INVESTIGATION OF PROBIOTIC PROPERTIES OF COLD BREWED AND FERMENTED COLD COFFEE BEVERAGE

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF ENGINEERING

in Food Engineering

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May 2023
İZMİR

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ACKNOWLEDGMENTS

I would first want to extend my sincere gratitude to my supervisor Associate Dr. Ayşe Handan Baysal for her guidance throughout my academic career and personal life. Her experiences and sincere support contributed a lot to my career, and she has always been helpful and has inspired me whenever I've felt unmotivated to pursue my research goal. I consider myself very fortunate to have studied for my Master's degree under her directions.

I want to express my sincere gratitude to Prof. Dr. M. Yekta Göksungur and Prof. Dr. F. Banu Özen for their assistance, for serving on my committee, and for their insightful feedback.

For their friendship and support, I would want to thank all the laboratory members, especially Damla Bulut and Şeyda Çancı.

Most importantly, I would like to take this opportunity to thank my family for their endless love, support, encouragement, and for being my pillars of strength without which it would not have been possible to achieve my endeavors. I give thanks to my brother Merthan Semiz for making sacrifices so that I may pursue a Master's degree and to my father Hüseyin Semiz for teaching me patience. I am grateful to Meral Semiz, my mother, for her guidance and for teaching me the meaning of limitless, unconditional love. I would also like to express my sincere thanks to my cousin, Sena Kızıldemir, who has always shed light and set an example for me in my chosen profession and my career plan. Finally, I want to thank my spouse Emrecan Akarsu for helping me stay grounded, always being there for me, and making me laugh even when things are tough. This thesis could not have been finished without their support, love, and tolerance.

ABSTRACT

INVESTIGATION OF PROBIOTIC PROPERTIES OF COLD BREWED AND FERMENTED COLD COFFEE BEVERAGE

The goal of the project was to produce a functional probiotic fermented cold brewed coffee that is perapered with medium roasted, grinded Colombian Arabica beans with patented probiotic yeast *Saccharomyces boulardii* that can survive in human gastrointestinal conditions. With pasteurization of the product, safe fermentation environment and long shelf life was achieved.

Coffee brew samples were prepared with different techniques to understand the effects of fermentation and glucose fortification to fermentation media on coffee chemistry, microbiota and sensory characteristic. One of the three coffee brews was only pasteurized (P), one of them fermented (F) and the other was fermented with glucose (FG). The cold brewing and fermentation were performed respectively at 22 °C and 30 °C for 24 hours. The brewed samples were pasteurized before fermentation at 65 °C for 30 minutes. Each sample was examined during their 120 days shelf life (at +4 °C).

Although the probiotic yeast enabled fermentation in coffee, it did not survive after the first week. Pasteurization was successful, so pathogen growth was not detected in any of the samples during their shelf life. While FG was evaluated as undrinkable, the other samples gave positive results in terms of sensory. Caffeine, chlorogenic acid, and ethanol levels were significantly altered during shelf life.

This is the first study in which chemical, microbiological and sensory analyzes of cold coffee that was brewed via cold immersion method, pasteurized and fermented with patented *S. boulardii* yeast during shelf-life were conducted, and will lead to new studies in this field.

ÖZET

SOĞUK DEMLENMİŞ VE FERMENTE EDİLMİŞ SOĞUK KAHVE İÇECEĞİNİN PROBİYOTİK ÖZELLİĞİNİN ARAŞTIRILMASI

Proje ile; orta kavrulmuş, öğütülmüş Kolombiya Arabica kahve çekirdekleri ile hazırlanan soğuk demlenmiş kahvenin, insan mide-bağırsak koşullarında hayatta kalabilen patentli probiyotik maya *Saccharomyces boulardii* ile fermente edilerek fonksiyonel probiyotik soğuk kahve içeceği hazırlanması amaçlandı. Pastörizasyon ile güvenli fermantasyon ortamı ve uzun raf ömrü sağlandı.

Fermantasyonun ve fermentasyon ortamına glikoz takviyesinin; kahvenin kimyası, mikrobiyal yükü ve duyusal özellikleri üzerindeki etkilerini anlamak için farklı tekniklerde kahve örnekleri hazırlandı. Üç kahve demlemesinden biri sadece pastörize edildi (P), biri fermente edildi (F) ve diğeri fermantasyon ortamına glikoz takviyesi yapılarak fermente edildi (FG). Soğuk demleme ve fermantasyon yöntemleri sırasıyla 22 °C ve 30 °C'de 24 saat süreyle gerçekleştirildi. Demlenen numuneler, fermente edilmeden önce 65 °C'de 30 dakika pastörize edildi. Her numune 120 günlük raf ömrü boyunca (buzdolabı sıcaklığında, +4 °C'de) analiz edildi.

Probiyotik maya kahvede fermantasyonu sağlasa da ilk haftadan sonra canlılığını koruyamadı. Pastörizasyon başarılı oldu, dolayısıyla raf ömrü boyunca numunelerin hiçbirinde patojen üremesi tespit edilmedi. FG örneği duyusal açıdan içilmez olarak değerlendirilirken, diğer numuneler olumlu sonuçlar verdi. Kafein, klorojenik asit ve etanol seviyeleri raf ömrü boyunca önemli ölçüde değişti.

Bu çalışma, soğuk daldırma yöntemiyle demlenmiş, pastörize edilmiş ve patentli maya olan *S. boulardii* ile fermente edilmiş soğuk kahvenin 120 günlük raf ömrü boyunca kimyasal, mikrobiyolojik ve duyusal analizlerinin yapıldığı ilk çalışma olup, bu alanda yeni çalışmalara yol gösterecektir.

Dedicated to my beloved family, who are always by my side and always encouraging me.

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LIST OF ABBREVIATIONS

P	:	Pasteurized
F	:	Fermented
FG	:	Fermented with glucose fortification
S. boulardii	:	*Saccharomyces boulardii*
S. cerevisiae	:	*Saccharomyces cerevisiae*
E. coli	:	*Escherichia coli*
L. monocytogenes	:	*Listeria monocytogenes*
C. difficile	:	*Clostridium difficile*
V. cholera	:	*Vibrio cholera*
H. pylori	:	*Helicobacter pylori*
LAB	:	Lactic acid bacteria
GI	:	Gastro intestinal
CDI	:	Clostridium difficile infection
UTI	:	Urinary tract infection
IBS	:	Irritable bowel syndrome
IBD	:	Inflammatory bowel diseases
AIDS	:	Acquired immune deficiency syndrome
spp.	:	Species
CFU	:	Colony forming unit
ND	:	Not detected
µm	:	Micrometer
mg	:	Milligram
g	:	Gram
mL	:	Milliliter
L	:	Liter
sec	:	Second
min	:	Minute
h	:	Hour
:	:	Ratio
/	:	Per

X, *	:	Multiple
%	:	Percentage
°C	:	Celsius
rpm	:	Rotation per minute
stdev	:	Standard deviation
ANOVA	:	One-way analysis of variance
DOE	:	Design of Experiment
CO_2	:	Carbon dioxide
ATP	:	Adenosine triphosphate
DNA	:	Deoxyribonucleic Acid
CGAs	:	Chlorogenic acids
CA	:	Caffeic Acid
TTA	:	Total titratable acidity
TDS	:	Total dissolved solid
EY	:	Extraction yield
PDA	:	Potato dextrose agar
MRS	:	de Man, Rogosa and Sharpe Agar
SDH	:	Succinate dehydrogenase
PGM2	:	Phosphoglucomutase-2
IMA2/3/4	:	Isomaltase-encoding geneS
NF- κB	:	Nuclear Factor kappa B
IL-8	:	Interleukin 8
HT-29	:	Human colonic epithelial
slgA	:	Immunoglobin A secretory form
lgA	:	Immunoglobin A
FAO	:	Food Agriculture Organization
WHO	:	World Health Organization
GRAS	:	Generally recognized as safe
USA	:	United States

CHAPTER 1

INTRODUCTION

The reality of the food industry is now to contribute to health with functional foods. While people cannot consume healthy foods without skipping meals on busy days, they cannot get the maximum benefit from foods and drinks, the value of consuming probiotics and prebiotics has become a necessity. The main driver in the probiotic market is probiotic fortified foods, and dominated by dairy products like kefir, yogurts, cultured milks etc. However, as veganism has become more popular and as health concerns regarding lactose intolerance, milk allergies, and cholesterol have increased, the manufacturing of probiotic products has grown to include non-dairy foods including grains, fruits, vegetables, teas, etc. By 2023, it is anticipated that sales of probiotic foods, components, and supplements, will total USD 69.3 billion, with a 7% Compound Annual Growth Rate from 2018 to 2025 (Chan, Liu and Toh 2021c). It would be advantageous to ferment coffee brews with probiotics in light of growing demand for non-dairy probiotic consumables and expanding worldwide coffee consumption trends (Chan, Liu and Toh 2020). The goal is to contribute to the immunity of the society by making coffee product, which is one of the most consumed nonalcoholic beverages in Türkiye, functional, probiotic and improving for health. The term "functional coffee" refers to coffee drinks that have been modified to provide additional functional benefits beyond those that are naturally present in coffee (Chan, Liu and Lu 2021b).

The aim of the project was to ferment of cold brewed coffee with the patented probiotic yeast *Saccharomyces boulardii* (Kelesidis and Pothoulakis 2012; Mitterdorfer and Viernstein 2001; Stewart 2020) and to prepare a functional probiotic and fermented cold coffee beverage. The coffee product prepared by fermenting with probiotic yeast will be an alternative product to probiotic drinks, which are mostly dairy products. A different production method and probiotic culture will be used than other probiotic coffees on the market. The culture will not only be added to the product; the coffee drink will be fermented with culture. The fermentation will be provided with probiotic yeast

that is resistant to human body temperature, gastrointestinal conditions, higher temperatures, low pH, and in short, more challenging conditions (Czerucka, Piche and Rampal 2007; de Paula et al. 2019; Kim et al. 2021; Pais et al. 2020) on brewed and pasteurized coffees. The fact that yeast does not produce acid and keeps the pH in balance is its distinguishing feature from most bacteria (Chan, Liu and Toh 2021c). The effects of *S. boulardii* on metabolism is due to its trophic effects, antimicrobial properties, and immunological modulations (Lazo-Velez et al. 2018), complementing the treatment of acute gastrointestinal conditions like antibiotic-associated diarrhea or chronic conditions like inflammatory bowel disease (Pais et al. 2020). Also, in order to improve the host's immune system, digestion, nutrient absorption, *Saccharomyces boulardii* works as a courier to release enzymes, proteins, and trophic factors (Buts and Keyser 2010).

Coffee is one of the most popular drinks in the world, and its popularity is growing every year. Coffee chemistry is influenced by a number of factors, including the origin of bean, growing conditions, roasting process, distribution of grind size, water chemistry, and temperature of the water used during extraction (Rao, Fuller and Grim 2020). Coffee that has been produced using low temperatures and extended brewing durations is known as "cold brew" and it has unique physicochemical and sensory properties (Bellumori et al. 2021). Coffee that has been cold brewed is typically consumed right away or after a brief period of refrigeration because of the potential microbiological problem and sensorial changes (Bellumori et al. 2021), for this reason, before fermentation the cold brewed coffees were pasteurized. Because many of the sweeter flavor components are soluble even in cold water, whilst the oils and the acids are not, extensive preparation times result in an entirely distinct flavor profile (smooth and mellow) (Kyroglou, Thanasouli and Vareltzis 2021). Hot extraction produces acidic and bitter beverages because the greater water temperature accelerates the breakdown and hot brew has a more caffeine level than cold brew (Kyroglou, Thanasouli and Vareltzis 2021) thus cold brew coffee beverage is targeting a wider consumer base.

The United States (USA) is where the cold brew method was developed, and it has spread quickly, it is unique from other varieties of coffee since it is made using a cold extraction process (Kyroglou, Thanasouli and Vareltzis 2021). Although consumers typically drink hot coffee, recent trends in northern European nations, the USA, and Japan have seen an increase in the consumption of cold coffee (Angeloni et al. 2019). In the survey conducted in Türkiye in 2021, it was determined that the coffee consumption frequency of 42.5% of the consumers was two to three cups a day and more than 82 % of

respondents consumed one or more cups of coffee each day (Dierks 2021). Turkish coffee is at the top of the most consumed (84%) and favorite (54.7%) coffee varieties (Kale 2022) due to its traditions and touristic nature. The second most popular coffee among the participants, with 41.3%, is filter coffee (Kale 2022). It indicates that the aimed product to be produced within the project will find demand.

General Manager of one of the cold coffee producers with a large share in the market in Türkiye, stated that the coffee market has a strong growth on the retail side as well, exceeding 3 billion TL in 2021 with a growth of 29% compared to 2020 (Milliyet. 2021). Consumption of cold coffee has increased, although it is a product that Türkiye is newly acquainted with and investments accelerated. Cold coffee constitutes approximately 10% of the 3 billion TL market and is the fastest growing category in the market (Milliyet. 2021).

Cold brew, fermented and probiotic ready to drink coffee product is not existing in Türkiye beverage market and there is no patent for probiotic cold coffee drink (Türkpatent n.d.). There are examples of cold coffee products fermented with probiotic microorganisms in the world, there is no patent (WIPO n.d.).

Fermentation was provided with and without sugar to observe yeast growth efficiency. Analyzes were carried out on coffee samples that unfermented pasteurized coffee that was coded as “P”, fermented “F” and fermented with sugar “FG”. A storage period of 120 days was determined and chemical, microbial, and sensory properties were monitored for 120 days (Bellumori et al. 2021, Chan, Liu and Toh 2021c). It was expected that the probiotic, sugar-free product to be produced according to the information in the literature will show good sensory properties. While the color values were expected to be close to each other, different taste was expected due to the higher alcohol production in the sample with added sugar (FG) because *S. boulardii* oxidizes and ferments sugars into CO_2, H_2O and ethanol (Lazo-Velez et al. 2018). It was expected that the taste and aroma properties of F and P samples will be close to each other and fermented coffee show better sensory properties compared to unfermented coffee. Balanced pH and no post-acidification were expected. And it was aimed that number of probiotic yeast in the final product will be at least 1×10^6 CFU/g.

CHAPTER 2

LITERATURE REVIEW

2.1. *Saccharomyces boulardii*

2.1.1. Introduction

Henri Boulardii, a French scientist, discovered the tropical yeast *S*accharomyces *boulardii* from lychee and mangosteen fruit in Asia in 1920 after noticing that people who drank tea made from tropical fruit skins did not get sick during a cholera outbreak, a potentially dangerous infection of the small intestine (Stewart 2020). It belongs to *Saccharomyces cerevisiae* species (Kaźmierczak-Siedlecka et al. 2020). A probiotic so, friendly creature called *Saccharomyces boulardii* CNCM I-745 aids in the fight against pathogenic bacteria in human gut (Kelesidis & Pothoulakis, 2012; Stewart, 2020). The Food and Agriculture Organization (FAO) and World Health Organization (WHO) defines probiotics as living microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al. 2014; Pais et al. 2020). *Saccharomyces boulardii* might be regarded as the most prominent representatives of "probiotic yeast" while not adhering to the current terminology (Mitterdorfer and Viernstein 2001). The first and only yeast that has been investigated for use as a probiotic strain in human medicine is *S. boulardii* CNCM I-745 (Kaźmierczak-Siedlecka et al. 2020; Pais et al. 2020). It has been used as a probiotic microorganism since it was discovered nearly 100 years ago, and it also has Food and Drug Administration (FDA) Generally Recognized as Safe (GRAS) status (Bagherpour et al. 2018; Douradinha et al. 2014). *S. boulardii* differs from bacterial probiotics that are prokaryotes since it is a member of the class of simple eukaryotic cells, which includes fungi and algae (Czerucka, Piche and Rampal 2007). *S. boulardii* are approximately 10 μm long by 5 μm wide oval cells with thick-

walled cells (Ta 2010). *S. boulardii* does not go through sporulation (Du et al. 2012), cannot make ascospores that the sexual propagules of ascomycetes and are necessary to describe the majority of fungus (Pitt and Hocking, 1997) and cannot turn haploid (Pais et al. 2020) but produces biofilms (Chan and Liu 2022b).

