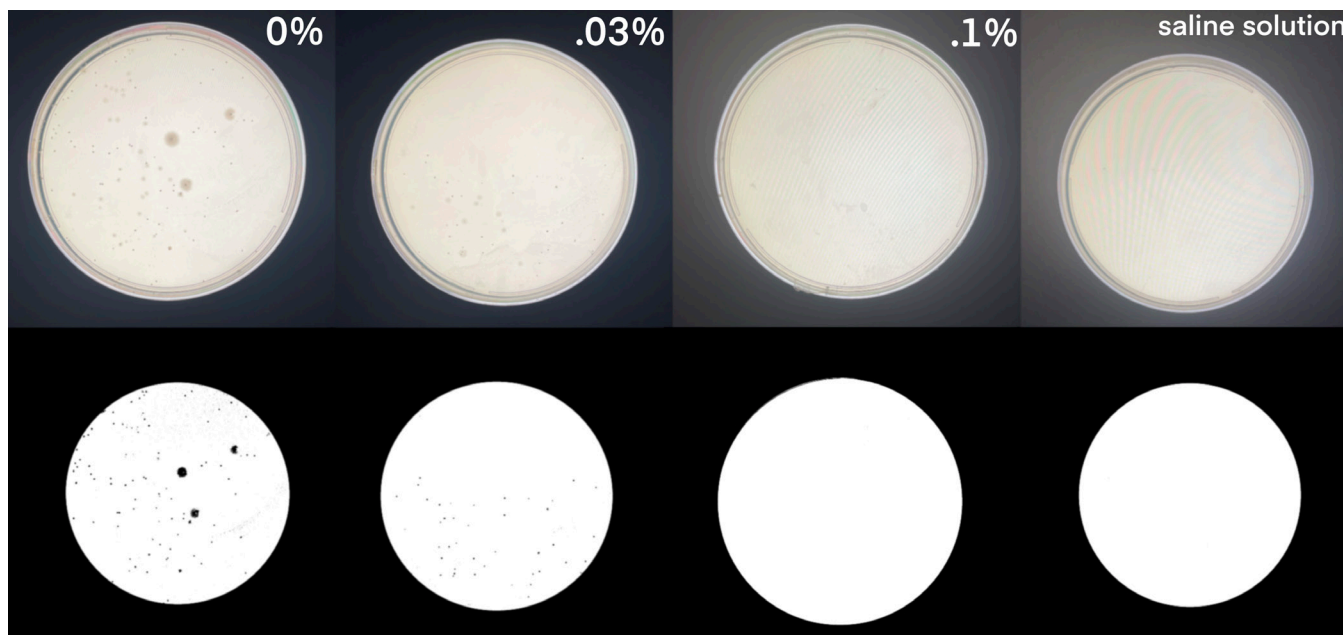

Figure 1. Plate colony comparison photos between different sorbic acid concentrations with and without ImageJ processing. This figure shows photos of one plate from each concentration of sorbic acid (0%, .03%, and .1%) as well as the plate with the sterilized saline solution. *L. casei* Shirota was grown in these agar concentrations for 36 hours at 33.5°C. The top row of images is without ImageJ processing, and the bottom row is with the ImageJ processing. Colonies were photographed by the iPhone 12's 12MP camera at a focal length of 26mm and *f*/2.4 aperture.
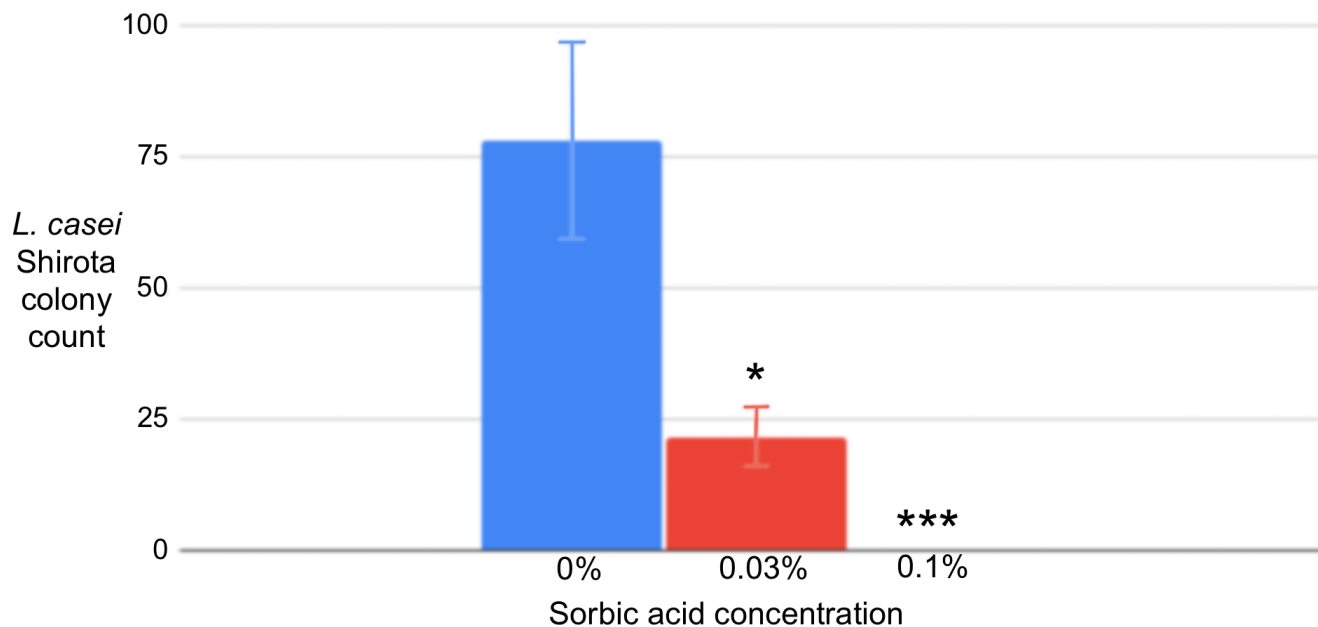
Figure 2. Average colony count comparison with different sorbic acid concentrations (n=3). Average colony counts of *L. casei* Shirota grown with each concentration of sorbic acid (0%, .03%, and .1%), with standard deviation shown via error bars. Bacteria were grown in these agar concentrations for 36 hours at 33.5°C. *p* < 0.05 is shown as * above error bars, and *p* < 0.01 is shown as ***. The 0.03% culture is significant compared to the control 0% culture. The 0.1% culture is also significant compared to the control.

available, so a makeshift sterile air box was made from a plastic box by melting two circles in the side of it and repeatedly sterilizing its inside and outside. This worked relatively well, as our sterilized saline solution plate and inoculated plates did not show any signs of contamination.

