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ALTERNATIVE SWEETENERS FOR CULTIVATING WATER KEFIR GRAINS

By

Mary Morgan Barnes

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford, MS

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DEDICATION

This thesis is dedicated to all the University faculty and staff who have guided me in my journey with education over the past four years.

ACKNOWLEDGEMENTS

Thank you to all of the people in Hom Lab who have made my three years of research unforgettable. Specifically, to Dr. Erik Hom and Xia Li who have always pushed me to be better, and have equipped me to expand the way I think. The skills that I have learned over these years will be skills that I use for the rest of my life, and I am forever grateful.

ABSTRACT

Water kefir is traditionally fermented using dark brown sugar due to a fast growth rate, however the use of other sweeteners, such as honey, is relatively unexplored. This paper describes the investigation of using alternative mediums made with honey, agave nectar, Truvia/stevia, and monk fruit sweetener, each supplemented with dark brown sugar on the biomass growth of water kefir grains (WKGs). Growth in these alternative mediums was compared to WKG growth in the standard medium prepared using only dark brown sugar. Two trials were conducted, each with three replicates for each experimental medium as well as three replicates of dark brown sugar medium controls. WKG biomass growth was measured every other day and recorded, and the culture medium was changed every other day to ensure adequate nutrients. Due to the higher vitamin content in honey and agave nectar (Cronometer, a nutrition analysis website was used to find this information), it was hypothesized that these two alternative mediums will have an increase in net biomass growth over the control. Because of the lack of vitamin supplementation in Truvia and monk fruit sweetener, it was hypothesized that these two mediums would have a decrease in net WKG mass compared to the control. I found that of the alternative mediums, agave nectar, Truvia, and honey did not support WKG growth as well as the control, with all of them recording a lower net increase of kefir grain biomass over time. Monk fruit recorded a slight increase of kefir biomass growth, although it was very modest.

Monk fruit recorded an average of 1.02% more biomass increase than the control, Truvia with 5% less increase in biomass compared to the control, agave nectar biomass increase was 5% less than the control, and honey with 11.6% less biomass increase compared to the control. While the monk fruit supplemented medium resulted in WKG growth closest to that of dark brown sugar medium, the observed growth was only a small percentage more than the control, and was not consistent with the hypothesized effect. These results suggest the notion that agave nectar, Truvia, and honey are either less nutritious or inhibitory to the growth of WKGs relative to the standard dark brown sugar medium, and monk fruit could be an adequate alternative with more research. It remains to be determined if WKGs could be adapted to grow better with these alternative sweeteners.

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LIST OF ABBREVIATIONS

MF+DBS	Monk Fruit and Dark Brown Sugar Medium
H+DBS	Honey and Dark Brown Sugar Medium
T+DBS	Truvia and Dark Brown Sugar Medium
A+DBS	Agave Nectar and Dark Brown Sugar Medium
DBS	Control/Dark Brown Sugar Medium

Introduction

Kefir

Kefir (pronounced kuh-FEER) is a widely known probiotic, traditionally fermented beverage that is believed to improve consumer's health. There are two types of kefir, milk kefir and water kefir. Water kefir is also known as 'aquakefir' or 'sugary kefir' due to how it is fermented using sugar water, and has a slightly acidic and fruity flavor. This project is focused on water kefir and the "grains" that are used to ferment it. Water kefir grains (WKGs) are small gelatinous aggregates—around 5 to 20 mm in diameter—and are translucent, taking on the color of whatever medium in which they are cultured. The "grains" that you visually see in WKG are polysaccharide matrices that are a byproduct of the microorganism growth. Some microorganisms are associated with this matrix, which is what allows grains to be reused for future cultures. The shape of WKGs is irregular, resembling that of rock candy (Fig. 1). Water kefir is made by adding WKGs to sugar water and fermenting between 21 and 30 °C for 2-3 days (Lynch et al., 2021). Occasionally, dried fruit or lemon is added to aid fermentation (Moretti et al., 2022). Depending on the type of fruit used and the process of fermentation, the microbial composition of the kefir can differ (Lynch et al., 2021).



Figure 1.A

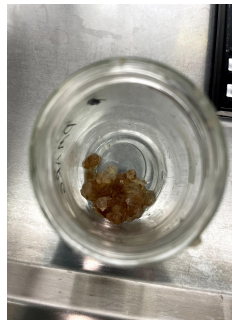


Figure 1.B



Figure 1.C

While undergoing fermentation, the microorganisms associated with WKGs get dispersed into the liquid culture medium (Pendón et al., 2021). In this context, the microorganisms grow and ferment sugar, decreasing the pH, even as many water kefir microorganisms stay associated with the grain. Within a WKG, select microorganisms use sucrose in the medium to synthesize glucans, which is a major component of the WKG matrix (Pendón et al., 2021). These kefir grains can be recovered by either removing the liquid medium from the mixture or removing the grains from the medium. These grains can then be reused to start a new fermentation while the freshly fermented water kefir can be immediately consumed or stored for 1 to 2 days at 4 °C to produce a stronger naturally carbonated drink in a stage known as “secondary kefir fermentation” (Pendón et al., 2021).

After fermentation, the kefir product will contain many microorganisms, released into the liquid medium used and through the multiplication of grains. In addition, other microbial metabolites generated from the fermentation process such as lactic acid, acetic acid, ethanol, carbon dioxide, mannitol, vitamins that are primarily B-complex, and amino acids will be present (Bengoa et al., 2021). The medium will also include polysaccharides that are mostly polymers of glucans and levans, although at a lower volume (Fels et al., 2018). Water kefir grains are considered natural reservoirs of probiotic strains such as *Lactobacillus kefiranofaciens*, *Lentilactobacillus kefiri*, *Lentilactobacillus parakefiri*, *Lacticaseibacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, as well as more than 23 species of yeasts (Bengoa et al., 2021).