2.1.2. Pharmaceutical Use

The yeast is traditionally used orally for medicinal purposes; it is a probiotic and a dietary supplement that is meant to be used by the general public who are in good health (Lazo-Vélez et al. 2018). There are a large number of pharmaceuticals (capsules, powders, tablets, and pellets) on the market that include yeast cells and these preparations are primarily sold through pharmacies and health food stores (Mitterdorfer and Viernstein 2001). In the past 30 years, the nonpathogenic yeast *Saccharomyces boulardii* has been recommended for both prevention and treatment of bacterial diarrheal illnesses (Kelesidis and Pothoulakis 2012). *S. boulardii* is also known to not be negatively impacted by antibiotics, inhibited by them, or change or negatively impact the usual microbiota (Czerucka, Piche and Rampal 2007; de Paula et al. 2019). It has demonstrated clinical and experimental benefit in digestive illnesses with a predominant inflammatory component, indicating that this probiotic may interfere with cellular signaling pathways prevalent in many inflammatory conditions (Kelesidis and Pothoulakis 2012). In intestinal treatments approximately 250 mg *S. boulardii* supplement is taken several times a day with antibiotics (RxList 2021). *S. boulardii* is the most potent probiotic for preventing or treating antibiotic associated diarrhea and recurrent *Clostridium difficile* infection (CDI) through a variety of mechanisms, either alone or in combination with other medications (Kelesidis and Pothoulakis 2012; Vandenplas, Brunser and Szajewska 2009).

Also, due to the registered cases of fungemia, the efficacy of *S. boulardii* as a biotherapeutic agent has been questioned, up to the present, nearly 100 cases of *S. boulardii*-associated fungemia have been reported and the origin of the fungemia is believed to be either a digestive tract translocation or a contamination of the central

venous line by the colonized hands of medical personnel (Kelesidis and Pothoulakis 2012).

Normal yeast elimination takes place 14 days after supplementation is stopped and does not colonize the digestive tract (Lazo-Velez et al. 2018; Pais et al. 2020). While modeling of the colon environment revealed the yeast is unable to colonize the colon but had an individual-dependent effect on the microbiotic profile, dynamic modeling of the stomach and small intestine conditions revealed *S. boulardii* to be resilient to gastric and lower intestinal conditions (Pais et al. 2020). However, after a single dose, it has been demonstrated to colonize the intestine of gnotobiotic mice (Pais et al. 2020). *S. boulardii* reaches steady-state colon concentrations in 3 days and is eliminated from the body 2–5 days later (Czerucka, Piche and Rampal 2007). Pharmacokinetic studies show that *S. boulardii* disappears from the colon three to five days after consumption is stopped (Pais et al. 2020).

2.1.3. Target Consumer

Saccharomyces boulardii is suitable for vegans, vegetarians also for Kosher and Halal diets (G&G Vitamins n.d.). The use of *S. boulardii* during pregnancy or lactation is recommended that healthcare professionals be consulted when evaluating its use because there is not enough reliable information about the safety of taking *S. boulardii* during pregnancy or breast feeding (Elias, Bozzo, and Einarson 2011) When taken by mouth properly, it may be safe for kids, but before using it, a doctor should assess whether or not a child's diarrhea is safe (Vandenplas, Brunser and Szajewska 2009). There is some concern that those who take *Saccharomyces boulardii* may be more susceptible to getting fungemia, a yeast infection that spreads to the circulation and the rest of the body, if they are critically ill, have a compromised immune system, or are using immune-altering medications (RxList 2021). Products containing *Saccharomyces boulardii* may cause an adverse reaction in those with yeast allergies, thus it should not be used (Lazo-Velez et al. 2018; *Saccharomyces boulardii* 2021). Also, *S. boulardii* has been contraindicated using in patients who are immunocompromised, have a central venous catheter in place, and are in poor health (Lazo-Velez et al. 2018).

2.1.4. Differences Between *S. boulardii* and *S. cerevisiae*

Saccharomyces spp. strains are facultative anaerobic bacteria so it is typically referred to as anaerobe, meaning that it can grow in both anaerobic and aerobic environments (Kim et al. 2021). Despite being genetically related to *S. cerevisiae* and sharing a similar genotype, the round and oval-shaped budding yeast known as *S. boulardii* is often referred to as separate species within the genus *Saccharomyces* (Pais et al. 2020). There are no apparent major benefits to human health that the baker's yeast *Saccharomyces cerevisiae* appears to offer (Pais et al. 2020), despite being identical to *S.accharomyces cerevisiae*, probiotic *S. boulardii* has probiotic and bio-therapeutic characteristics that set it apart (Ta 2010). According to average nucleotide identity, *S. boulardii* CNCM I-745 has more than 99% more genomic similarity to non-probiotic *S. cerevisiae* strains but there are some genetic differences between *S. cerevisiae* and *S. boulardii* (Kaźmierczak-Siedlecka et al. 2020). The greater rate of acetic acid generation in *S. boulardii* compared to *S. cerevisiae* is associated with antibacterial action and has been connected to altered gene copy number and mutations in the SDH1 and WHI2 genes (Kaźmierczak-Siedlecka et al. 2020; Pais et al. 2020). By creating stress response proteins, ribosomal proteins, kinases, transporters, and fluoride exports, *S. boulardii* may be better able to adapt to harsh environments as a result of the majority of its genes being duplicated or triplicated (Kaźmierczak-Siedlecka et al. 2020).

2.1.4.1. Carbon Source

S. boulardii has also been distinguished as a separate genus from *S. cerevisiae* since it cannot digest galactose (Du et al. 2012); despite having all of the genes necessary for galactose absorption and fermentation, *S. boulardii* cannot utilize galactose (can be assimilated but not fermented) as a carbon source (Pais et al. 2020). The ineffective utilization of galactose was also linked to a mutation in the PGM2 gene, the inability of *S. boulardii* to utilize palatinose may be due to the loss of three isomaltase-encoding genes (IMA2, IMA3, and IMA4), which are essential for the uptake and metabolism of

palatinose (Pais et al. 2020). Many enzymes are activated by yeast to carry out the full metabolic process, which involves the breakdown of glucose and fructose into pyruvic acid, which is subsequently decarboxylated to produce acetaldehyde and CO_2 through glycolysis; acetaldehyde is then further transformed to alcohol (Lazo-Velez et al. 2018). Also, it uses prebiotics that lactulose, fructooligosaccharides etc. to modify gut microbiota (Kaźmierczak-Siedlecka et al. 2020).

S. boulardii absorbed a variety of carbon and nitrogen sources, including glucose, fructose, mannose, maltose, sucrose, lactose and galactose, sucrose, trehalose, glycerol, raffinose, starch, ammonium sulfate, urea, potassium nitrate, and peptone (Du et al. 2012; Lazo-Velez et al. 2018).

2.1.4.2. Growth Conditions and Stability

S. boulardii and *S. cerevisiae* are very different from one another in terms of metabolism and physiology (Czerucka, Piche and Rampal 2007); *S. boulardii* is more resistant to temperature and acidity stressors (Kim et al. 2021; Pais et al. 2020). It is a thermotolerant yeast that grows best at 37°C, which is the physiological temperature of the human host (Czerucka, Piche and Rampal 2007). *S. boulardii* retains 65% viability after one hour at 52°C (Pais et al. 2020). *S. boulardii*'s thermal death temperature was 55–56°C and that 20% alcohol was the greatest quantity it could take (Du et al. 2012).

It is a distinct probiotic and bio-therapeutic yeast that has been shown to survive in gastric acidity (de Paula et al. 2019). Low pH commonly causes the cytosol to become acidified, which damages cellular components (such as proteins and cell membranes) and inhibits metabolic processes, ultimately leading to cell death (Chan, Liu and Toh 2020). The lowest pH at which *S. boulardii* could survive is pH 2.0; as a result, its high tolerance to lower pH levels protects it from gastric acid as it passes through the digestive tract and takes effect as a probiotic in the gut (Du et al. 2012) and it seems to be more resilient than the *S. cerevisiae* strain (Czerucka, Piche and Rampal 2007; Kim et al. 2021). The presence of bile and pancreatic juice as well as the short transit time in the duodenum create a hostile environment as the pH rises of the GI tract (Czerucka, Piche and Rampal 2007). Pais et al. (2020) showed that *S.boulardii* was still alive after exposure to artificial gastric

juice containing pepsin and hydrochloric acid, and was also resistant to the effects of bile salts of probiotics. The comparison of *S. boulardii* and *S. cerevisiae* was given in Appendix A.

The yeast is an excellent option for probiotics since probiotics entering the GI system must be resistant to local stresses such the presence of GI enzymes, bile salts, organic acids, and major fluctuations in pH and temperature (Czerucka, Piche and Rampal 2007).

2.1.5. Health Effects of *S. boulardii*

During its intestinal transit, *S. boulardii* functions as a courier to liberate enzymes, proteins, and trophic factors to enhance the host's immune system, digestion, and nutrient absorption (Buts and Keyser 2010). It has a positive impact on food's nutritional value; phytates and other anti-nutrients are eliminated and folate is synthesized by the yeast (Lazo-Velez et al. 2018). A few of the mechanisms of action that have been identified that affect both the host and pathogenic microorganisms include inhibiting pathogens' capacity to colonize and infect the mucosa, managing local and systemic immune responses, stabilizing the function of the gastrointestinal barrier, and inducing enzymatic activity that promotes absorption and nutrition (Kelesidis and Pothoulakis, 2012; Moré and Swidsinski 2015; Pais et al. 2020). However, the genetic basis and mechanistic details are not fully understood yet (Pais et al. 2020).

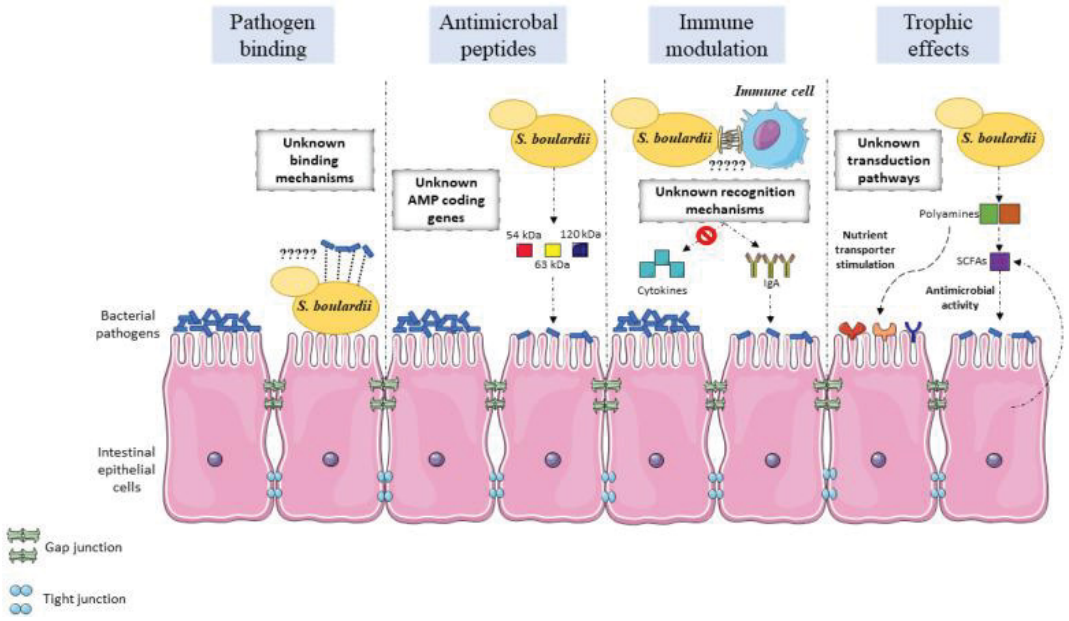

Figure 2. 1. Primary mechanisms of *S. boulardii* probiotic activity in gut epithelium (Source: Pais et al. 2020)

Among the illnesses for which *S. boulardii* is given include lactose intolerance, urinary tract infections (UTIs), vaginal yeast infections, high cholesterol, hives, and teenage acne (RxList 2021). *S. boulardii* can release the polyamines spermine and spermidine to control gene expression, which in turn controls protein synthesis (Buts and Keyser 2010).

S. boulardii capacity's to reduce post-acidification in coffee brews, either by lowering citric acid levels or preventing lactic acid formation, may lessen acid stress for Lactic acid bacteria (LAB) as well as for itself (Chan, Liu and Toh 2021c). It has been demonstrated that yeasts can increase the survivability of probiotic LAB in a variety of dietary systems, including milk, goat yogurt, and grape juice (Chan, Liu and Toh 2021c).

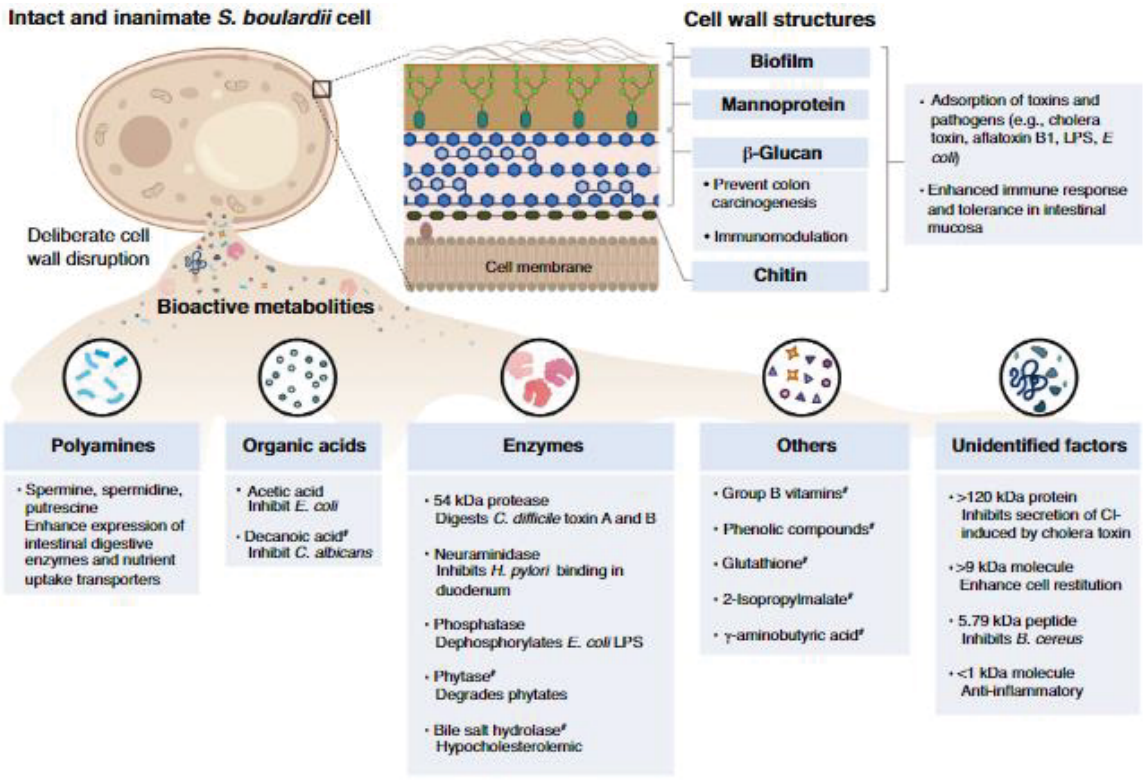

Figure 2. 2. Various bioactive metabolites produced by *S. boulardii*

(Source: Chan and Liu 2022b)

It was discovered that *S. boulardii* inhibits inflammation via modifying pro-inflammatory gene expression and host cell signaling. As illustrated in Figure 2.3, in particular, NF- κB activation and NF- κB-mediated IL-8 gene production in the intestinal epithelial cells are able to be blocked by probiotic yeast, reducing inflammation (Sougioultzis et al. 2006).