As stated previously, more acidic pH levels have been previously found to influence sorbic acid's antimicrobial efficacy. This could mean that the same level of sorbic acid in foods with different pHs would have different effects on inhibiting bacterial growth. Adding to this, sorbic acid may have influenced the pH of the growth medium that the *L. casei* samples were on, as it is an acid.

The species of *L. casei* Shirota was chosen for several reasons. Firstly, its wide commercial availability makes these results potentially applicable for all consumers. Additionally, *L. casei* Shirota is present in the human gut, acting as another reason why it was the optimal choice. Additionally, as mentioned in the results section, some of the *L. casei* Shirota colonies appeared larger than others (**Figure 1**). However, we feel these differences were most likely due to some colonies growing sooner than others or possibly mutation. This could have affected the data as this may have inhibited other colonies from growing or made extra colonies grow that were sorbic acid resistant.

Overall, our findings suggest that even under FDA-approved levels of sorbic acid, statistically significant decreases in *L. casei* Shirota colony count were found. This could be important as even with food carriers following the federal restrictions, *L. casei* Shirota growth may be inhibited by sorbic acid; furthermore, this may be applicable to the human gut microbiota as sorbic acid could negatively impact the microbiome diversity of the gut. This data could be cause for further experimentation to find if this is a pattern throughout other preservatives and probiotics. If this is the case, then it is important to realize the negative health effects that could be associated with ingesting these common preservatives so frequently.