History of Kefir

The word “kefir” originates from the Slavic *Keif* that means “well-being” or “living well”, and reflects the overall cultural sense of health and well-being derived from consuming the fermented beverage. The most well-accepted origin of kefir is from the Caucasus, Tibetan, or Mongolian mountains around 2,000 BC. Here, early kefir grains were passed down traditionally from generation to generation as a source of family wealth (Rosa et al., 2017). These grains were used to ferment milk and to make milk kefir.

While the historic origin of water kefir grains and relation to milk kefir grains is not entirely clear (Rosa et al., 2017), there have been multiple different ideas of its historical origin (Moretti et al., 2022). The first theory involves the leaves of the Mexican cactus *Opuntia* spp., on which it is believed that the grains spontaneously formed through microorganisms feeding on the sugary excretions of the plant (Pendón et al., 2021). Another theory is based on the first scientific report on water kefir, published in 1889 by the Dutch microbiologist Martinus Beijerinck (Beijerinck, 1889). Beijerinck linked water kefir grains to ginger beer plants that English soldiers had brought back from the Crimean War in 1855. A similar system of fermenting the grains was described from prickly pear cactus fruits in Mexico and was described by Lutz in 1899 (Fiorda et al., 2017). The actual name of “sugary kefir grains” (which has more broadly become referred to as “water kefir grains”) was assigned to differentiate them from the milk fermented grains by Pidoux in 1989 (Pidoux, 1989) (Fiorda et al., 2017).

Use of Kefir Today

Milk Kefir can be found easily in grocery stores across the United States, commonly beside dairy products like yogurt, and kefir's probiotic health benefits are of growing interest to many consumers. Some benefits reported from kefir consumption include: immune system stimulation, anti-inflammatory, antioxidant, anti-obesity, anti-proliferative, lipid metabolism improvement, hypocholesterolemia stress modulator effects, and the enhancement of intestinal bacterial microbiota (Bengoa et al., 2021). While these benefits are broad, they are believed to be strain-specific, and can depend on how the kefir was fermented (Bengoa et al., 2021).

Kefir is commonly marketed as a probiotic in many stores where it is sold, even though it officially does not fit the formal definition of a probiotic. Probiotics need to have well-defined and characterized living microorganisms with health benefits that stem directly from them (Marco et al., 2021). Fermented foods are described as “foods made through desired microbial growth and enzymatic conversions of food components” (Marco et al., 2021). Because of this marketing as a probiotic that contains health promoting microorganisms, kefir has grown in prominence and popularity (Marco et al., 2021). Recent studies have shown the lactic acid bacteria present in kefir fermented beverages have therapeutic effects in many different diseases such as rheumatoid arthritis and cancer (Sharifi et al., 2017). These studies suggest that the probiotic-containing materials may have anti-proliferation and anti-inflammation properties (Sharifi et al., 2017). Because of this, kefir may act as an effective agent in cancer treatment and prevention, as well as in treatments for other inflammatory diseases (Sharifi et al., 2017).

This experiment was conducted in efforts to discover alternative ways to culture water kefir for those who may have sugar dietary restrictions and to explore the consequences of using a different sugar source on the growth of kefir grains. By finding new sweeteners to culture WKGs, a wide range of improvements could be made such as added nutrients, new flavor compounds, and could help market kefir. Hopes of discovering alternative sweetener mediums would also expand flavor profiles of kefir, and could eventually lead to new water kefir variants that could satisfy a larger population.

Materials and Methods

This study was conducted to determine the effects of alternative sweeteners on kefir grain multiplication/biomass growth as a measurement of microbe replication. The sweeteners studied were as followed: Latvia Monk Fruit Sweetener (Lakanto, Orem, Utah), Agave In The Raw Organic Agave Nectar (Cumberland Packing Corp., Brooklyn, New York), Truvia Naturally Sweet Calorie Free-Stevia Sweetener (The Truvia Company, Stacy, Minnesota), Kroger Clover U.S. Grade A Honey (Kroger, Cincinnati, Ohio), and Domino Dark Brown Sugar (Domino Foods Inc., Yonkers, New York). Four alternative mediums were prepared using 40 g of the alternative sweetener, 40 g of dark brown sugar (DBS), and 800 mL of water (50% (weight) alternative sweetener + 50% dark brown sugar), as shown below in Table 1. These media were compared to a control group of DBS (50% dark brown sugar + 50% dark brown sugar). One hundred mL of each of these mediums were added to 8 g of WKG, and changed every Monday, Wednesday, and Friday for the experimental period of 2 weeks.

Table 1. Contents of the alternative mediums used

A+DBS	50% agave nectar + 50% dark brown sugar
T+ DBS	50% Truvia + 50% dark brown sugar
H + DBS	50% honey + 50% dark brown sugar
MF+DBS	50% monk fruit sweetener + 50% dark brown sugar

The grains used in this study were a “master mix” of different water kefir grains (WKGs) of undetermined microbes that were previously adapted in the Hom Lab to grow on autoclaved

100% DBS medium (80 g of dark brown sugar mixed in 800 mL of water). This was decided in efforts to generate sufficient water kefir grains needed to carry out this project, and to avoid potential idiosyncratic grain-specific variations.

Two trials were conducted at separate times with fresh WKGs from the “master mix”. For each trial, 3 replicate kefir cultures were prepared of 100 mL for each medium with 8 g of WKGs. In Trail 1, tall and skinnier jars were used for the cultures, and for Trial 2 shorter and fatter jars (were made to hold baby food) were used. Kefir biomass of each replicate was measured every Monday, Wednesday, and Friday before the medium was changed. The same scale was used each time to measure the kefir grains’ biomass each time across both trials. Each Monday, Wednesday, and Friday, prior to placing in fresh media, WKG biomass for each replicate culture was measured and recorded.