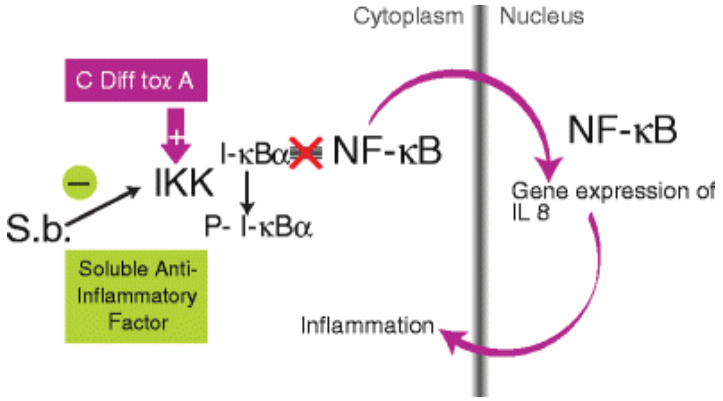

Figure 2. 3. NF-κB inflammation pathway blocked with anti-inflammatory factor secreted by *S. boulardii* (Source: Vandenplas, Brunser and Szajewska 2009)

T cells that gather in the lymph nodes infiltrate the large intestine and occasionally the small intestine, causing inflammation; by regulating and reducing T cell infiltration in the colon, *S. boulardii* lowers inflammation (Dalmasso et al. 2006). By specifically altering the migratory behavior of T cells, which assemble in mesenteric lymph nodes, *S. boulardii* has a special effect on inflammation and it prevents the synthesis of pro-inflammatory cytokines (Dalmasso et al. 2006). Findings imply that the use of *S. boulardii* may be helpful in the management of inflammatory bowel disease. In order to determine whether the inhibitory activity performed by *S. boulardii* was due to a toxic effect, it was investigated whether *S. boulardii* affected cell viability in Human colonic epithelial (HT-29) colonocytes and it was found that *S. boulardii* did not adversely affect cell viability (Sougioultzis et al. 2006). Due to their capacity to stimulate the immune system, the yeast cells are a great source of β (1,3) d-glucan, a component of fungal walls that serves as "biological response modifiers" (Moré and Swidsinski 2015). *S. boulardii* appears to have a beneficial effect on amebiasis, giardiasis, and *Blastocystis hominis* infection, despite the fact that its potency against protozoal illnesses is poorly understood (Kelesidis and Pothoulakis 2012).

2.1.5.1. Gut Microbiome and Epithelium

In a healthy intestine, a thick layer of mucus separates the intestinal wall from the highly populated intestinal contents, which include approximately 10^{12} bacteria/g of feces in the colon (Moré and Swidsinski 2015). Various causes, such as antibiotic use or surgical procedures, can have detrimental effects on the gut microbiome and inhibit defensive function in the host epithelial lining (Pais et al. 2020). In the brush border of the microvilli, *S. boulardii* increases the production of glycoproteins (Vandenplas, Brunser and Szajewska 2009). Release of polyamines, which drive intestinal cell repair and colonic mucosa synthesis, as well as an increase in the formation of short-chain fatty acids and enzymes that hydrolyze various disaccharides, are some of the methods by which *S. boulardii* produces its therapeutic effects (Lazo-Velez et al. 2018; Moré and Swidsinski 2015). *S. boulardii* has natural qualities that enable it to protect the host mucus layer and mucosa further while setting that is advantageous for the development of the healthy gut microbiota (Moré and Swidsinski 2015); indirectly or directly improves the environment of the usual gut microbiota, strengthens the intestinal immune system and anti-microbial activity, thereby improving intestinal function (Chan et al. 2021a; Chan, Liu and Toh 2021c).

Beneficial bacteria in the human gut are harmed by infections, but research indicates that *S. boulardii* can aid in their recovery so they can resume their productive roles in creating crucial short-chain fatty acids that maintain the gut lining (Stewart 2020). The most significant changes in the fecal composition caused by *S. boulardii* include a rise in the number of bacteria that produce short chain fatty acids (Moré and Swidsinski 2015). The primary microbial population is known to collapse in a dysbiosis that unfavorable dysbalance of the intestinal microbiota, such as during diarrhea, and this gap typically results in temporary increases in pioneer-type bacteria; treatment with *S. boulardii* in dysbiosis accelerates the formation of a healthy microbiome, as shown by numerous human and animal models (Moré and Swidsinski 2015).

2.1.5.2. Gastrointestinal Diseases

S. boulardii is beneficial in enhancing the treatment of digestive disorders (such as antibiotic-associated diarrhea), whether they are chronic or acute (bowel disease) (Pais et al. 2020), Crohn's disease (Sougioultzis et al. 2006); it was investigated for clinical efficacy and it had shown that significant efficacy in preventing them (Kelesidis and Pothoulakis 2012). *S. boulardii* is prescribed for illnesses like irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) (such as ulcerative colitis), lyme disease, recurrent *Clostridium difficile* colitis, and bacterial overgrowth in short bowel syndrome (RxList 2021). *S. boulardii* also is effective at curing diarrhea in patients with AIDS-related diarrhea; after one week of treatment, 61% of the patients were clear of diarrhea (Czerucka et al. 2007; de Paula et al. 2019).

2.1.5.2.1. Pathogens and Toxins Mechanisms

The many advantages of *S. boulardii* come from its ability to prevent intestinal infections of pathogens and their toxins, which often result in diarrhea and inflammation (Stewart 2020). The primary causes of diarrhea and colitis are *C. difficile* Toxins A and B, which are known to be inhibited by *S. boulardii* proteases (Lazo-Velez et al. 2018). A 308-kDa cytotoxin and enterotoxin called toxin A causes mucosal damage, fluid discharge, and severe intestinal inflammation and a 270-kDa protein called toxin B is cytotoxic to mammalian cells and causes the production of inflammatory cytokines that from monocytes (Qamar et al. 2001). The ability of the yeast to stop *C. difficile* recurrent colitis and diarrhea may be directly linked to an increase in gut antitoxins A levels in mice treated with *S. boulardii* (Qamar et al. 2001). Additional health mechanisms used by *S. boulardii* include neutralizing the toxins produced by *C. difficile*, *V. cholera*, and *E. coli*, producing inhibitory molecules, altering the cellular pathways of enterocytes, suppressing the production of inflammatory molecules, and increasing levels of secretory immunoglobin A (IgA) (Douradinha et al. 2014). It can activate lgA responses to *Clostridium difficile* toxin A and greatly increases intestinal immunoglobin A secretory

form (slgA) levels, respectively (Kaźmierczak-Siedlecka et al. 2020). Since slgA release is the first line of defense against pathogens in the intestine, it is important to stimulate slgA after *S. boulardii* administration and this may be a key mechanism for *S. boulardii*-mediated protection against diarrheal disorders (Kaźmierczak-Siedlecka et al. 2020).

Qamar et al. (2001) showed that by promoting the release of the antibody IgA, which is effective against pathogenic bacteria in the digestive and respiratory systems, *S. boulardii* reduced symptoms of intestinal damage and inflammation caused by a variety of pathogens in the rat jejunum. Several clinical investigations have demonstrated the effectiveness of the probiotic yeast *S. boulardii* in treating acute infectious diarrhea and preventing diarrhea brought on by antibiotic use (Chan, Liu and Toh 2021c).

E. coli O55B5 endotoxin is inhibited by yeast cells secreting enzymes such as sucrase, a-atrehalase, leucine-aminopeptidase, and a new protein phosphatase into the intestinal lumen (Buts and Keyser 2010). 24 kids between the ages of 36 and 192 months participated in a study using commercial supplements containing 5 billion *S. boulardii* colony-forming units (CFU) and the number of *Escherichia coli* (*E. coli*) colonies detected in stools samples of the patients decreased significantly (Akil et al. 2006).

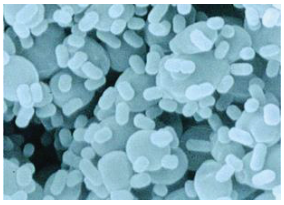

Figure 2. 4. Adherence of *E. coli* 0157:H enterohemorrhagic bacteria to *S.boulardii* surface (electron microscopy, X 5,000) (Source: Vandenplas, Brunser and Szajewska 2009)

By directly attaching to intestinal pathogens such *C. difficile*, *V. cholera*, *Salmonella*, *Shigella*, and pathogenic *E. coli*, it has a wide range of advantageous effects (Douradinha et al. 2014). It also helps eliminate germs, prevents relapses, lessens unpleasant reactions, and lessens treatment-associated diarrhea in patients with *Helicobacter pylori* (*H. pylori*) (Pais et al. 2020).

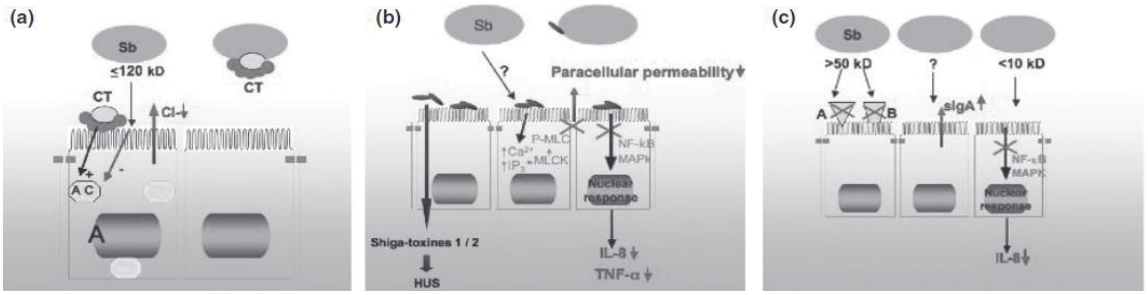

Figure 2. 5. Proposed model for the mechanism of action of *Saccharomyces boulardii* against *Vibrio cholerae* (a), *Clostridium difficile* (b) and pathogenic *E. coli* (c) (Source: Czerucka, Piche and Rampal 2007)

2.1.6. Using *S. boulardii* in Fermentation

S. boulardii differs from other probiotic bacteria by carrying the features described above such as being resistant to the stomach and intestinal environment, matching the optimum growth temperature with human body temperature, maintaining its viability at high temperatures, being viable at low pH, and it is considered advantageous. Functional fermented foods fermented by *S. boulardii* can produce bioactive chemicals (Lazo-Vélez et al. 2018; Ryan et al. 2011). Yeast is commonly used in the manufacturing of fermented drinks due to its potential to turn carbohydrates into alcohol and CO_2; radish, carrot, and beet juices that have been fermented with *S. boulardii* have been found to be rich in phenols and have a high antioxidant activity (Lazo-Vélez et al. 2018). It can produce tasty compounds such sulphur lactones, higher fusel alcohols, organic acids, aldehydes, ketones, esters, and ketone esters from sugars, peptides, and amino acids (Lazo-Vélez et al. 2018). Also, in order to enhance livestock productivity, *S. boulardii* is used as a feed additive and biological therapeutic agent (Kim et al. 2021), however, although its use in animal feeds is extensive, it has not been widely used in functional products for the processing of human food (Lazo-Vélez et al. 2018). In recent analyses evaluating *S. boulardii*'s technical application in foods, along with its genetic and phenotypic characteristics, health advantages, and mechanisms of action, these distinct physiological qualities have put *S. boulardii* at the forefront (Chan and Liu 2022b).

The most important point is that the probiotic microorganism maintains its viability at a certain rate during the planned shelf life of the probiotic food or beverage. Probiotic viability is the primary technological factor to be taken into account while creating probiotic products, probiotics must continue to be present in beverage at an effective dose throughout production and the product's shelf life in order to provide the claimed health benefits (Chan and Liu 2022a). According to the Turkish Food Codex (2006), there should be 1.0×10^6 CFU/g probiotic microorganisms in functional probiotic products, so the minimum amount of *S. boulardii* required in the final product 1.0×10^6 CFU/g (Chan, Liu and Toh 2021c; Pais et al. 2020).

There are several other *Saccharomyces* products that are sold commercially as probiotics; *S. boulardii* is typically offered in lyophilized form, which is stable at room temperature, advantageous for mobility, and maintains high viability numbers for extended periods of time (Lazo-Vélez et al. 2018) or heat-dried capsules (Kelesidis and Pothoulakis, 2012). Moreover, the probiotic yeast is easily and cheaply generated in large amounts (Douradinha et al. 2014).

2.2. Coffee

2.2.1. Introduction

Coffee is one of the most popular non-alcoholic beverages in the world and is an important plantation crop farmed in more than 50 developing countries (de Melo Pereira et al. 2014). Approximately 60 tropical and subtropical nations produce a lot of coffee, with several of them using it as their primary agricultural export (Esquivel and Jimenez 2012). By using a variety of techniques, coffee beans are removed from the fruit, dried, ground, and utilized to make coffee drinks.

2.2.2. Coffee Health Effects

Coffee use has been linked in reverse to total mortality, depression, diabetes mellitus, and several cancer types (Fuller and Rao 2017). It inhibits the growth and spread of cancer cells is one of the most notable benefits of coffee; an animal model published in the journal Anticancer Research found that after just 10 days, coffee extract was able to significantly reduce tumor growth in mice by almost 54 % (Ajmera, 2020).

Coffee increases motility, which can enhance digestion and excretion, by causing the muscles in the colon to move more quickly. This encourages peristalsis, which results in bowel motions (Pratt 2020), and its use in probiotic products is also promising in this respect. Also, coffee has an antimicrobial effect; a number of microorganisms, including pathogens, can be inhibited by the antibacterial compounds found in coffee (Nonthakaew, Matan and Aewsiri 2015). Flavonoids, chlorogenic acids (CGAs), caffeic acid (CA), trigonelline, caffeine, and protocatechuic acid play a vital role as potential natural antibacterial agents against intestinal bacteria (Rawangkan et al. 2022). It has an effect against *Streptococcus mutans*, intestinal pathogens and it significantly inactivates and inhibits the growth of *E. coli* O157:H7 (Akhlaghi et al. 2019; Nonthakaew, Matan and Aewsiri 2015). It exhibits antibacterial activity against both gram positive (as *Staphylococcus aureus* and *Bacillus cereus*) and gram negative microorganisms (*Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumonia*) (Nonthakaew, Matan and Aewsiri 2015). It has been claimed that caffeine prevents mold and germ growth (Nonthakaew, Matan and Aewsiri 2015).

2.2.3. Coffee Composition

A large number of phytochemical components, flavonoids (catechins and anthocyanins), caffeic and ferulic acid are the most important functional components found in coffee and other active compounds are nicotinic acid, trigonelline, quinolinic acid, tannic acid, pyrogallic acid (Esquivel and Jimenez 2012; Hasni et al. 2021). Coffee beans are rich in essential oils and one well-known health advantage of coffee drinking

is its high antioxidant capacity (Kang et al. 2020) and it contains rutin and beneficial antioxidants such as chlorogenic, protocatechic and gallic acids and polyphenols that help optimize health and avoid disease, it contains enough antioxidants in each serving (Liang and Kitts 2014; Ajmera, 2020). Antioxidants are chemicals that can resist free radicals and protect the biological systems of humans from damage brought on by activities or reactions that result in excessive oxidation (Hasni et al. 2021). 0.15-0.30 mg/g of total antioxidants are present in coffee (Sualeh, Tolessa and Mohammed 2020). Phenolics, one of the main bioactive compounds, have a high antioxidant effect and slow the oxidative process of coffee oil rich in essential fatty acids, including a high amount of linoleic acid (Fernandes et al. 2022). Also, coffee contains melanoidins that displayed increased antioxidant activity (Esquivel and Jimenez 2012; Kyroglou, Thanasouli and Vareltzis 2021). To extract an antioxidant molecule from coffee, process variables such as brewing time, water temperature, (Asiah et al. 2019) and roasting & grinding degree are important (Hasni et al. 2021).

Carbohydrates in coffe improve the organoleptic quality, reduce the risk of colon cancer, and have various biological activities (Tian et al. 2017). Coffee contains oligosaccharides, certain proteins and minerals (Esquivel and Jimenez 2012). The majority of the oligosaccharides discovered were hexoses, which may also include galactooligosaccharides and manno-oligosaccharides and contain a heterogeneous blend of glucose, arabinose, xylose, and rhamnose (Tian et al. 2017). The oligosaccharides have prebiotic activity that is selective for certain strains that can break down glycosidic linkages and use them as a source of carbon; the kind and origin of the beans, the level of roasting, and the brewing conditions all affect how much oligosaccharides are transferred into the brewed coffee (Tian et al. 2017). Probiotics and prebiotic substrates working in concert would increase yeast survival and boost the effectiveness of helpful bacteria.