MATERIALS AND METHODS

Different amounts of sorbic acid were tested on *L. casei* Shirota to determine whether it had an inhibitory effect. This sorbic acid (TalsenChem) was diluted several times in a sterilized saline solution until 1 mL of the solution contained the desired amount of sorbic acid. The sorbic acid-saline solution was then added to two premade yeast malt agar bottles (Carolina Biological Supply Company, Item #: 777200), with 37.5 mg being added to the .03% sorbic acid mixture and 125 mg to the .1% agar mixture, respectively. Since the agar liquid was pre-made, the preparation only required heating in a microwave (G&E Electronics, 1100W) for approximately 2 minutes before pouring in petri dishes (Ysanciuu, YJKSDPJ755). Prior to the plates used in data, experimentation was done to determine how much to dilute the Yakult for prime colony viewing. This value was found to be 10-7mL of Yakult per 1mL of saline solution, and this was to be expected as Yakult is marketed to have more than 20 billion CFUs (colony forming units) per 65mL bottle (19). This allowed us to view under 100 colonies per plate, which were hand-countable due to *L. casei* Shirota colonies being so characteristically small.

A makeshift sterile air box was created so that the possibility of accidental inoculants would be diminished. This was created by using a 60 cm x 41.6 cm x 33.7 cm Sterilite® box and making two hand-sized holes in the side so operations could be done inside. Elmers® glue fastened the base of the box to the lid, as well as gloves to the hand holes. During inoculation, this box was placed with the lid facing down. All surfaces or exposed areas were extensively sterilized with 70% isopropyl alcohol. Inside, all plates and tools were also sterilized.

The Yakult (*L. casei* Shirota) was kept in cool areas to ensure that their CFU would not decrease. Since the *L. casei* Shirota was already found in a 20 billion CFU 80 mL measurement by the manufacturer (Yakult®), dilution of 10-7mL of Yakult per 1mL of the solution was required to properly isolate individual colonies. 1 mL of the *L. casei* Shirota solution was poured in 90 mm x 15 mm plates. All plates were then evenly inoculated with *L. casei* Shirota using the pour plate method. Once liquid inoculants had dried on the agar medium, they were sealed with Scotch™ tape and placed in a 24 cm x 22.5 cm x 33 cm cooler with a 4W incandescent light bulb. The inside of this cooler measured an average of 33.5°C. All plates were put upside down and sheltered from the light by a sheet of cardboard. All three trials were simultaneous and continually monitored in 12-hour increments until clear results were recorded at the 36-hour mark.

Colonies were then photographed by an iPhone™ 12 camera at a focal length of 26mm and *f*/2.4 aperture. An 11" 2020 iPad Pro at the maximum 600 nits was used as an illuminator with a black background and 90 mm circumference white circle in the center. These photos were all taken at a distance of 14 cm before being analyzed to find the colony count.

Colonies formed in petri dishes were determined to be *Lactobacillus casei* Shirota by using several online images of L. casei Shirota and other *Lactobacillus* colony morphology (20, 21). Authors also tested the sterile saline solution for any potential contaminants by using the pour plate method on several agar plates.

Images were processed using ImageJ, an application developed by the National Institute of Health. A main use of ImageJ is colony counting, which is done by estimating the surface area of the colonies on a plate. However, in this process, individual colonies are also separated from the background of the plate to make the colonies expressed in **Figure 1** easier to view, which was our main purpose. A more detailed step by step procedure is found on the Lynbrook HS Research Program YouTube channel (22). Briefly, we selected the agar plate by using the circle tool and deleting the background. Then, we made the image black and white and used the threshold tool to finally separate the colonies from the background.

Statistical analysis was then performed using an online ANOVA calculator on VassarStats (23). Two separate Tukey's HSD post-hoc tests. The first compared the control data to the .03% concentration group, and the other compared the control to the .1% concentration. Both were found to be statistically significant.

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