- Agave replicate 1-3: 8 g of kefir “master mix” + 100 mL of A+DBS medium
- Honey replicate 1-3: 8 g of kefir “master mix” + 100 mL of H+DBS medium
- Truvia replicate 1-3: 8 g of kefir “master mix” + 100 mL of T+DBS medium
- Monk Fruit replicate 1-3: 8 g of kefir “master mix” + 100 mL of MF+DBS medium
- Control replicate 1-3: 8 g of kefir “master mix” + 100 mL of DBS medium

Results

Trial 1	Net Growth	Average Growth	Trial 2	Net Growth	Average Growth
Monk Fruit 1	2.989	MF Ave:	Monk Fruit 1	-0.4	MF Ave:
Monk Fruit 2	1.717	2.014	Monk Fruit 2	0.076	-0.182
Monk Fruit 3	1.336		Monk Fruit 3	-0.222	
Truvia 1	1.675	T Ave:	Truvia 1	-1.069	T Ave:
Truvia 2	1.488	1.424	Truvia 2	-0.259	-0.584
Truvia 3	1.108		Truvia 3	-0.423	
Agave 1	1.388	A Ave:	Agave 1	-0.748	A Ave:
Agave 2	1.549	1.312	Agave 2	-0.402	-0.474
Agave 3	1		Agave 3	-0.272	
Honey 1	0.98	H Ave:	Honey 1	-0.109	H Ave:
Honey 2	-0.301	0.248	Honey 2	0.006	-0.617
Honey 3	0.065		Honey 3	-0.767	
Control 1	0.826	C Ave:	Control 1	0.19	C Ave:
Control 2	2.547	1.728	Control 2	0.392	-0.098
Control 3	1.81		Control 3	-0.876	

Table 2. Data of Trial 1 and Trial 2

WKG Growth with 100% Dark Brown Sugar (Control)

The control group of trial one had an average of 1.73 g of biomass increase after 2 weeks of growth (Figure 2). All three replicates had a large range of WKG biomass increase, from replicate 1 gaining 0.83 g, replicate 2 gaining 2.55 g, and replicate 3 gaining 1.81g. All of the graphs illustrating growth patterns of the control group showed the relatively consistent increase in biomass. Only replicate 1 had a significant dip in biomass on day 5.

DBS control Group Growth Trial 1

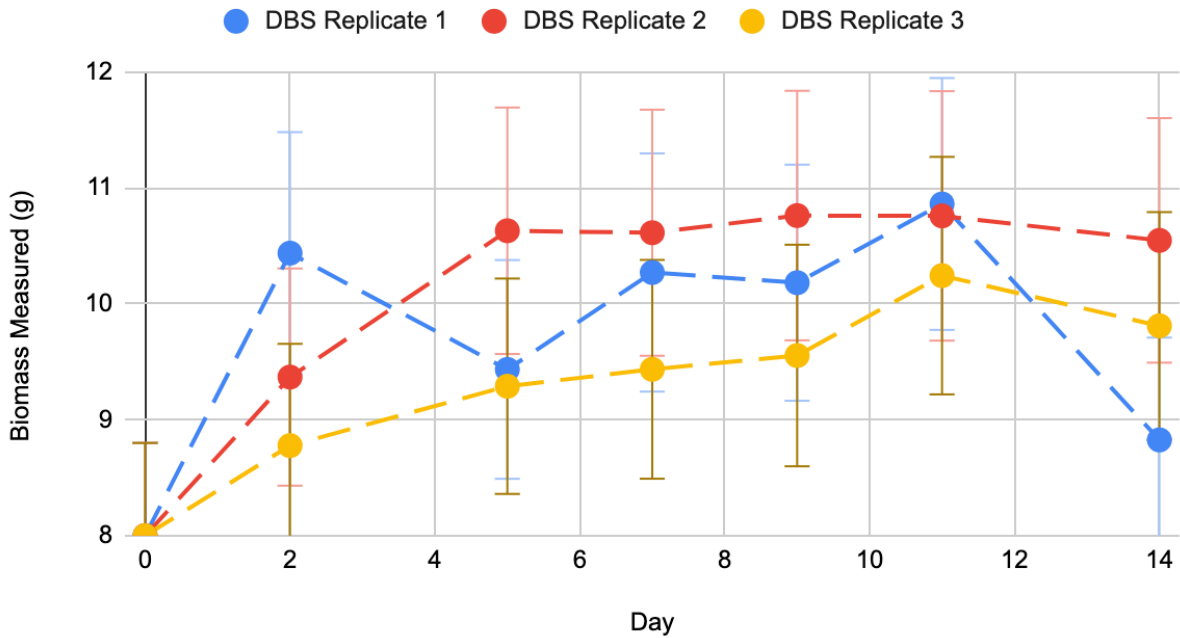


Figure 2. DBS Growth recorded over Trial 1

Trial 2 of the control group showed a general trend of biomass decrease over two weeks (Figure 3). Average biomass loss was 0.098 g, with replicate 1 increasing 0.19 g, replicate 2 increasing 0.39 g, and replicate 3 losing 0.88 g of biomass. These growth charts showed very inconsistent growth from trial 1, but are consistent across all replicates and groups in trial 2. These control replicates often lost significant biomass one day of measuring to have drastically increased in biomass the next day of measuring. This resulted in very unpredictable growth.