2.2.3.1. Caffeine in Coffee

Coffee contains caffeine and CGAs, which have antioxidant properties on roasted coffee (Esquivel and Jimenez 2012; Ajmera 2020), they are the main bioactive components of coffee drinks and have biological effects, affecting flavor and aroma

(Bellumori et al. 2021). In brewed coffee caffeine associated with 10% of its properties and bitterness.

The caffeine is best extracted with hot brew methods thus hot brew has a much higher caffeine concentration than cold brew for the same coffee-to-water ratio (da Silva Portela et al. 2021; Kyroglou, Thanasouli and Vareltzis 2021) but there are some studies that states the opposite (Fuller and Rao 2017).

2.2.3.1.1. Caffeine Digestion and Absorption

The lining of the mouth, throat, esophagus, and stomach allow caffeine to pass through epithelial tissue and into the bloodstream. The membranes and organs absorb 99% of the caffeine after 45 minutes, and peak plasma concentration is reached between 15 and 120 minutes later (Kallmyer 2019). Depending on the metabolic rate, the residual caffeine metabolites are then removed from the body by the kidneys and excreted in the urine (Kallmyer 2019). Theophylline, theobromine, and paraxanthin are broken down by the molecule as soon as it reaches the liver, which then starts to digest it (Echeverri et al. 2010). Not all caffeine is promptly metabolized by the liver; some free caffeine is still present in the bloodstream and some reaches the brain (Kallmyer 2019). Adenosine, a neurotransmitter, and the caffeine molecule have a similar structure; due to their structural similarity, caffeine molecules can attach to adenosine receptors in the brain and prevent them from responding to adenosine (Kallmyer 2019). The sleep-wake cycle is influenced by adenosine; it alerts the brain that it is time for rest or sleep when it attaches to enough receptors, while caffeine cannot replace the need for sleep, it might conceal weariness because adenosine is no longer able to function as it should (Kallmyer 2019). When caffeine blocks adenosine, more dopamine, the "feel-good" neurotransmitter, is released, which results in an increase in sensations of happiness and well-being (Kallmyer 2019). Moreover, caffeine is a xanthine that has a variety of effects on the vascular wall, especially the endothelium and smooth muscle cells (Echeverri et al. 2010). It is also known to have an impact on arterial pressure and the autonomic nervous system; regular use may cause tolerance to develop (Echeverri et al. 2010). Caffeine's impact on the cardiovascular system has been the subject of numerous studies, all of which have shown

conflicting results. While some studies have shown that caffeine use raises cardiovascular risk, others have suggested that it has a positive or neutral impact on the same (Echeverri et al. 2010). The cardiovascular reaction to this chemical depends on a number of variables, including the quantity consumed, the timing of consumption, the frequency, the degree of absorption, etc. and all of which affect how each person reacts to caffeine differently.

2.2.3.1.2. Caffeine Side Effects

In addition to the positive effects of caffeine, some people may be more sensitive to its harmful effects. These people include young adults, pregnant and breastfeeding women, children and teenagers, as well as people with other health conditions including underlying heart or mental illness (Temple et al. 2017). Caffeine use up to 400 mg per day for a healthy adult has no effect on overall toxicity, cardiovascular effects, bone status, or calcium balance. Up to 400 mg of caffeine per day is healthy (almost as much as 5 cups of coffee) (Pratt 2020). However, it is recommended that women and children consume no more than 300 mg daily (Nonthakaew, Matan and Aewsiri 2015). The FDA has stated that toxic effects have been observed with rapid consumption of approximately 1,200 mg of caffeine (U.S. Food and Drug Administration [FDA] 2018). FDA recommends reducing caffeine intake in pregnant women and for children and The American Academy of Pediatrics advises against children and teenagers consuming caffeine and other stimulants (FDA 2018).

2.2.4. Coffee Bean Types, Arabica Coffee Beans

Coffee beans are grown in about 80 countries such as South and Central America, Africa, the Caribbean, Asia etc. and all coffee varieties are variations of the four main beans; Arabica, Robusta, Liberica and Excelsa; each variety has a distinct flavor profile and needs a different environment to grow well (Caya et al. 2020; Clark 2022). The taste

of Liberica beans is more sooty than the others, it is not preferred much, and because of their complex flavors, excelsa that is a subspecies of Liberica and Liberica are frequently utilized in coffee blends (Nair 2021). Coffee Arabica and Coffee Robusta are the two main species used for making commercial coffee and the two type of beans exhibit different morphological and compositional traits, which are related to how different compounds are extracted from each matrix when making coffee (da Silva Portela et al. 2021). Espresso, instant coffees, and other types of ground coffee are produced by Robusta beans (Clark 2022). The most machinable bean is Arabica and it dominates global production; represents approximately 64.5% of global coffee production and is the most popular type (Collazos-Escobar et al. 2022; Sualeh, Tolessa and Mohammed 2020), followed by Robusta. Coffee made from Arabica beans has a more complex flavor profile (Clark 2022), when compared to the harsh and bitter flavor of Robusta, most people believe that Arabica coffee is of higher quality and taste (Moreno 2021). Compared to Arabica coffee beans, these Robusta beans are less fruity, less sweet woodier, lower in acidity and more bitter (Clark 2022). When coffee is brewed, its acidity, which comes from the carboxylic acid group in coffee beans and comprises formic acid, acetic acid, oxalic acid, citric acid, lactic acid, malic acid, and quinic acid, gives it a sour flavor (Asiah et al. 2019).

Compared to Arabica coffee, Robusta coffee contains more CGAs and caffeine (Clark 2022; da Silva Portela et al. 2021; Hasni et al. 2021). It is adored for its varied flavors and soft texture and is the most common specialty grade coffee offered (Clark 2022). At first, Robusta coffee has more antioxidants than Arabica, however after roasting, Arabica has more antioxidants than Robusta beans (Hasni et al. 2021). Due to its higher solids content and the fact that cold brew production often uses low temperatures, Arabica coffee is a substance of potential interest because cold brew production typically results in a lesser extraction of chemicals (da Silva Portela et al. 2021). The project aimed to obtain coffee beverage with rich flavors, sweet taste and contain less caffeine inside thus Arabica coffee beans are more suitable for cold brewing process.

2.2.4.1. Colombian Arabica Coffee Beans

The Arabica coffee beans is quite sensitive; it is affected by the aromas of the fruits and spices found in the lands where it grows, therefore, coffees prepared with beans grown in other parts of the world have different smells and tastes (Collazos-Escobar et al. 2022). Arabica coffee beans grown in Colombia have a sweet taste and balanced acidity compared to other regions; it provides a bright brew (Espresso and Coffee Guide 2020), it has outstanding sensory qualities, including its flavor and body (Özdestan et al. 2013). It can be counted among the flavor notes of red fruits, tropical fruits etc. and has a sweetness of its own (Espresso and Coffee Guide 2020). Colombian coffee contains roughly the same amount of caffeine as other coffee beans (Eldorado Coffee Roasters 2019). In addition; Colombian coffee is ideal for almost all roasting methods (Espresso and Coffee Guide 2020). Thanks to its soft taste, it can be consumed with milk and similar flavors without the need for softening; Colombian Arabica coffe was preferred to get sweet and balanced flavor.

2.2.5. Roasting of Coffee Beans

In order to obtain a highly flavored beverage, moderate roasting should be provided. Roasting coffee beans causes many changes in chemical composition; oxidizes most organic compounds and affects coffee color (Liang and Kitts 2014). Only around 40 of the approximately 850 volatile chemicals found in roasted coffee that have been identified so far contribute to the aroma (Esquivel and Jimenez 2012). It is well known that the caramelization of sucrose during roasting and the production of melanoidins which are polymeric byproducts of the Maillard reaction are what give roasted coffee its distinctive brown hue (Yeager et al. 2022). The process of roasting causes a decrease in bean density and an increase in bean volume (Rao, Fuller and Grim 2020). Release of gases during heating; the physical and chemical changes that take place in the bean matrix during roasting affect the final beverage's antioxidant activity in addition to the acidity of the resulting coffee (Rao, Fuller and Grim 2020). Coffee's antioxidant activity level

reduces as roasting intensity rises (Sualeh, Tolessa and Mohammed 2020). Acids, caffeine, lipids, and carbohydrates are just a few chemical components that are produced or degraded as a result of roasting (Collazos-Escobar et al. 2022). Coffee's unique aroma is produced during roasting via the Maillard process, pyrolysis, and degradation (Sualeh, Tolessa and Mohammed 2020). A decrease in extractable chlorogenic acid concentrations and an increase in caffeine concentrations are correlated with higher roasting temperatures (Fuller and Rao 2017). Compared to low-temperature roasted beans, coffee beans with higher roasting temperatures have less porosity and more volume so, the high heat of roasting causes microstructural changes that make chemicals easier to extract (Cordoba et al. 2021). Roasting also increases the soluble dietary fiber in the beans (Esquivel and Jimenez 2012) so brewed coffee is a source of dietary fiber in addition to phytochemicals, contains high soluble dietary fiber and their metabolic activities indicate that they may have prebiotic effects (Esquivel and Jimenez 2012). It was predicted that providing probiotic fermentation with prebiotic-containing coffee will create a mutual relationship.

Medium-roasted coffee has the highest activity because it balances the simultaneous generation of Maillard reaction products and the degradation of phenolic components (Esquivel and Jimenez 2012). For cold brewing medium roasted colombian arabica coffe beans provides desirable results in certain parameters in coffee drink (Rao, Fuller and Grim 2020) and medium roasting degree was preferred to not get too much caffeine but get more extractable coffee, easier extraction.

2.2.6. Coffee Grinding Size, Medium-Coarse Grind

The extraction, and consequently the overall flavor and quality of the coffee, are impacted by the grind size; grinding the coffee beans increases the overall surface area, which increases the coffee's contact with the water and makes it possible for the compounds it contains to dissolve in the water more rapidly and effectively, resulting in extraction (Yeatts 2021). Coffee beans are typically ground to a degree between 10 (extremely coarse) and 1 (very fine) (Cold Drip Coffee n.d.). When using finely ground coffee (1-3) the ground coffee is exposed to (usually hot) water for a very short time and

this usually happens by machine and under high pressure (Cold Drip Coffee n.d.). If the contact time of water and coffee is medium, the grinding size should be medium (4-6) and coarse ground coffee (7-10) is particularly suitable for brewing methods where the coffee powder is in contact with water for a long time, it is generally preferred in cold brew coffees (Cold Drip Coffee n.d.). The coarse fraction can be as large as 2 mm, while the fine fraction is less than 200 μm (Microtrac n.d.). But in cold brew, if the coffee is ground too coarsely, the extraction process will be insufficient; low water temperature (cold water) and very coarse ground coffee should not be used to obtain high bioactive compounds (Chlorogenic Acids (CGAs), caffeine and melanoidins) and high antioxidant activity (da Silva Portela et al. 2021). Brewing with very fine grinding can create bitter, ashy flavors and a muddy mouthfeel (MasterClass 2021).

Wider particle distributions are present in medium grind coffees, which include 5% of the mass of particles larger than 3350 μm and coarse grind coffees have a smaller particle distribution and no 3350 μm section, with more than 70% of the total mass of the particles remaining on the 841 μm sieve. Coffee beans have pores by nature; the intra-granular pores that the spaces found between coffee grains are those found inside each individual coffee grain (Fuller and Rao 2017). The amount of caffeine significantly increased in samples of medium- and dark-roasted coarse grains (Fuller and Rao 2017). To get cold brews with higher antioxidant activity the use of coarser grind coffee should be avoided (da Silva Portela et al. 2021).

Physicochemical properties of cold brew coffee, including as extraction yield, total dissolved solids, total phenolic content, and pH, are significantly influenced by the degree of grinding (Kyroglou, Thanasouli and Vareltzis 2021). Cold extraction of medium-coarse ground coffee becomes faster and easier, resulting in sweet coffee beverage (Adams 2017); medium (near coarse) ground and medium roast coffee was used in the project.

2.2.7. Chemical Properties of Cold Brewed Coffee

2.2.7.1. Coffee pH

Both organic acids and chlorogenic acid are present in coffee, each of these acids adds a distinct flavor to the beverage; they not only raise the acidity of the coffee but also give it a lot of flavor and taste (Preiato 2019). The acidity is essential to the flavor and taste of coffee. Coffee beans are acidic, with an average pH of 4.85 to 5.10; the type of coffee beans, the degree of roasting and brewing temperature, time affect the pH of the coffee (Preiato 2019). Most of the acids formed in roasting are formed in the early stages, the brewing process releases nine key acids (chlorogenic, quinic, citric, acetic, lactic, malic, phosphoric, linoleic and palmitic) that contribute to the flavor profile (Preiato 2019). Cold coffee that prepared with medium roasted Arabica beans has pH average values of 5.17 ± 0.03 (da Silva Portela et al. 2021).

2.2.7.2. Brix and Total Dissolved Solids (TDS)

°Brix grade is the grade of dissolved sugar in an aqueous solution and brix value of cold brew higher than hot brew (Asiah et al. 2019). The °Brix percentage in the coffee industry is a good indicator of a coffee's normal strength, the °Brix scale has become the accepted benchmark for the coffee, and a refractometer is used to calculate readings (Gant 2022; Sim 2022). TDS is, by definition, the ratio of the mass of the liquid to the amount of non-organic suspended solids in water and it measures the quantity of coffee compounds per unit water in coffee (Sim 2022). A cup of coffee with a strong flavor will have a high TDS%, while one with a milder, watered-down flavor will probably have a low TDS% (Sim 2022). TDS value of cold brew less than hot brew (Asiah et al. 2019).

2.2.7.3. Coffee Color

Color is typically the first quality assessment a consumer makes and is the primary indicator of quality. The color is measured by CIE method, it indicates L*a*b* color values are 3-dimensional color coordinates, respectively; L* is the aperture, a* value indicates red/green and b* is the yellow/blue coordinate (Aldosari et al. 2021; Cordaba et al. 2021). The color of the coffee varies depending on factors such as the type of bean, the degree of roasting, and the brewing method (Cordaba et al. 2021; Esquivel and Jimenez 2012; Yeager et al. 2022). For medium-roasted beans, the brew temperature and roast level had the greatest effects on the color of the coffee brewed using the immersion method (Yeager et al. 2022). Medium roast coffee beans showed desirable color quality in brews (Yeager et al. 2022). In terms of quality, the cold-brewed coffees had a tendency to be redder while the hot-brewed coffees had a darker color (Yeager et al. 2022).

2.3. Process

Probiotic coffees are a very recent addition to the category of probiotic foods, hence studies examining their production process are scarce and unreliable. Here, it is considered how probiotic coffee formulations might be adapted to current coffee production techniques for simpler implementation and more industry receptivity. The process flow-chart illustrating potential probiotic coffee production is shown in 3.2.

2.3.1. Brewing

The coffee bean is made up of components/solutes which are both volatile and non-volatile substances like different oils, acids, and other aromatic compounds (Erbabi 2021). According to the extraction method, both soluble and non-soluble chemicals are dissolved in the extraction water during the solid-liquid extraction that occurs during

coffee brewing and process variables have a significant impact on the kinetics of extraction of the numerous chemical components found in roasted coffee (Cordaba et al. 2021). In brewing methods, the temperature of the water used and the time factor are the determining factors in the balance of coffee components (Erbabi 2021) thus brewing method affects the composition of the final coffee beverage (Bellumori et al. 2021). Substances in coffee become dissolved or suspended solids in the extract, greatly enhancing the sensory qualities of the coffee brew (Cordaba et al. 2021).

Coffee extraction is a complex process that depends on several factors, including water volume, water temperature, coffee grind size, coffee grind matrix porosity, coffee grind network, and coffee grind brewing duration (Fuller and Rao 2017). At varying temperatures, various aromatic molecules are extracted (Angeloni et al. 2019). Different brewing temperatures may result in different compositions in cold brew coffees since temperature frequently has a considerable impact on a compound's aqueous solubility (Fuller and Rao 2017). The temperature of the water used to remove acidic molecules from the coffee matrix during the aqueous phase has a considerable impact on the overall titratable acidity and antioxidant activity of the final coffee beverage (Fuller and Rao 2018).

Additionally, agitation disrupts tissue and cell walls, which makes it easier for caffeine to cross the cell membrane and enter the solution and agitation considerably increases the amount of caffeine extracted compared to non-agitation (Kyroglou, Thanasouli and Vareltzis 2021). For this reason, to not increase caffeine content in coffee, agitation was not considered during brewing. 24 hours of brewing will be sufficient to obtain coffee brews with the cold brew method (Kyroglou, Thanasouli and Vareltzis 2021; Yeager et al. 2022).

2.3.1.1. Comparison of Cold and Hot Brewing Methods

Cold brew is characterized by low energy, prolonged water-coffee contact times; 20-25°C or colder water used for 8 to 24 hours, while in the hot brew method, the high extraction temperature is considered the driving force for extraction (Kyroglou, Thanasouli and Vareltzis 2021). Since temperature frequently has a significant impact on

a compound's aqueous solubility, different brewing temperatures may result in noticeably different compositions in hot brew and cold brew coffees (Fuller and Rao 2017). In cold brew, the main extraction of compounds occurs during the first 3-5 hours of infusion (da Silva Portela et al. 2021). The health benefits of cold-water extraction include increased shelf life, better retention of secondary metabolites, and volatiles (Asiah et al. 2019). The flavor can change based on the brewing process (the strength of the sweet flavor and its related properties are affected by the extraction technique), and cold brew coffee can display stronger toasted, sweetness, and sourness flavors than hot extraction (Bellumori et al. 2021; Cordaba et al. 2021) this is because most of the sweet flavor compounds dissolve in cold water (da Silva Portela et al. 2021). High water temperature causes rapid degradation and oxidation of coffee acids and oils, so coffee drinks produced by hot extraction are acidic and bitter whereas cold brew coffees have higher pH values overall (Fuller and Rao 2018; Kyroglou, Thanasouli and Vareltzis 2021). Also, cold brew coffee shows more antioxidant activity than hot brew method (Kang et al. 2020). Between cold and hot brews, there are no discernible variations in the amounts of CGAs (Fuller and Rao 2017). Caffeine levels in cold brew samples were discovered to be comparable to hot brew samples (Rao, Fuller and Grim 2020); for the same coffee-to-water ratio, hot brew contains more caffeine than cold brew due to the oxidation of the coffee acids and oils as well as the solubility of caffeine at high temperatures (Kyroglou, Thanasouli and Vareltzis 2021).

2.3.1.2. Comparison of Cold Brewing Methods

Cold brewing methods are basically divided into two as immersion or (steeping/dipping) and dripping (or pour over) (Kang et al. 2020). When immersion method is used, flavor differences occur in terms of chocolate, acidity, sweetness, bitterness, body and aftertaste (Bellumori et al. 2021). Dripping method is provided by slowly dripping cold water and its aroma is quite strong (Angeloni et al. 2019; Cordaba et al. 2021), it extracts coffee at a higher rate than immersion brewing, indicating that the configuration and functioning of the cold brewing system have an impact on the extraction process (Cordaba et al. 2021). In contrast, in cold immersion systems, the

coffee is dipped directly into the aqueous enviroment (Cordaba et al. 2021; Fuller and Rao 2017; Rao, Fuller and Grim 2020). Coffee beverages made by cold immersion are primarily distinguished by their sweetness (Angeloni et al. 2019; da Silva Portela et al. 2021) that are remarkably intense and exhibited a negative association with the levels of caffeine, trigonelline, CGAs, and TDS whereas those made by cold dripping have a higher level of bitterness (Cordoba et al. 2021). The brewing procedure has an impact on the final coffee beverage's composition; in particular, there are discernible differences in the extraction of polyphenols, caffeine content, total solids, antioxidant activity, and volatile profile (Angeloni et al. 2019). Cold brew coffees produced by immersion are principally distinguished by the presence of volatile compounds such as ketones, furans, furanones, and pyrazines, which are linked to sensory qualities found in the same quadrant as nutty, caramel, and malt (Cordoba et al. 2021). These flavor variations could result from the caffeine concentration, which is higher in cold dripping than immersion (Cordaba et al. 2021).

With the cold dipping method, it was aimed to be an alternative coffee product containing less caffeine, that consumers who are sensitive to caffeine, who do not want to consume flavored coffee with milk, but who find black coffee bitter, can prefer the product. One of the most important reasons why the product can be suitable for a wide range of consumers is its smmoth tatse and low caffeine content. The fermented product targeting production in the project also appeals to women during pregnancy and teenagers & children. For these reasons, cold brewing process with immersion technique was found to be suitable for this project.

2.3.2. Filtration of Brewed Coffee

After brewing, the brewed coffee must be filtered to remove settled or suspended coffee particles. Coarse -7 (near medium) ground coffee has a particle size of 0.75-1 mm (MasterClass 2021) and fine filtration under pressure is not needed. Paper filters are costly and must be folded/inserted into the filter head. Since the coffee grounds to be used is not fine grind, metal filters provide successful filtration.

However, in the project, since the filtered coffee's amount was small; the coffee samples were filtered by filter paper to prevent remaining too small particles inside samples to provide brief observation of colonies on agars.

2.3.3. Heat Treatment

Cold brew coffee has the potential to pose food safety hazards because of the prolonged brewing procedure, which may increase microbial activity. These beverages' low acidity also promotes microbial growth, which can lead to development of infections or spoiling organisms. *Salmonella*, *Listeria monocytogenes*, and *E. coli* may survive in cold brew (Kyroglou, Thanasouli and Vareltzis 2021). Also, *Bacillus cereus* is pathogen that is potential to be found in roasted ground coffee (Chan and Liu 2022a). A study showed that untreated cold brewed sample was spoiled by microorganisms and microbial load exceed acceptable limits; during shelf life lactic acid bacteria, yeast and *B. cereus* contamination was expected, while the pasteurized sample remained below the acceptable limits for 120 days (Bellumori et al. 2021). Besides the effect of *S. boulardii* against pathogens, heat treatment, pasteurization, was required. In a study, it was concluded that heat treatment at 65°C for 35 minutes was appropriate for pasteurization of cold brewed coffee products (Bellumori et al. 2021). Another study suggested 95°C for 35 minutes for pasteurization (Chan, Liu and Toh 2020). To not lose heat sensitive components in coffee, low heat treatment temperature that the lowest temperature for successful heat treatment was preferred. Also, according to Bellumori et al. (2021), pasteurization at 65°C for 30 minutes did not affect sensory properties; non-treated and pasteurized coffee samples showed nearly same properties.

Heat treatment has no discernible effects on the caffeine and chlorogenic acids (CGAs) concentration (Bellumori et al. 2021). Some of major aromatic components that present in cold coffee beverages are pyrazines, furans, aldehydes, ketones, and phenols and there are no discernible variations in the level of this chemical between the pasteurized and untreated samples (Bellumori et al. 2021). The sensory descriptors used to describe heat treated (pasteurized) and untreated coffee samples were fruity, flowery,

low bitter, sour, and toasted and these samples exhibited a high degree of similarity in the two-dimensional representation and pH values was not change (Bellumori et al. 2021).

2.3.4. Fermentation of Cold Brewed Coffee with *S. boulardii*

After cold brewed coffee drink was pasteurized and become suitable for fermentation; the fermentation process will be start with probiotic yeast that is targeted to be minimum at 1×10^6 CFU/g in the final product.

2.3.4.1. Glucose fortification in Fermentation Medium

In addition to enhancing sensory characteristics, adding a prebiotic or synergistic substrate to foods containing *S. boulardii* may increase its viability and effectiveness (Chan and Liu 2022b). For the efficiency of *S. boulardii* and to promote probiotic growth, glucose was fortified to fermentation environment to observe its effects on *S. boulardii* growth (Chan, Liu and Toh 2021c). It thoughed that *S. boulardii* consumes glucose per hour per *S. boulardii* cell and is in transformation; the yeast converse the substrate to biomass (de Paula et al. 2019). By the way, non-supplemented glucose coffee brews, insignificant changes in levels of lactic acid or pH were observed (Chan, Liu and Toh 2021c). Synbiotic formulations, fructo-oligosaccharides and therapeutic yeast can be combined since it is anticipated that both the probiotic yeast and the colon's natural good bacteria will be promoted (Mitterdorfer and Viernstein 2001).

2.3.5. Final Product

A study showed that the population of *S. boulardii* in pasteurized brewed coffee was stable during 120 days of cold storage (+4 °C) (Chan, Liu and Toh 2021c), and

sample showed an unaltered flavor profile (Bellumori et al. 2021). Thus, the coffee samples that F, FG and P shelf lives are decided as 120 days at +4 °C. Probiotic survival during storage was closely checked, it was important to monitor the growth, survival, and metabolic processes of *S. boulardii* during storage of coffee brews. Because of the decreased metabolic activity, slower post-acidification rates under refrigeration may lessen the probiotic's exposure to acid stress (Chan, Liu and Toh 2021c).

Probiotics in coffees must not only be able to withstand lengthy periods of storage but also maintain their sensory appeal in order to be considered shelf-stable. Consumer acceptance restrictions have a significant impact on the organoleptic qualities of taste, scent, and texture, which similarly determine a product's shelf life.

It was thought that *S. boulardii* used in fermentation will prevent the presence and growth of unwanted microorganisms, make it last longer without spoiling, and provide a healthier and safer drinking experience. Fermentation of the probiotic *S. boulardii* in coffee will convert many difficult-to-digest compounds into simpler molecules that can be treated more effectively by the body and has a lower concentration of certain chemical compounds associated with gastrointestinal irritation, which will make it easier to digest than standard coffee (Lazo-Velez et al. 2018).

S. boulardii produces volatile phenols, esters, and alcohols that are the most prevalent compound in terms of quantity, with ethanol and fusel alcohols contributing less significantly (Lazo-Velez et al. 2018). The ability to deacidify the environment is a survival strategy for *S. boulardii*; under simulated gastric conditions (pH 1.1) it increased the pH to 3.2, minimizing post-acidification (Chan, Liu and Toh 2021c). Citric acid consumption by the yeast was likely the cause of the deacidification of coffee brews, as depletion coincided with pH increases not seen at 4 °C (Chan, Liu and Toh 2021c) and this is actually one of the aspects where using probiotic yeast is more advantageous than using probiotic lactic acid bacteria. Without glucose, citric acid probably acted as a substitute carbon source for the probiotic yeast, allowing it to use the Krebs cycle to produce ATP and other important metabolic intermediates (Chan, Liu and Toh 2021c).

For fermented product, fermentation is important to establish key sensory quality characteristics; will generally remove most of the bitter notes of coffee in terms of taste, making it a sweeter, less bitter beverage that most people prefer. In this respect, the F and FG samples were different from other products prepared by adding probiotic culture.

CHAPTER 3

MATERIALS AND METHODS

3.1. Experimental design

The experiment was designed to evaluate the effects of the fermentation on cold coffee brews that were prepared at room temperature via immersion cold brewing method and glucose effects on probiotic yeast fermentation over 120 days.

The samples were prepared by the production steps as given in the flow chart (Fig. 3.1). Although the samples were prepared as lab-scale in the laboratory environment, the flow chart was prepared in accordance assuming industrial production. Until the aseptic filling all the process steps were validated for the current design.

Chemical, microbiological and sensorial analysis were carried out and changes in the chemical and sensory profile of samples that were codded as P (pasteurized), F (fermented), and FG (fermented with glucose) were evaluated. A shelf-life study was also conducted by keeping coffee at 4 °C, at refrigerator temperature and taking samples at 0^{th}, 7^{th}, 30^{th}, 60^{th} and 120^{th} days, corresponding t_1, t_2, t_3, t_4 and t_5 respectively. At t_1, t_3 and t_5, all the samples were subjected to the all-specified analysis. At t_2 and t_4, only chemical properties and probiotic yeast availability were checked. Two replicates were carried-out for each treatment and mean values are given.

3.2. Flow Chart

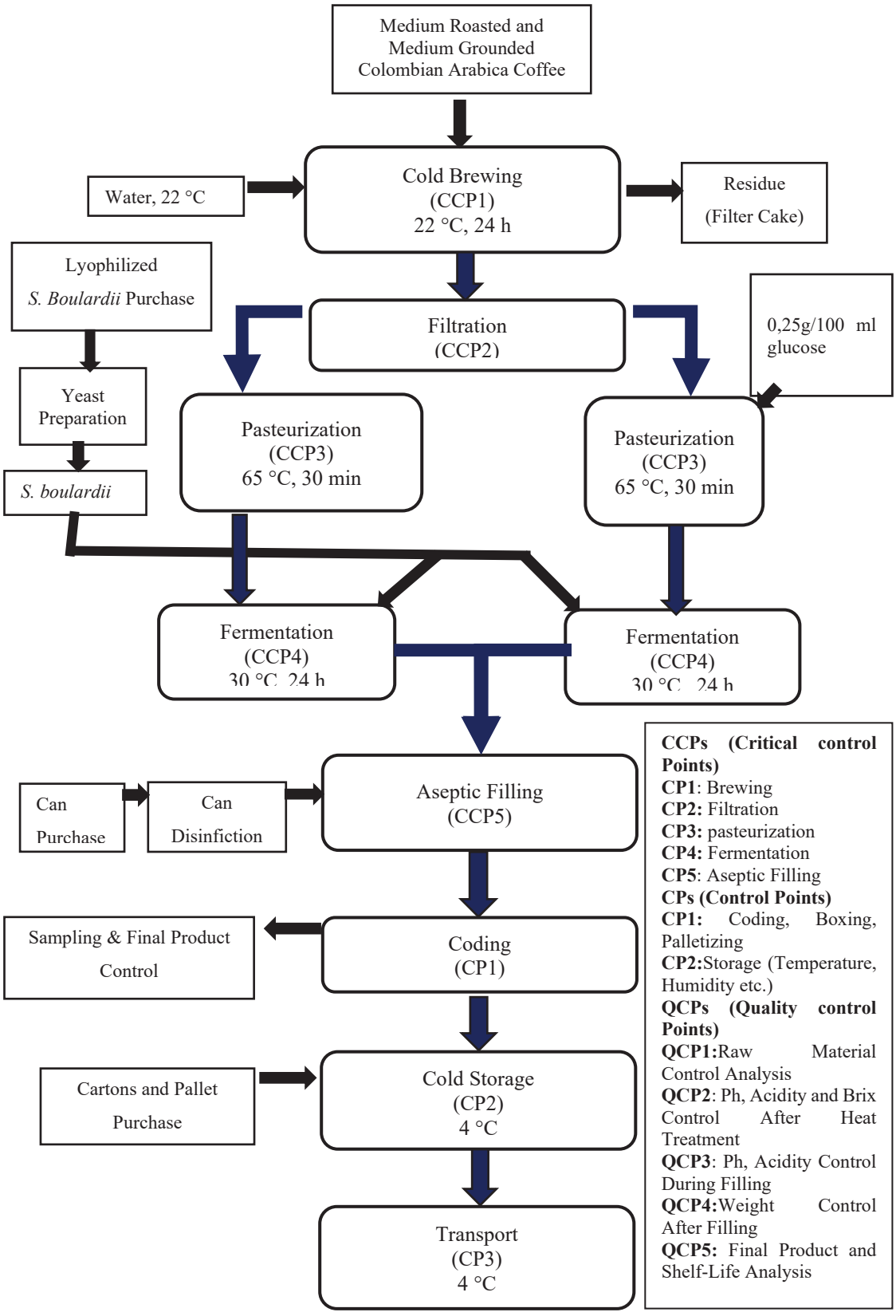

Figure 3. 1. Flow Chart of Pasteurized (P), Fermented (F) and Fermented with glucose (FG) samples

3.3. Brewing and Filtration Procedure

Colombian Arabica coffees that were medium roasted and medium-coarsely ground were kindly provided by Kuru Kahveci Mehmet Efendi Tic. Ltd. Sti. (Kurukahveci Mehmet Efendi n.d.) and was used for the cold brew extraction. Erikli purified water with pH 7.86 was used for all brews (Erikli n.d.). The water was taken to incubator at 22°C before brewing (Yeager et al. 2022). 16000 ml water and 1600 g of coffee (1:10) (Angeloni et al. 2019; da Silva Portela et al. 2021; Kyroglou, Thanasouli and Vareltzis 2021) were brewed in an incubator at 22°C for 24 hours (Fuller and Rao 2017; Kyroglou, Thanasouli and Vareltzis 2021; Rao, Fuller and Grim 2020).