DBS Control Group Growth Trial 2

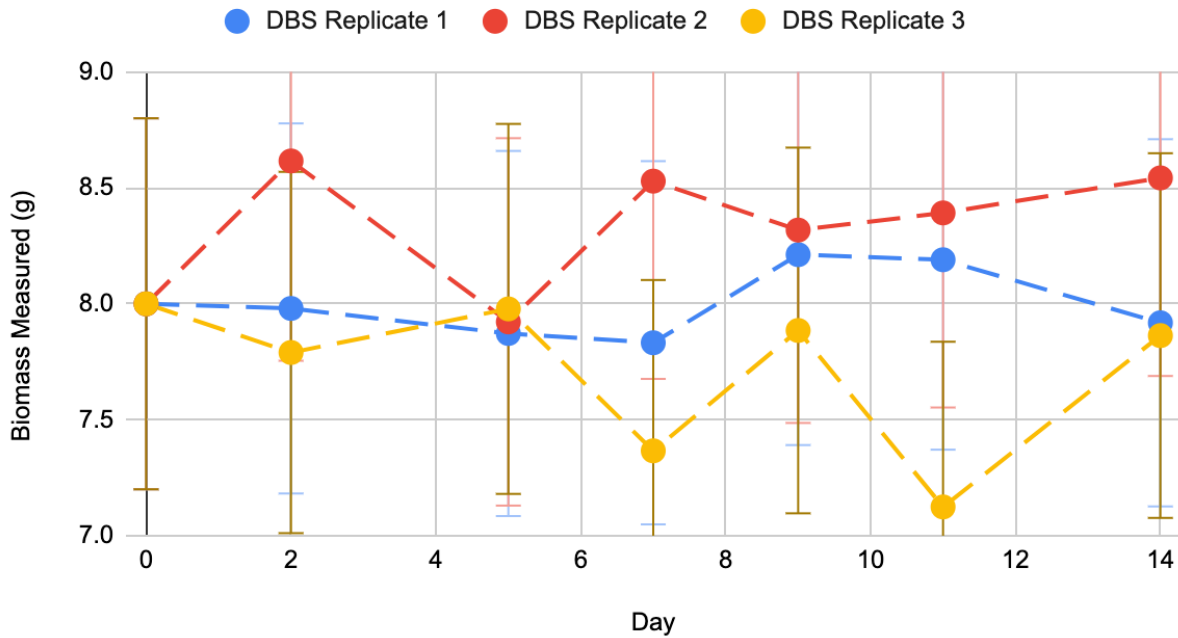


Figure 3. DBS Growth recorded over Trial 2

WKG Growth with 50% Monk Fruit Sweetener

Trial 1 on MF+DBS medium showed a growth pattern and biomass increase most similar to the control group DBS (Figure 4). In trial 1, replicate 1 and 2 showed a comparatively constant growth rate, and the average biomass increase was 2.01g after 2 weeks. Compared to the average control group growth of 1.73 g after 2 weeks, this data initially showed an increase in culturing ability to the DBS control medium.

MF+DBS Trial 1 Growth vs. Control Average Growth Trial 1

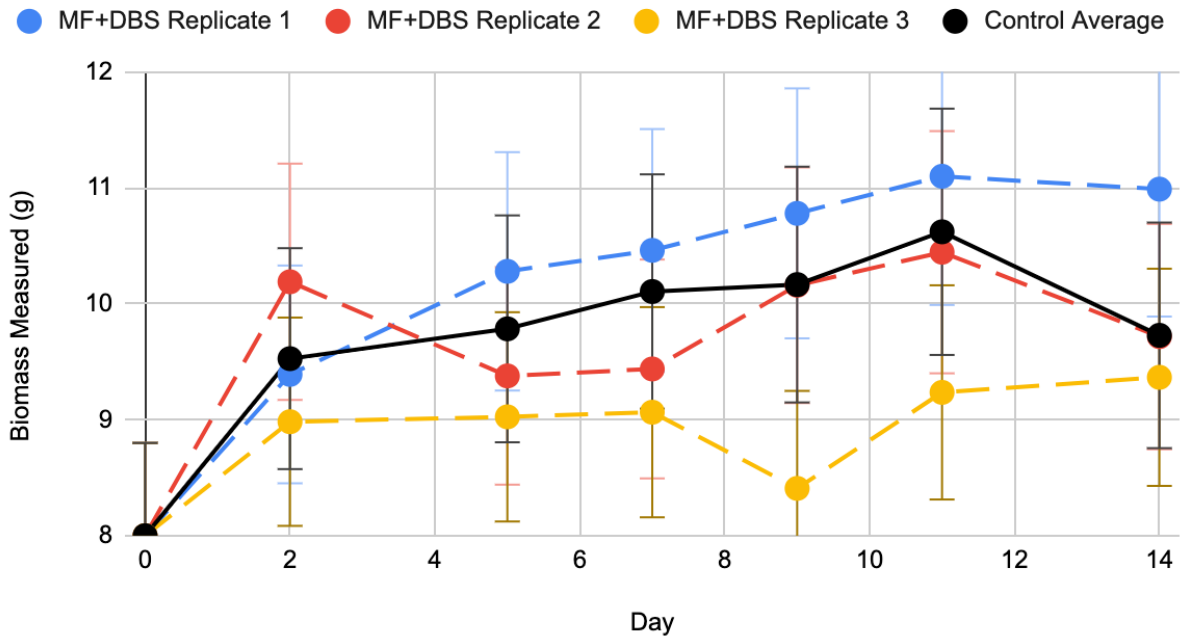


Figure 4. MF+DBS Growth recorded over Trial 1 compared to the Control Group

Trial 2 of MF+DBS was very erratic and did not follow this same pattern (Figure 5).

Replicate 1 was the closest growth curve to anything from trial 1, but still overall resulted in a loss of biomass over 2 weeks. This loss of biomass was small in comparison to replicates 2 and 3, and the average biomass *decreased* by 0.18 g after 2 weeks. A loss of biomass was very different from trial 1, but compared to the control group's average growth of -0.10 g, the loss of biomass was not unexpected.