After extraction, the beverage was filtered and immediately put through the processes. The particle size of the coffees was assumed as nearly 0.75-1 mm (MasterClass 2021). To avoid coffee particles remaining in the beverage, all brewed coffee was filtered using a standard filter paper supplied from Tchibo Kahve Mamülleri Dağıtım ve Pazarlama Tic. Ltd. Şti (Tchibo n.d.).

3.4. Pasteurization

All glasswares were sterilized at 121°C for 30 minutes (Centers for Disease Control and Prevention n.d.). Pipettes, spatulas, centrifuge tubes, petri dishes etc. were supplied sterile and disposable. Three 5000 mL erlenmeyer flasks were used to hold the cold-brewed coffees. In order to better understand how sugar affects probiotic growth; 12.5 grams of powdered glucose (Merck) were added to 1 x 5-liter erlenmeyer flask (0.25 g/100 mL) (Chan, Liu and Toh 2021c), whereas 2 x 5-liter erlenmeyer flasks were pasteurized without sugar additive. By adding powdered glucose before pasteurization, the risk of microbial contamination was eliminated. The flasks were pasteurized in an autoclave at 65°C for 30 minutes (Bellumori et al. 2021). At the end of the heat treatment, 10 liters of P and 5 liters of pasteurized coffee with sugar were obtained. A 5-liter sample of P was kept for analyses.

3.5. Fermentation

3.5.1. *S. boulardii* Culture Preparation

Lyophilized *S. boulardii* CNCM-I745 was kindly provided by Assist. Prof. Ayşe Handan BAYSAL (Department of Food Engineering, İzmir Institute of Technology). The lyophilized form of the yeast was suspended, colonized, cultured and added to the medium under appropriate conditions (Chan, Liu and Toh 2021c). *S. boulardii* commercial lyophilized powder was suspended in sterile 0.1 g/100 mL peptone solution (Biomark). The suspension was cultivated on potato dextrose agar (PDA, Merck), and incubated at 30°C for 48 hours (Chan, Liu and Toh 2021c). Thus, the viability of the powder yeast was determined after incubation. A colony was selected and grown in yeast malt broth (Biomark) and incubated at 30°C for 24 hours (Chan, Liu and Toh 2021c). After incubation, it was ready for fermentation.

Total probiotic starter broth was used for 10.000 ml pasteurized coffee samples. The amount of broth containing the microorganisms to be prepared was divided into two flasks to be added to two 5 liter erlenmeyer flasks. In this way, two fermentation media containing equal CFU/g of *S. boulardii* were obtained. It was aimed to obtain at least 1.0 $\times 10^6$ CFU/g probiotic yeast on each sample at the end of fermentation.

3.5.2. Culturing Pasteurized Samples for Fermentation

The liquid fraction was removed after being centrifuged for 10 minutes at 10,000 g at 4 °C (Chan, Liu and Toh 2021c), and the remaining cell pellets were then suspended in 50 ml of pasteurized coffee brews. The pasteurized coffee sample, P, for F and the pasteurized sample with glucose for FG were used to dissolve the yeast pellets. Good mixing was achieved by using vortex (Velp Scientifica) and cultured coffee samples were added to the main fermentation medium to obtain F and FG. The flaks were kept for 24 hours at 30°C for fermentation in an incubator under anaerobic conditions (Chan, Liu and

Toh 2021c). Each sample was stored at refrigerator (4°C) for 120 days (Chan, Liu and Toh 2021c) for final product & shelf-life analyses.

Cultured coffee samples (not be fermented yet) were controlled by analysis that was explained in 3.5.2.6 to determine yeast viability in coffee.

3.6. Chemical and Microbial Analyses

3.6.1. Chemical Analyses

The chemical analyses conducted in this research were pH, total titratable acidity (TTA), brix, total dissolved solids (TDS), color, caffeine, chlorogenic acids (CGAs), and ethyl alcohol (ethanol) concentration for each sample.

3.6.1.1. pH and Total Titratable Acidity (TTA) Measurements

The pH values of the coffee samples were measured at room temperature using a pH meter (WTW ProfiLine pH 3110) (Fuller and Rao 2017). The electrode was dipped into the samples and waited for a stable reading on the pH meter display and the values were recorded.

For TTA, 50 mL of the coffee extract were titrated with 0.1 mol/L NaOH (Merck) solution to a pH of 6.5 (Cordaba et al. 2021). The results were expressed as used amount of 0.1 N NaOH (mL) per 50 mL coffee (Yeager et al. 2022).

3.6.1.2. Brix and Total Dissolved Solids (TDS) Measurements and Extraction Yield (EY)

Brix value shows the concentration of sugar found in liquid phase. Digital refractometer (Atago), was used to measure the brix of each coffee brew (Rao, Fuller and Grim 2020). First, the refractometer was calibrated by using distilled water, then a clean plastic pipette was used to transfer nearly 10 drops of the samples at room temperature into the sample well of the refractometer. The brix values were recorded.

To find out solid percentage the following modified equation was used (Moreno, Raventós and Hernandez 2015);

$$\%TDS=0.87 \text{ X Brix} \quad (3.1)$$

By multiplying the product TDS by the entire quantity of water (Ww) divided by the weight of ground coffee (Wc) used for extraction, extraction yield (EY) was obtained (Stayte 2022).

$$EY=TDS \text{ x } (Ww/Wc) \quad (3.2)$$

3.6.1.3. Color Measurement

All samples were measured for the CIE-L*a*b values, which are used to characterize color, to provide the numerical values for the 3D color measurements (Yeager et al. 2022) at room temperature (22°C) (Aldosari et al. 2021). Color studies of the brews were performed by Chroma Meter (Konica Minolta CR 400) and three-color parameters that L*, a*, and b* were measured directly by a colorimeter. Before each measurement, the device was standardized with a white reference plate (Yeager et al.

2022). In a glass petri dish placed on a white printer paper, 50 mL of each sample was added before the measurement was taken.

The color difference (ΔE) was calculated by using the following equation (Cai et al. 2022).

$$\Delta E=[(L^{*1}-L^{*2})^2+(a^{*1}-a^{*2})^2+(b^{*1}-b^{*2})]^{1/2} \quad (3.3)$$

During calculation the following combinations were considered; cold brewed and no-treated & fermented samples F and FG.

3.6.1.4. Caffeine and Chlorogenic Acids (GCAs) Measurements

The caffeine analyses were carried out using the modified method (Tanner and Mountford 1993: 399-413.) and the chlorogenic acid analyses were made using the modified method (Cui et al. 2005, 3882-3887) in HPLC-DAD-UV-Vis. Prior to measurements, cold brew coffee samples were diluted 1:10 with distilled water and centrifuged at 16,900 g for 5 min. The system was equipped with an auto sampler, column heater module, and quaternary pump, linked to a diode array detector (DAD), and UV visible detector (UV-Vis). These were used for HPLC measurements, all from SIA Laboratory (Izmir, Türkiye).

For caffeine analyses, C18 reversed phase column (Hypersil Gold) (250 mm × 4.6 mm, 5 μm) was used. The mobile phase A was Monopotassium phosphate and mobile phase B was acetonitrile. The samples were homogenized and kept in a water bath at 65 °C. Then samples were cooled to room temperature and were filtered and given to the HPLC-DAD-UV-Vis system.

For chlorogenic acids analyses, C18 reversed phase column (Inertsil ODS) (250 mm × 4.6 mm, 5 μm) was used. The mobile phase A was water with %1 formic acid and mobile phase B was methanol. The homogenized sample was taken into a falcon tube. Ethanol (95%) and phosphoric acid were added. The samples were thoroughly mixed and

were degassed in an ultrasonic water bath. The extracts were centrifuged, a certain amount was taken from the supernatant and evaporated to dryness under nitrogen. Water was added to the tube containing the residues, filtered, taken into the vial and given to the HPLC-DAD-UV-Vis device.

By comparing retention times, caffeine and CGAs were determined. To evaluate concentrations; a six-point calibration curve for 5-O-caffeoylquinic acid at 330 nm was used for chlorogenic acids and a six-point calibration curve at 278 nm was used for caffeine concentration by HPLC-DAD-UV-Vis.Caffeine and CGAs quantitative values were expressed as mg/L of cold brew coffee.

3.6.1.5. Ethyl Alcohol / Ethanol Measurement

Headspace Gas Chromatography was used to analyze volatile ethanol in coffee samples according to method used by Wachelko, Szpot and Zawadzki (2021) with some modifications. Using the Headspace GC sampling technique, volatile sample components migrate into the gas phase in the headspace above the sample inside the bottle after being collected from the sample and fed into the gas chromatography system for analysis. First, the sample was degassed by Ultrasonic Bath (Hydra Ultrasonic) to remove the gas inside. 1 mL sample at 25 °C was taken into the headspace vial with a micropipette, and diluted by adding 9 ml of distilled water. 2 mL of the diluted sample was taken into the headspace vial and it was sealed tightly. After heating to 80 °C for 8 minutes, the ethanol in the sample was transferred to the gas phase in the headspace vial and given to the column. The carrier gas was helium.

The device plotted the detector signals against time (in the form of a peak) and calculated the area under the peaks. Thus, the ethanol concentration in the sample was given in g/L.

3.6.2. Microbial Analyses

P, F and FG samples were analyzed for pathogenic microorganisms (*Salmonella*, *Listeria monocytogenes*, *Escherichia coli*, *Bacillus cereus*) as they are risky and likely to be present in the cold brewed coffee samples (Chan and Liu 2022a; Kyroglou, Thanasouli and Vareltzis 2021) during their shelf life (t_1, t_2, t_3, t_4 and t_5) according to the standard methods given in the following sections. Also, viability of yeast and growth of lactic acid bacteria (Chan, Liu and Toh 2020; Chan, Liu and Toh 2021c) were analyzed. All media and supplements used were prepared and sterilized in an autoclave according to their instructions. Two replications were made for each dilution, broth and petri.

3.6.2.1. *Salmonella* Analysis

Salmonella analysis was performed by the MPN method adapted from ISO (2007). To prepare the first dilution, 25 ml of each sample was mixed with 225 ml of buffered peptone water (BPW, Difco) and incubated at 37 °C for 18–24 h. Afterwards, 0.1ml from each BPW tube was added to 10 ml of Rappaport–Vassiliadis modified broth (Difco), with incubation at 41.5 °C for 24 h, and 1.0 ml to 10 ml tetrathionate broth (Difco), with incubation at 37 °C for 24 h. Thereafter, cultures were streaked on xylose lysine deoxycholate agar (XLD, Difco) and bismuth sulfite agar (BS, Difco) and the plates were incubated at 37 °C for 24–48 h. Presumptive *Salmonella* colonies were picked up and identified by biochemical tests (triple sugar iron, lysine decarboxylase Voges–Proskauer, urease and indole). Afterwards, the serotype of each confirmed colony was identified by serological tests using somatic (slide agglutination test with O-antigen) and flagellar antiserum groups.

3.6.2.2. *Listeria monocytogenes* Analysis

L. monocytogenes was determined according to ISO-11290-1:1996 (1996).

3.6.2.3. *Escherichia coli* Analysis

10 mL of coffee samples were homogenized with 90 mL of 0.1 % peptone water. Serial dilutions in peptone water were prepared, and transferred onto specific agar media. Coliforms, and *E. coli* in the samples were enumerated on Violet Red Bile Agar (VRB; CM0968, Oxoid) and Chromocult TBX Agar (CM0945, Oxoid), respectively. The incubation periods with VRB agar at 37 °C for 24 h and TBX agar 44 °C for 24 h under aerobic conditions (Irigoyen et al. 2005). The typical colonies were counted after incubation, and the results were expressed as log cfu/mL. The MPN of coliforms and *E. coli* was determined as described previously according to ISO-4831:2006 (2006) and ISO-7251:2005 (2005), respectively.

3.6.2.4. *Bacillus cereus* Analysis

B. cereus counts were assayed in the samples according to the colony count technique ISO 7932 (2004). The samples were transferred under sterile conditions in the amount of 10 mL and placed in sterile plastic bags containing 90 mL of peptone saline solution. The mixtures were homogenized, 10^{-1} dilutions were obtained, which were subjected to further dilution. To enumerate *B. cereus*, the plates were inoculated by spreading the samples on the surface of the selective Mossel (MYP Agar, Merck) medium and incubated at 30°C for 24-48 h. Presumptive colonies are sub-cultured onto sheep blood agar for confirmation by haemolysis. The number of presumptive *B. cereus* per mL of sample is calculated from the number of confirmed colonies obtained on plates at dilution levels chosen and confirmed according to the test specified.

3.6.2.5. Lactic Acid Bacteria (LAB) Analysis

Man, Rogosa, and Sharpe (MRS) Agar (Neogen) was used to count the amount of lactic acid bacteria present in the samples according to ISO 15214:1998. Under aseptic conditions, a decimal serial dilution up to 10^{-6} was prepared using 0,5 g of samples that were homogenized in 4,5 ml of sterile% (w/v) peptone solution. MRS agar, which had been cooled to 45°C, was added to 1 ml of the dilutions in a petri dish. For a petri dish with an 8–9 cm radius, 12.5–15 ml of medium was placed into the dish. It used circular mixing motions. The culture medium was incubated anaerobically for 72 hours at 37 °C using anaerobic atmosphere generating bags, AnaeroGen (Thermo Fisher Scientific). Following incubation, the number of white opaque colonies that grew on agar were counted, multiplied by dilution factors, and used to estimate the amount of lactic acid bacteria in a 1 ml sample. The data were acquired by plating two samples in parallel, and they were presented as an average standard deviation.

3.6.2.6. *S. boulardii* Analysis

The pour-plate method was used according to the colony count technique ISO 7954:1987. First, 4.5 ml of peptone water was used to dilute the 0.5 ml sample up to 10^{-6} and 1ml solvent form each dilution was added to petri dishes. PDA agar (Merck) that had been melted and cooled to 45°C was added to petri dishes 12.5–15 ml and mixed. For 48 hours, the culture medium was incubated at 37 °C (Chan, Liu and Toh 2021c). Colonies that formed on agar after incubation were counted and calculated with consideration of the dilution factor.

3.6.3. Sensorial Evaluation

Before the sensory analysis panel, an application was made to the Sensory Analysis Committee of Izmir Institute of Technology for ethical approval of the test and

each panelist was informed about the samples. Signed consent was obtained from each panelist for their agreements to participate to the analysis. Panellist Informed Consent Form and Sensory Analysis Evaluation Form are given in Appendix D.

3.6.3.1. Sensory Analysis with Panellists

Sensory analyzes were carried out with 31 pannelists in simply standard sizes tasting cabinets, in a panel room that was uniform and glare-free lightened, painted in neutral colors, noiseless, odorless (with ventilation system) at 20–22ºC (Thiemt n.d.). The sensory test was applied to a total of 3 different coffee samples; P, F, FG. The samples were coded with independent 3-digit numbers as following; P:845, F:563, FG:237. Samples prepared for a lab scale but were considered as representing the main production. The panelists were instructed to rinse their mouths with water both before and after the tasting in order to get rid of any remaining flavors from the sample they had just tasted. All of the samples to be presented are given at the same temperature (20ºC) and amount; about 50 mL of each coffee samples were provided individually to the panelists (Degirmencioglu et al. 2016). Plastic glasses in which the panel samples were presented were transparent for well observation of color and identical in shape and size (Asiah et al. 2019) (Appendix E).