MF+DBS Growth Trail 2 vs. Control Average Growth Trial 2

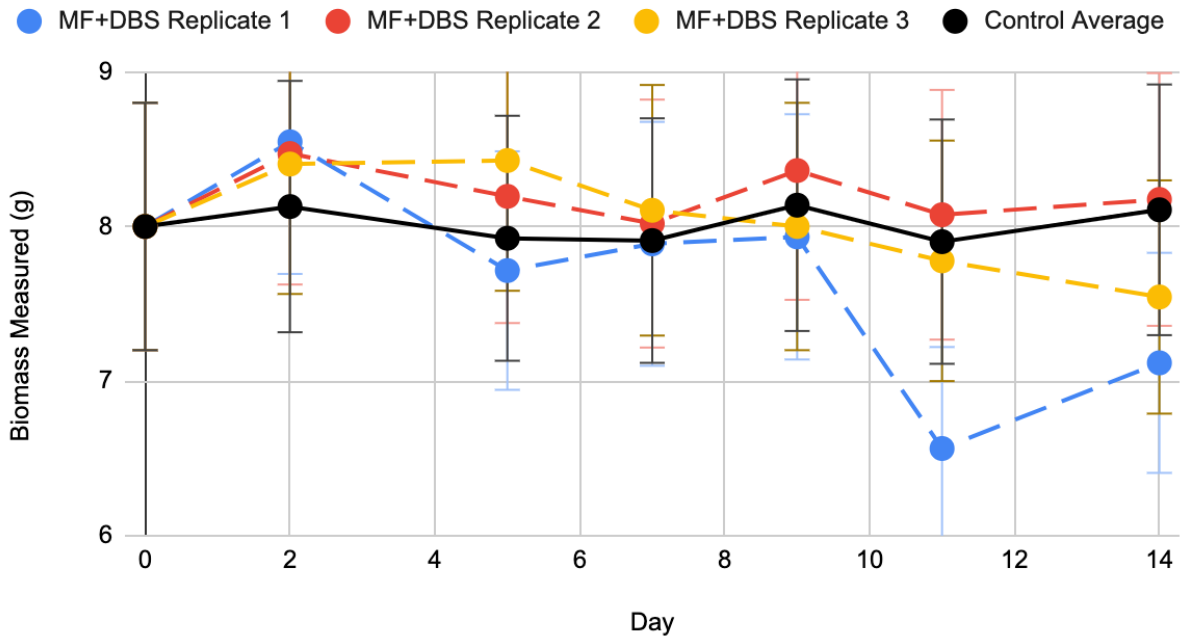


Figure 5. MF+DBS Growth recorded over Trial 2 compared to the Control Group

WKG Growth with 50% Honey

Trial 1 of H+DBS showed the worst growth out of all of the experimental groups (Figure 6). Replicate 1 only had a net increase of biomass of 0.98 g after 2 weeks, replicate 2 a decrease of biomass of 0.30 g, and replicate three a small increase of 0.07 g. The average growth of WKGs in the H+DBS medium was a small increase of 0.25 g. The honey medium also had a unique look compared to the other alternative sweetener media; it was cloudy instead of a clear amber color. There was also a noticeable increase in biofilm that accumulated on the top of the culture medium. While such a biofilm is common, the thickness and prominence of the H+DBS biofilm was noticeable.

H+DBS Growth Trial 1 vs. Control Growth Average Trial 1

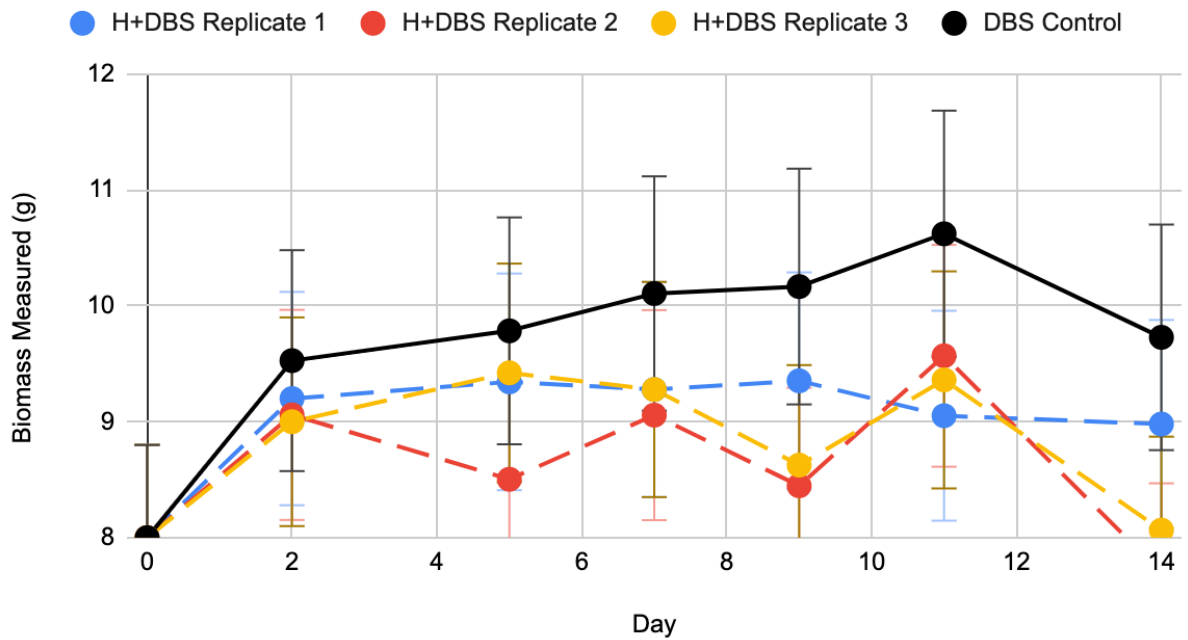


Figure 6. H+DBS Growth recorded over Trial 1 compared to the Control Group

Trial 2 using H+DBS medium showed similar trends but there was even less overall biomass growth (Figure 7). Most of the replicates in trial two for H+DBS showed loss of WKG biomass. When compared to the overall trends of trial 2, this was not surprising as all of the experimental groups showed either loss of biomass or significantly less growth than trial 1. Replicate 1 had a loss of biomass of 0.11 g, replicate 2 had a net growth of 0.01 g, and replicate 3 lost 0.77 g of biomass. Averaged together, H+DBS growth for trial 2 was a net loss of 0.62 g

after 2 weeks. Trial 2 also created the same type of biofilm as was observed in trial 1.

H+DBS Trial 2 vs. Control Average Trial 2

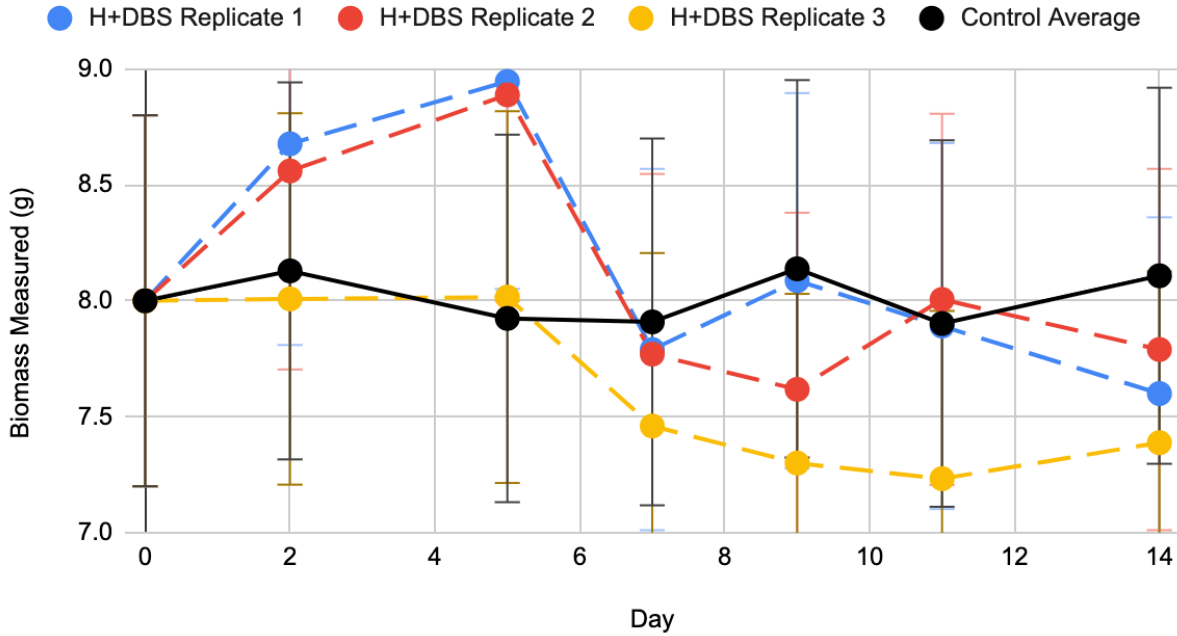


Figure 7. H+DBS Growth recorded over Trial 2 compared to the Control Group

WKG Growth with 50% Agave Nectar

A+DBS growth in trial 1 was modest but was comparatively consistent with respect to the other experimental trials conducted (Figure 8). Replicate 1 had a net growth of 1.39 g, replicate 2 of 1.55 g, and replicate 3 of 1.0 g. Averaged together, the A+DBS growth for trial 1 was 1.31 g after 2 weeks. This was the second smallest increase of biomass across all of the experimental groups, and was significantly lower than the control group.

A+DBS Trial 1 vs. Control Average Growth Trial 1

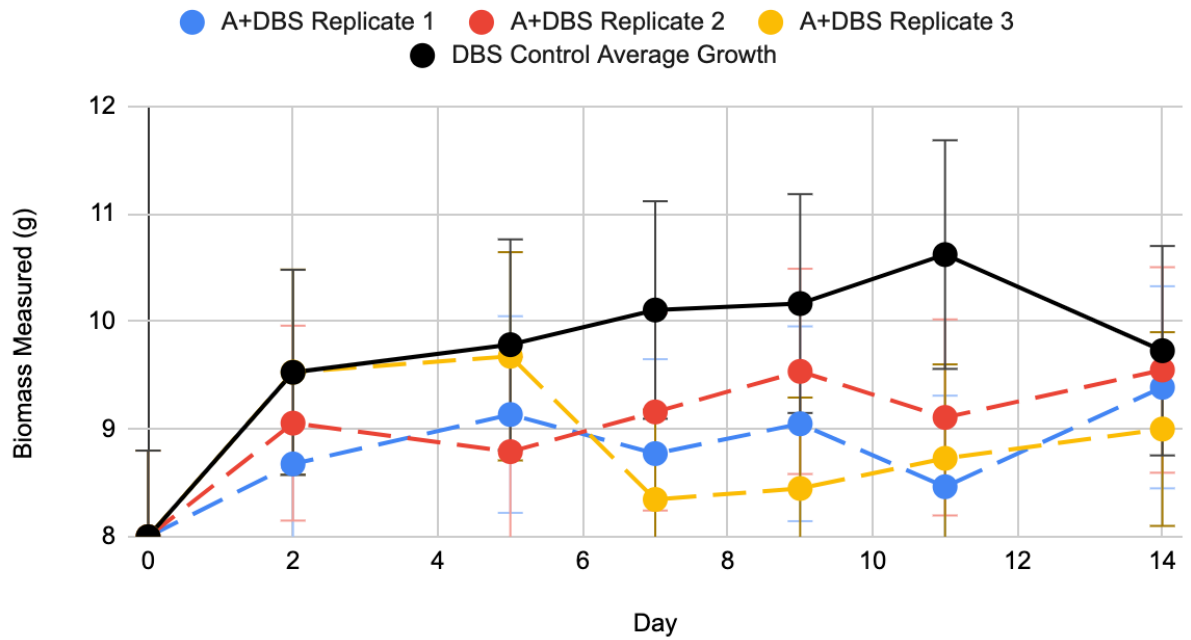


Figure 8. A+DBS Growth recorded over Trial 1 compared to the Control Group

Trial 2 for A+DBS followed like most groups from trial 2, and also resulted in a net loss in biomass (Figure 9). Trial 2 A+DBS average biomass change was a loss of 0.47 g. While none of trial 2 experimental groups yielded significant positive WKG biomass increase after 2 weeks, only H+DBS lost more biomass than A+DBS.