Numerical scoring test that is a technique used to rate the quality characteristics of the samples using a numerical scale of sensory properties was used (Log10 n.d.). Sensory attributes as color, viscosity, odor, aroma, taste, coffee strength sweetness, bitterness, and sourness that characterized cold brewed coffee beverage were evaluated by panelists (Cordaba et al. 2021). Assessors evaluated the samples and assigned scores using a 9-point scale panel with a range of 1 to 9 (where 1 is equal to "dislike" and 9 is equal to "like extremely") (Degirmencioglu et al. 2016). Participants were asked to rank the samples in terms of their preference or the intensity of each sensory attribute. In the scoring tests, the scores given by the panelists were averaged. The results were shown with radar/spider plot (Degirmencioglu et al. 2016).

3.6.3.2. Sensorial Evaluation During Storage

During the shelf life of P, F and FG samples' color, odor and taste controls were made. Each sample was filled in an amount equal to 50 ml in transparent glass and their colors were observed on a white table & background under sunlight. Each sample was then smelled and tasted (samples were checked for microbial safety before tasting). Before tasting, a small amount of water was drunk to remove the aftertaste from the sample before tasting. To better understand the odor differences, three minutes were waited before sniffing the next sample. The samples were scored by using 5-point scale (where 1 is equal to "dislike extremely", and 5 is equal to "like extremely").

3.7. Consumer Survey

In order to measure the consumers' coffee consumption rates & preferences, and awareness of probiotics, a general survey was conducted with Google Surveys without mentioning the product; the questions are given at Appendix B.

The survey was sent to people via WhatsApp groups and Instagram stories without any age & gender restriction. With the survey, a prediction was made about whether the probiotic coffee product would attract the attention of the consumers.

3.8. Statistical Analysis

The F and FG samples' ethanol level differences was examined with Minitab (Version 17 Statistical Software). Factors were storage time and availability of sugar source in the fermentation medium. The ethanol concentration (response) was examined for factors as storing time (t_1 & t_5) and fortifier existence (glucose presence & absence) with Design of Experiment (DOE), "two-factor, two-level full factorial design" and eight experiments with two replications were considered (the data used were the results of the

ethanol analysis). The level of statistical significance was fixed at 0.05; the effects of factors and their interactions on response were examined for alpha 0.05. No block variables were assumed for the experiments. Within-group comparisons were made using repeated measures; data from F and FG were reported.

Also, the sensory analysis results were evaluated using one-way analysis of variance (ANOVA). Calculations were performed using Minitab. The comparison was made by consideration of p value, F value, residual plot and normality plot.

CHAPTER 4

RESULTS AND DISCUSSION

To better understand the effects of *S. boulardii* fermentation and the addition of glucose to the fermentation medium on the chemistry, microbial load and sensory characteristic of cold brewed coffee, Columbian Arabica beans (medium roasted/ground) were brewed with drinking water (1:10) at room temperature for 24 hours.

In the brewing process, a total of 16000 ml of coffee was prepared with 1600 g of coffee. After 24 hours of brewing, the coffee volume decreased by 1000 mL to 1500 mL. Since 1600 g of coffee absorbs approximately 1000 mL of water after 24 hours and the coffee grounds settle to the bottom, it has been deduced that the volume of brewed coffee decreases after brewing.

4.1. Microbial Analyses

İnitially, no microbial load was observed on both not heat treated and pasteurized cold brewed coffees as also found in literature (Bellumori et al. 2021). Yeast growth was not observed in any of the samples. The microbial analysis results are given in Table 4.1.

Table 4. 1. Microbiological Analyses of Pasteurized (P), Fermented (F) And Fermented with Glucose (FG) During Shelf Life

Samples	**Pasteurized (P)**					**Fermented (F)**					**Fermented with Glucose (FG)**				
Interval times/Days	**t1** **1st**	**t2** **7th**	**t3** **30th**	**t4** **60th**	**t5** **120th**	**t1** **1st**	**t2** **7th**	**t3** **30th**	**t4** **60th**	**t5** **120th**	**t1** **1st day**	**t2** **7th**	**t3** **30th**	**t4** **60th**	**t5** **120th**
Salmonella	ND***	*	ND		ND	ND		ND		ND	ND		ND		ND
L. monocytogenes	ND		ND		ND	ND		ND		ND	ND		ND		ND
B. cereus	<10		<10		<10	<10		<10		<10	<10		<10		<10
E. coli	<10		<10		<10	<10		<10		<10	<10		<10		<10
LAB	ND	ND	X**	X	X	$>10^3$	ND	X	X	X	2.6×10^3	ND	X	X	X
S. boulardii	ND	ND	X	X	X	3.38×10^6	ND	X	X	X	1.1×10^6	ND	X	X	X

*The grey painted areas: Related analyses were not conducted according to shelf-life plan.

**X: Since there was no growth of *S. boulardii* on t2; t3, t4 and t5, the analyses were not conducted.

***ND: Not detected

Bellumori et al. (2021) found that not heat-treated cold brews' microbial load increased after 5 days of storage at room temperature, mold and total bacterial count were detected above the microbiological acceptability limits; for all of the pasteurized coffee samples, P, F and FG, microbial load remained below the acceptable limits (no growth of *Salmonella*, *L. monocytogenes*, *B. cereus*, *E. coli*) during 120 days' shelf-life time. These results showed that pasteurization process (65°C for 30 minutes) was successful (Bellumori et al. 2021).

Before fermentation, *S. boulardii* colonies were added to pasteurized cold-brewed coffees to understand the relationship between yeast and coffee. The yeast colonies were observed; it was found that the yeast colonies were alive on coffee medium thus the fermentation could be made successfully.

According to the literature, the samples were fermented for 1 day (Chan, Liu and Toh 2021c). Fermentation was confirmed according to the results of chemical and sensory analysis.

S. boulardii colonies were observed as 3.38 x 10^6 CFU/mL for F and 1.1 x 10^6 CFU/mL for FG as shown in Figure 4.1. In a study, *S. boulardii* provided fermentation and was alive after weeks (Chan, Liu and Toh 2021c). However, the yeast could not be survived during shelf life.

Interestingly the population of *S. boulardii* was more than 6 Log CFU/mL on F while 6 Log CFU/mL was counted on FG at the first day after fermentation. In opposite of the expectations; the *S. boulardii* colony numbers were the highest on F; but it was thought that *S. boulardii* would consume glucose and its colonies would increase rapidly on FG. When sensory comparison was made between F and FG; it was observed that FG had some compounds that led to unsweet, bitter and strength sensory profile and its taste was unfavorable. The growth of *S. boulardii* was not accelerated despite the fact that it used glucose and produced fermentation products, including alcohol, sulphur lactones, higher fusel alcohols, organic acids, aldehydes, ketones, esters, and ketone esters (Lazo Velez et al. 2018). According to literature, a higher glucose concentration can sustain a high level of yeasts' biomass, a low level of organic acid generation, and thus a lower pH (Sweechin et al. 2015). Additionally, de Paula et al. (2019) founded that *S. boulardii* cells consume 3.13 × 10^{-12} g of glucose per hour during exponential treatment, and that transformation and conversion of the substrate to biomass led to 1.42 X 10^{10} cell growth per gram of glucose consumed. Due to the increasing alcohol level (Figure 4.6), the media's pH was lower than its initial value of pH 5.23 however, despite alcohol and

organic acid production by glucose utilization the yeast did not have any biomass increase for FG.

After the first day of fermentation, the probiotic yeast viability in the fermented samples decreased and ended; after one week, the fermented samples could not maintain their probiotic properties. Sweechin et al. (2015) stated that unfavorable media that create a lot of alcohol led to significant decrease in cell growth rate, additionally, it has been demonstrated that ethanol alters the physiology and redox balance of yeast. Thus, as a result of rapid increasing of alcohol levels the rate of cell growth significantly decreased. This is thought to be the reason why *S. boulardii* did not grow in the fermented samples.

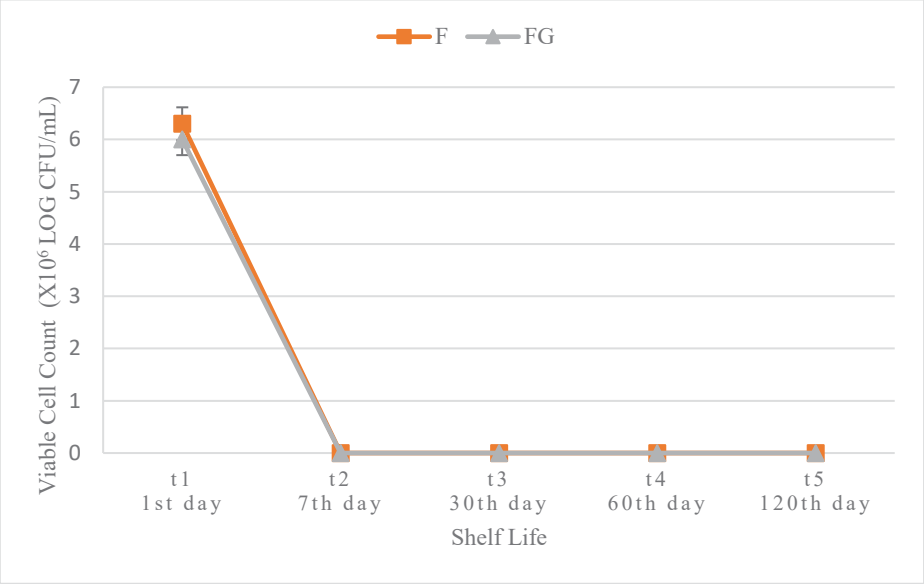

Figure 4. 1. *S. boulardii* growth in Fermented (F) and Fermented with glucose (FG) samples during shelf life

It was observed that *S. boulardii* enhanced the growth of LAB in both F and FG. In a study, starter cultures of the *S. boulardii* were combined with probiotic LAB, and it was found that these starter cultures increased the viability of the probiotic LAB and dramatically increased the lactic acid bacteria's survivability during storage (Figure 4.2) (Chan, Liu and Toh 2021c).

At the first day, t_1, the LAB were greater than 10^3 CFU/g (very high count than excepted thus the dilutions were insufficient) in F and 2.6×10^3 CFU/g in FG.

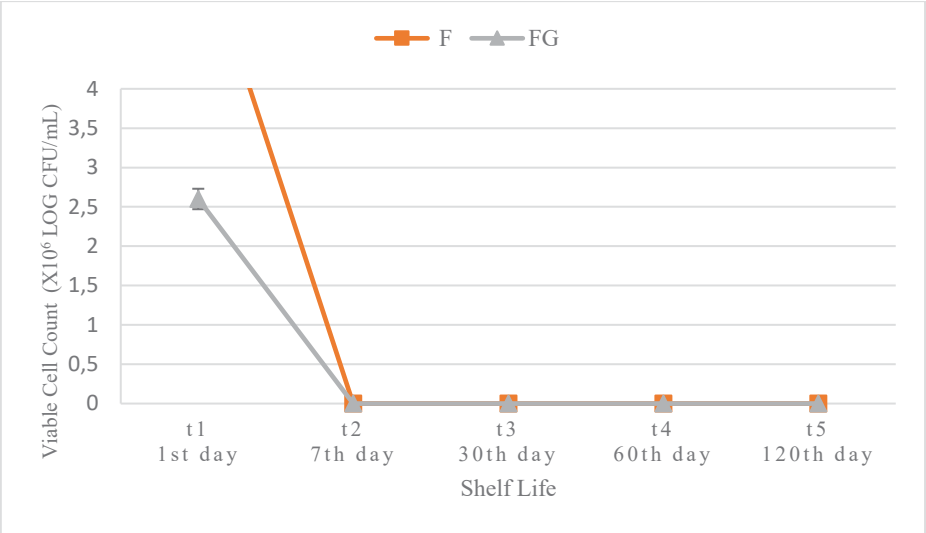

Figure 4. 2. Lactic acid bacteria growth in Fermented (F) and Fermented with glucose (FG) samples during shelf life

After *S. boulardii* cell counts were depleted, LAB growth proportionally decreased. At t_2, no LAB colonies were detected on both F and FG. It was showed that LAB directly affected by yeast presence. Chan et al. (2021 c) fermented hot-brewed coffees with *S. boulardii*, *L. rhamnosus* GG, and a combination of the two, and found that the bacteria survived for 10 weeks in single-cultured *L. rhamnosus* GG coffee brews but viable counts decreased below 10^6 CFU/mL. In samples combined with two microorganisms, both microorganisms survived for 14 weeks; LAB colonies showed survival over 6 Log CFU/ml. Lactic acid bacteria count was proportional to *S. boulardii* presence, with *S. boulardii* cells death, LAB count depleted.

There may be various reasons why probiotic yeast and LAB microorganisms could not survive in this study. Cold brewing method and the selected coffee type may not be suitable for fermentation with *S. boulardii*. To achieve favorable fermentation media decreasing alcohol levels is too important. In a study, it was founded that in the presence of *L. rhamnosus* GG on fermentation medium containing *S. boulardii*, ethanol levels were much lower, which may be related to the probiotic LAB's ability to use glucose to limit the amount of pyruvate that may be used to produce ethanol (Chan et al. 2021 c). Also, the bitter taste caused by ethanol production on FG can be prevented by adding *L. rhamnosus* GG to the fermentation medium to limit alcohol production. Also, sugar supplement could be added after fermentation to prevent rapid growth and ethanol accumulation (Chan and Liu 2022a). To prevent the undesirable features, symbiotic

formulations, fructo-oligosaccharides and therapeutic yeast can be combined instead of glucose since it is anticipated that both the probiotic yeast and the colon's natural good bacteria will be promoted (Mitterdorfer and Viernstein 2001). It may be an option to encapsulate the yeast against depletion, ethanol release and ensure its viability throughout 120-day shelf life.

Another reason why the *S. boulardii* cannot survive after fermentation may be the quality of the cells. The culture could be taken from the collection and viability of the samples could be monitored for one week prior to fermentation. Yeast viability could be checked with other media and/or techniques other than potato dextrose agar (PDA) agar. Yeast viability and cell growth rate could be detected in case of a decrease in viability by monitoring every day for 1 week, the reason for the decrease could be understood.

4.2. Chemical Analysis

Chemical properties of each coffee sample were characterized by pH, total titratable acidity (TTA), brix, total dissolved solids (TDS), color, caffeine concentration, chlorogenic acid (CA), and ethyl alcohol (ethanol). Results are given in the Table 4.2.

Table 4. 2. Chemical Analyses of Pasteurized (P), Fermented (F) And Fermented with Glucose (FG) During Shelf Life

Samples		**Pasteurized (P)**					**Fermented (F)**					**Fermented with Glucose (FG)**				
Interval times/Days		**t1 1st**	**t2 7th**	**t3 30th**	**t4 60th**	**t5 120th**	**t1 1st**	**t2 7th**	**t3 30th**	**t4 60th**	**t5 120th**	**t1 1st**	**t2 7th**	**t3 30th**	**t4 60th**	**t5 120th**
pH		5.23	5.07	5.05	4.93	4.94	5.24	5.28	4.93	5.06	4.95	5.23	5.14	4.92	4.99	4.84
TTA (mL)**		5.5	11.3	12.5	15.1	16.3	5.7	5.8	14.0	13.1	15.8	5.8	7.9	13.8	15.2	18.4
Brix		2.9	2.8	3.0	2.1	3.4	2.6	2.7	2.9	1.7	3.1	2.8	2.9	3.0	1.9	3.5
TDS* (%)**		2.52	2.43	2.56	1.82	2.95	2.26	2.34	2.52	1.47	2.69	2.43	2.52	2.65	1.6	3.04
Color	**L***	17.72	16.83	15.93	15.72	16.06	17.47	16.26	15.94	15.89	16.90	17.50	16.99	16.00	16,05	16.33
	a*	-0.15	-0.65	-1.11	-1.07	-1.12	0.50	-0.65	-1.14	-1.09	-1.21	0.05	-0.57	-1.14	-0,88	-1.22
	b*	3.95	3.61	2.99	2.86	2.74	5.50	3.61	2.90	2.61	2.79	5.09	3.84	3.12	2.79	2.26
Caffeine (mg/L)		997.33	1150.18	1156.92	*	1150.64	1091.28	723.58	1046.3		1081.36	983.34	1009.47	1060.04		1092.75
Chlorogenic Acids (CGAs) (mg/L)		872.96	723,77	752.43		1532.57	919.88	758,40	747.08		1590.6	786.44	836.72	840.38		1530.67
Ethyl Alcohol (g/L)		<0,04	<0,04	ND		0,23	0,07	0,05	1.02		0.2	0.87	0.79	0.07		1.3

*The grey painted areas: Related analysis were not conducted according to shelf-life plan.