A+DBS Trial 2 Growth vs. Control Average Trial 2 Growth

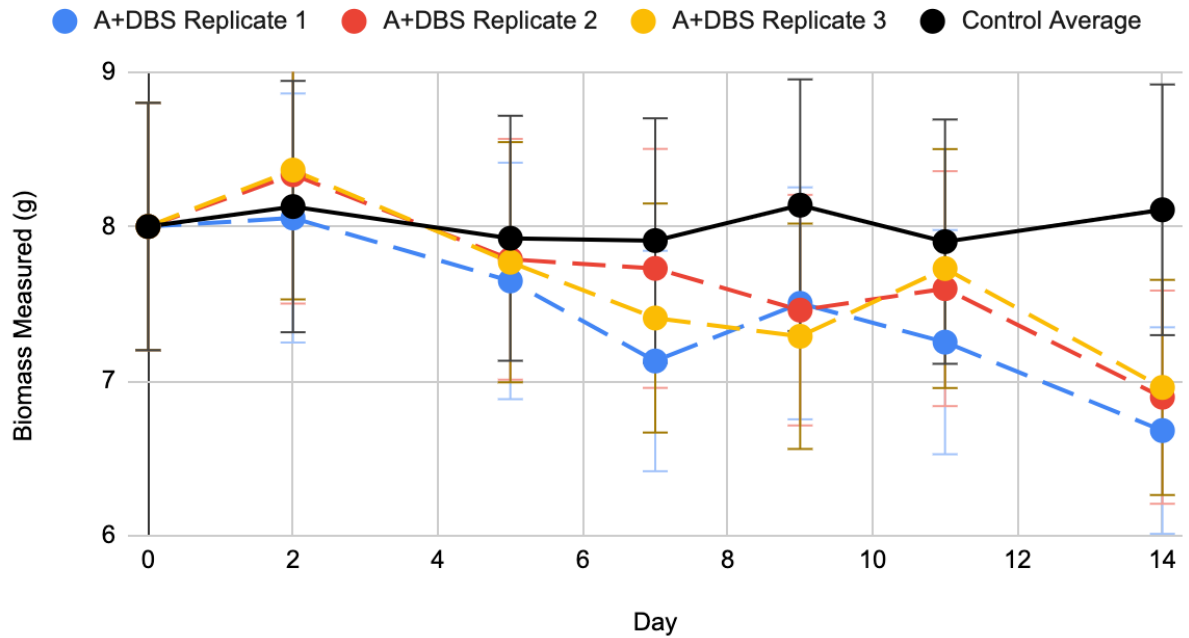


Figure 9. A+DBS Growth recorded over Trial 2 compared to the Control Group

WKG Growth with 50% Truvia

In trial 1, the growth curves of the T+DBS mediums were very similar to the control groups' growth patterns. The net increase of biomass was not quite as much as the control, but was the closest experimental group to the control, discounting MF+DBS, which exceeded the control biomass growth in trial 1 (Figure 10). This was of particular interest because I hypothesized that Truvia would have the worst effect on kefir growth because it is an artificial sweetener. Replicate 1 showed a net increase of 1.68 g after 2 weeks, replicate 2 with 1.49 g, and replicate 3 with 1.11 g. All of this growth averaged out to be a net increase in biomass of 1.42 g—only 0.30 g away from the control average growth.

T+DBS Growth Trial 1 vs. Control Average Growth Trail 1

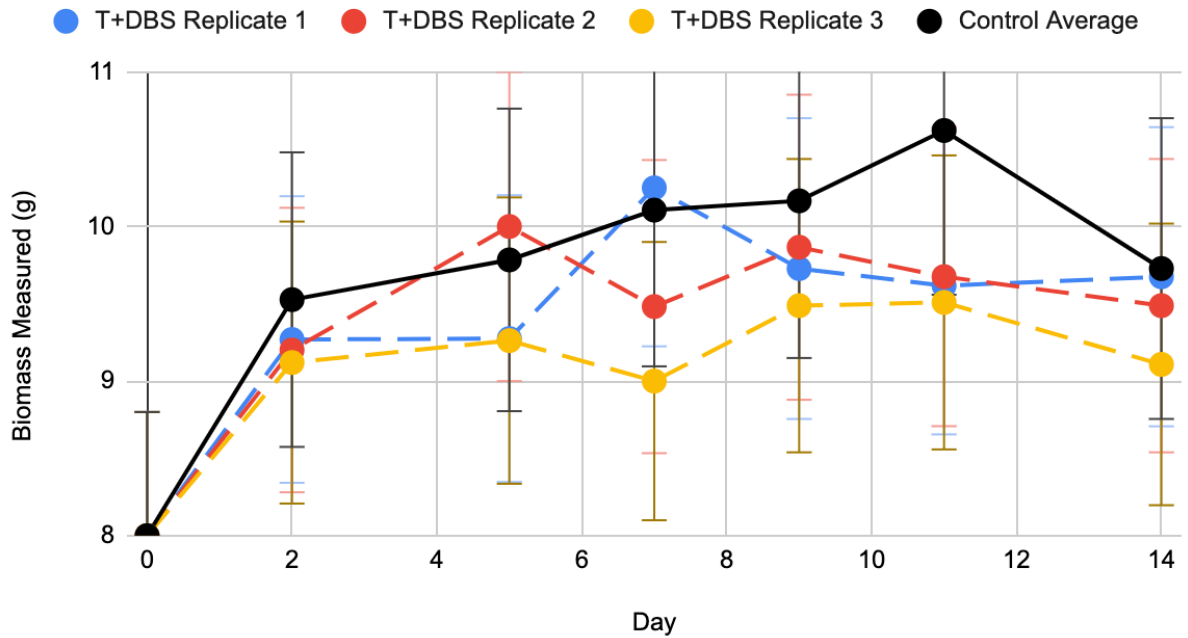


Figure 10. T+DBS Growth recorded over Trial 1 compared to the Control Group

Trial 2 for T+DBS was very inconsistent with trial 1, as T+DBS had the second worst growth rate compared to the control, only behind H+DBS (Figure 11). On average, T+DBS lost 0.58 g of biomass after 2 weeks, with replicate 1 losing 1.07 g, replicate 2 losing 0.26 g, and replicate 3 losing 0.43 g. While all of the data in trial 2 showed a net decrease in biomass, T+DBS showed the biggest change in medium effectiveness.

T+DBS Growth Trial 2 vs. Control Average Growth Trial 2

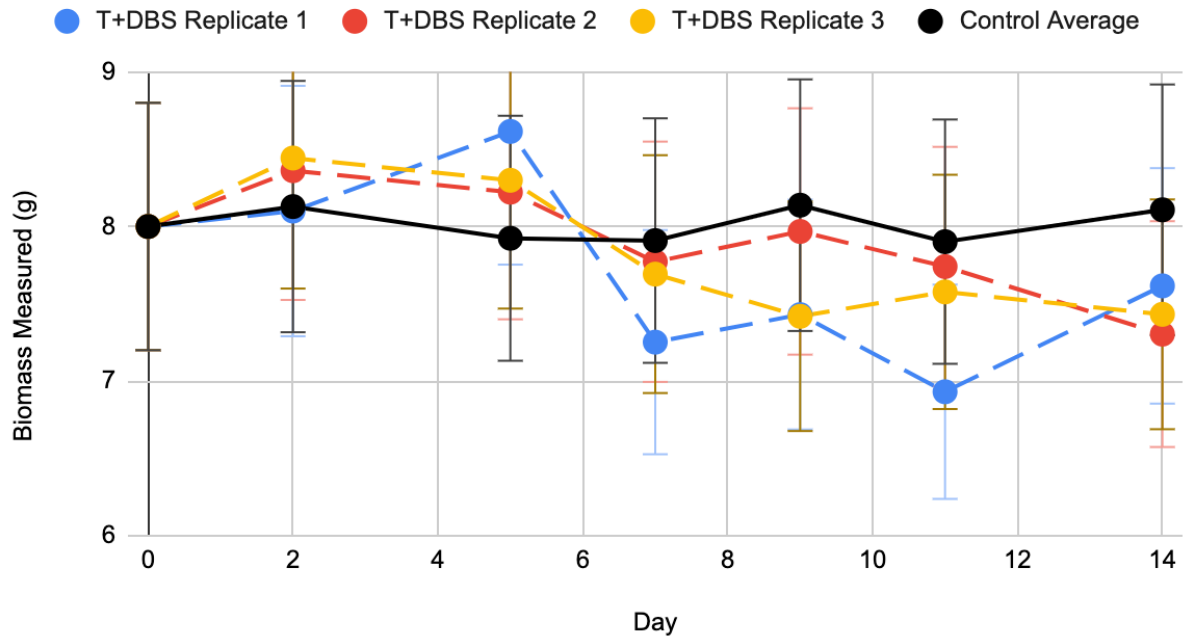


Figure 11. T+DBS Growth recorded over Trial 2 compared to the Control Group

Discussion

When designing this experiment it was decided that the alternative mediums would swap out 50% of the DBS sugar for the alternative sweetener of choice. This was chosen instead of using 100% alternative sweetener because this medium would be better for observing any inhibitory effects of the alternative sweeteners. Because of the 50% DBS in the medium, the nutrients required for kefir biomass growth would be present and any inhibitory growth relative to growth in DBS could be attributed to potentially inhibitory compounds present in the sweeteners or generally lower nutrient value compared to DBS. We reasoned that this would allow for a more clear way of determining if a decrease or increase of WKG growth was due from a lack or excess of nutrients or if it was due to boosting effects of the alternative sweeteners added into the medium.

The hypothesis that the mediums of H+DBS, and A+DBS would result in an increase of WKG biomass compared to control 100% DBS conditions was not supported by the findings of this study. While MF+DBS biomass increase in trial 1 did seem to support the idea that it could support growth as well as the control group due to its 16.6% increase, the inconsistency of trial 2 results threw this into question and more studies would be needed.

The second hypothesis that T+DBS and MF+DBS would result in less biomass increase than the control group was also not supported by the findings of this study. Trial 1 showed 17.59% less biomass increase than the control group. Trial 2 resulted in a net loss of biomass for T+DBS. Even comparing this to the net loss of the control group, the T+DBS lost 6% more

biomass than the control DBS group did. MF+DBS recorded positive growth in trial 1 of 2.01 g, but a loss of 0.18 g in trial 2.

The inconsistency of trial 2 could be due to outside factors that were not accounted for in this study. Contamination could have occurred in the master mix in between trial 1 and trial 2 that resulted in a predisposition to lose biomass. A different set of jars with a larger volume and wider sides was also used in trial 1 than trial 2 and the difference could have affected how the grains interacted, resulting in less biomass increase.

These results from this study support 100% DBS as the most effective medium for kefir growth. While MF+DBS seemed effective also, more studies would be needed in order to generate sufficient support of MF+DBS as an effective tradeoff for DBS medium. It is suggested that A+DBS, MF+DBS, H+DBS, and T+DBS do not have the appropriate nutrients or contain inhibitory compounds that compromise WKG growth with changing medium every other day for 2 weeks.

Because of the inconsistency between trial 1 and trial 2, another study of this nature would be beneficial. The results of this study suggest that H+DBS may not be a good medium for fast water kefir grain growth. While sensory effects were of interest in this study, time did not permit for any exploration on this. No sensory analysis was conducted in this study, but could be combined with the above research to explore a wider range of sensory profiles for kefir products.

In future studies of this nature, it would be beneficial to make some of these suggested changes. If possible, changing medium and measuring biomass daily would permit for a more clear idea on when exactly the grains are growing. As shown in this study, large jumps and

decreases are shown between two days and this could help draw a clearer picture of when this is happening. It would also be interesting to measure the amount of microbes in the kefir liquid that is removed from the culture. Because the grains mix with this liquid, measuring the microbes would show if any alternative sweetener encourages kefir dissociation from the grain or not. This could also help create a more clear understanding of the culture effectiveness, as measuring biomass is an approximation to microbial growth. It is also possible that the kefir growth rates could be influenced because they were previously adapted to grow on DBS. Because of this, elongating trials longer than 2 weeks would be useful to see if there is a possibility that the grains could adapt to grow on the new medium, and eventually surpass the growth rate on DBS.

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