**TTA: Total titratable acids to a pH 6.5 expressed in ml of 0.1 N NaOH per 50 mL of coffee,

***TDS: Total dissolved solids, per liter of coffee,

L*a*b* color values of 3-dimensional color coordinates. L*: aperture; L*:0 indicates black, L*:100 indicates white, a*: red/green, b*: yellow/blue.

4.2.1. pH and Total Titratable Acidity (TTA)

The cold brewed coffee sample that was non-treated had an average of pH 5.56, TTA 4.45 mL of 0.1 N NaOH/50 mL coffee. The literature showed that the pH value of cold brew samples prepared with Arabica coffee beans was approximately 5.15 (da Silva Portela et al. 2021), 5.20 (Asiah et al. 2019), 4.79 (Cordaba et al. 2021) and 4.94 (Bellumori et al. 2021). The acidity of non-heat treated cold brew sample prepared with Colombia Arabica coffee beans (at 22°C, 14h) was founded as 3.78 ± 0.17 mg CGAs/g (Cordaba et al. 2021). Yeager et al. (2022) determined that the titratable acidity of a medium roasted, cold brewed coffee samples prepared with Arabica coffee beans (non-Colombian origin, at 22°C, 12h) was approximately 9.13 mL NaOH/50 mL coffee. In addition, Bellumori et al. (2021) found the TTA values of cold brewed samples prepared with Ethiopian Arabica coffee beans (at 20°C, 16 h) and subjected to different processes like UV irradiation, pasteurization, and microfiltration process were 5.07 ± 0.12. Generally, the literature findings for both pH and acidity were significantly different than the analysis outputs. This difference can be caused by coffee beans type and/or origin, brewing type, brewing time and temperature, experiment conditions etc.

The pH and TTA values of P, F and FG during 120 days of shelf life were showed in Figure 4.3 and Figure 4.4.

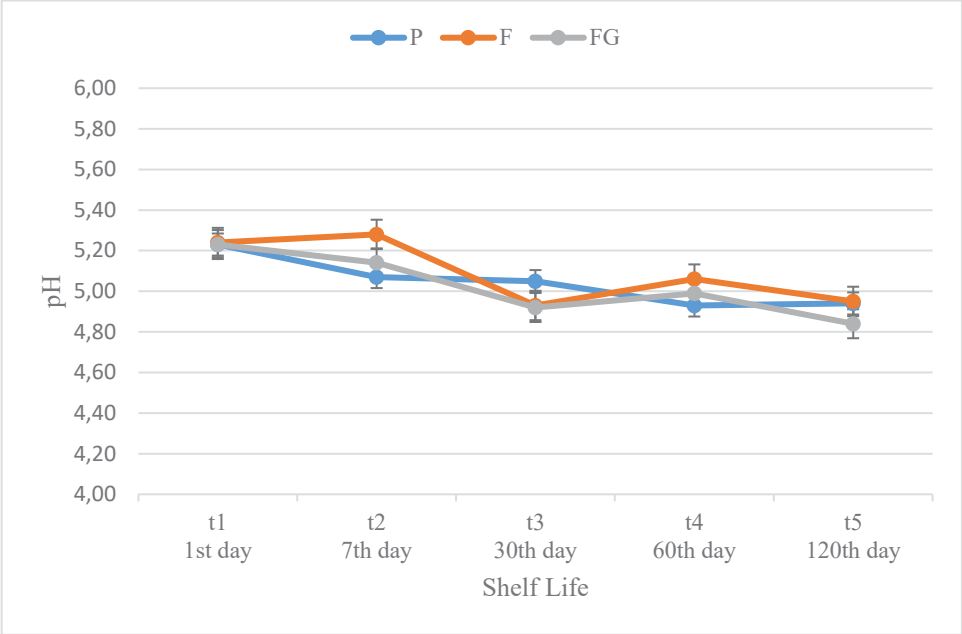

Figure 4. 3. pH values of pasteurized (P), Fermented (F) and Fermented with glucose (FG) samples during shelf life

At first day, t_1, all cold brew coffee samples had pH values in the range of 5.23-5.24 and TTA from 5.5-5.8 mL of NaOH 0.1 M in 50 mL of coffee. pH and TTA values dd not change by fermentation in both F and FG at first day but Chan et al. (2021 c) found that 24 hours after fermentation, the pH value of not fermented cold coffee was 4.92, while the pH value of coffee with added *S. boulardii* (FG) was 4.85; this decrease was not observed after fermentation in the current study and the pH values were significantly higher than values founded by Chan et al. (2021c). The difference may be caused by coffee bean type and brewing method differences; they studied with hot brewed coffee prepared with Ethopian Yirgacheffe Kochere coffee beans.

After t_1, the pH value of each sample did start to decrease (only at t_4 F and FG samples' pH values slightly increased but then decreased). As founded previously (Bellumori et al. 2021), a significant decrease was observed on pasteurized sample's pH with time at the end of 120 days and FG sample's pH level decreased more than P an F samples since acid production was higher because of sugar content of the fermentation medium. pH value of P sample decreased from 5.2 to 4.94 at the end of shelf life. Chan et al. (2021c) stated that because of citric acid utilization by *S. boulardii* there were insignificant changes in acidity or pH values on fermented coffee samples however both

F and FG sample's pH decreased significantly during shelf life because of yeast death. The mean pH value of both F and FG was the same, 5.23, at t_1 but pH of F decreased to 4.95 and pH of FG decreased to 4.84 at t_5. The FG pH level decrease was higher than F because of glucose utilization led to higher acid production on FG.

Fuller and Rao (2018) stated that between pH and TTA no correlation was observed. However, in the current study TTA and pH were adversely proportional. The results showed that P, F and FG samples' acidity increased significantly with time respectively from 5.5 mL NaOH/50 mL coffee to 16.3 mL NaOH/50 mL coffee, 5.7 mL NaOH/50 mL coffee to 15.8 mL NaOH/50 mL coffee and 5.6 mL NaOH/50 mL coffee to 18.4 mL NaOH/50 mL coffee at 4°C. TTA levels of each samples increased at the end of the shelf life, if *S. boulardii* stay alive, it was expected that TTA values of F and FG samples were much higher than P sample's (Sweechin et al. 2015; Lazo-Vélez et al. 2018).

Bellumori et al. (2021) showed that the TTA of pasteurized samples did not increase in 120 days of storage at room temperature however, a very significant increase in TTA and decrease in pH were measured at the end of the shelf life of the pasteurized sample.

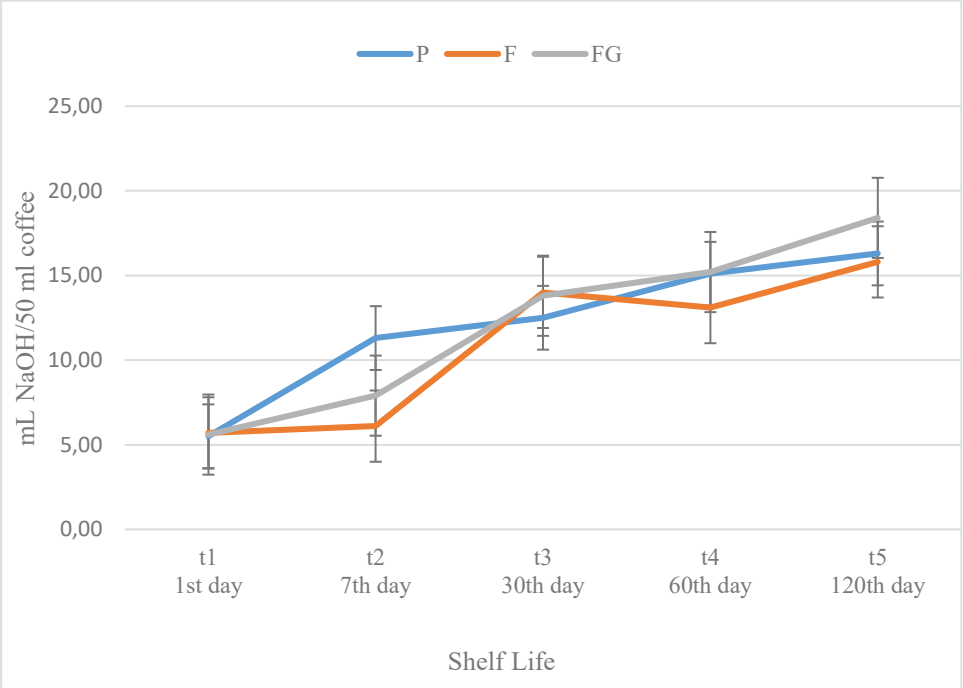

Figure 4. 4. Total titratable acidity values of pasteurized (P), Fermented (F) and Fermented with glucose (FG) samples during shelf life

There is not enough information about TTA of *S. boulardii* fermented coffee samples in Literature.

According to Chan et al. (2021c), *S. boulardii*'s ability to inhibit the production of lactic acid or reduce citric acid concentrations in coffee brews lowered acid stress. Both F and FG samples showed fluctuations on TTA and it was not fully understood exactly why the total acidity increased & which acid was produced on samples. Acidity increase can have many causes; further studies are needed to understand factors having effects acidity such as the acid production by fermentation.

4.2.2. Brix, Total Dissolved Solids (TDS), Extraction Yield (EY)

The soluble solid concentration of sugar solutions was measured by the °Brix (Moreno 2021), in other words brix value represented the concentration of sugar from coffee samples and measured with °Brix refractometer (Asiah at al., 2019). One-degree °Brix showed one gram of sucrose per 100 g of coffee (Gant 2022).

Total dissolved solid (TDS) is percentage of the mass of the liquid; the amount of suspended solids in water. TDS% measured the quantity of coffee compounds per unit water in coffee representing the strength of a cup of coffee (Sim 2022). Brix was used as a proxy to calculate the TDS% of P, F and FG samples. The relationship between degrees of °Brix and TDS% was calculated with Equation 3.1 and the equation enabled calculation of TDS% via refractometer measurement (Moreno 2021).

Temperature has an impact on the solubility and volatility of the coffee soluble component. Fructose is more soluble in cold water than sucrose and glucose and the best temperature range for solubilizing coffee soluble is between 90-96 °C (Asiah et al. 2019). Coffee's total sugar and non-reducing sugar generate sweet and caramel smells and aromas and this explains why cold brew sweeter than hot brew (Asiah et al. 2019). A cup of coffee with a strong flavor will have a high TDS%, while one with a milder, watered-down flavor will probably have a low TDS% (Sim 2022).

Figure 4.5 shows the correlation between °Brix and TDS% of samples and linear relationship was found (Moreno 2021). The non-treated cold brewed coffee sample showed °Brix 2.8 that significantly lower than 4.07 that Asiah at al. (2019) found and

TDS% was 2,43 that lower than 4.03 Yeager et al. (2022) found. The diversity was high probably caused by coffee bean origin and cold brewing method differences.

The °Brix and TDS % values increased until t_3 on each sample. In the range of t_1 to t_3, the °Brix value of the P sample was increased from 2.9 to 3.0, for the F sample from 2.6 to 2.9, and for the FG sample from 2.8 to 3.0. At day 60, the °Brix values decreased but then each sample's °Brix values increased sharply. At t_5 P, F and FG samples' °Brix values were respectively 3.4, 3.1 and 3.5. TDS% values of P, F and FG samples increased with time respectively from 2.52 to 2.95, 2.26 to 2.69 and 2.43 to 3.04 at 4°C.

By measuring the refraction of light passing through a liquid sample, a refractometer calculates degrees brix. Since they are denser than water, liquids containing sugars and/or other dissolved particles cause more light to be refracted when it passes through. The device calculates the °Brix value and compares this to light refraction through water. The obtained refractive index is directly transformed into the weight percent solids content (°Brix). Alcohol (ethanol) has a higher refractive index than water, thus alcohol existence increases brix value. With the fermentation, the brix value of FG was expected to decrease with glucose consumption. But alcohol produced during fermentation prevented °Brix reduction by reducing sugar (Gosset 2012). Although brix calculated as % sugar in the samples, the amount of sugar that the yeast consumed or sugar existence had no impact because alcohol caused an increase in the measurement values (Gosset 2012). Therefore, the brix values of the samples were found to be close to each other; sugar consumption was balanced by alcohol production on FG. In addition, since it contains the most ethanol, the highest °Brix value belongs to FG.

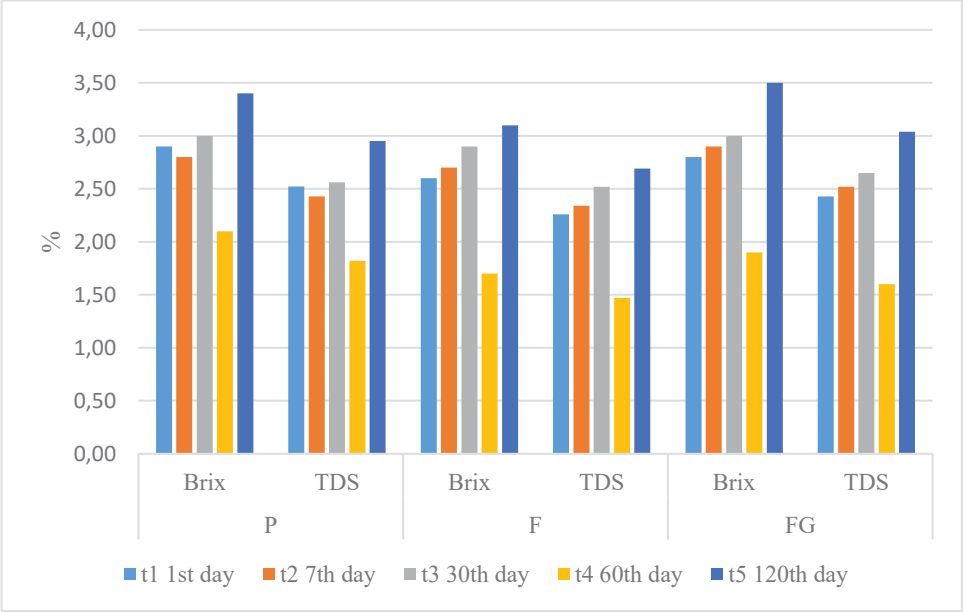

Figure 4. 5. Brix and Total Dissolved Solids % values of pasteurized (P), Fermented (F) and Fermented with glucose (FG) samples during shelf life

There is not enough information about coffee TDS% of *S. boulardii* fermented coffee samples in Literature. P, F and FG showed increased TDS%. After t_5 with higher °Brix and TDS%, each sample became thicker.

TDS is a useful measure of how thoroughly the coffee was extracted (Stayte 2022). By multiplying the product TDS by the entire quantity of water (Ww) divided by the weight of ground coffee (Wc) used for extraction, extraction yield (EY) was obtained (Equation 3.3)

The EY of coffee sample that was non-treated, cold brewed via immersion was 24.3. Cordoba et al. (2021) compared EY of coffee samples that were brewed with different techniques and it was found that the most efficient brewing technique was cold dripping (at 19 ± 2 ◦C, 6.5 h), the EY was 20.66. Cold immersion (at 19 ± 2 ◦C, 14 h) technique is more efficient than hot brewing (at 90 C, 5 min); cold immersion's EY was 19.68 while hot brewing was 18.58 (Cordoba et al. 2021). The extraction yield found in the study was found to be higher than those in the literature. Thus, it can be concluded that the brewing efficiency of the coffee brewed with water at room temperature for 24 hours by immersion met the expectations.

4.2.3. Color

All samples were tested for the CIE-L*a*b* values, which are used to characterize color, to provide the numerical values for the 3D color measurements (Yeager et al. 2022) at room temperature (22°C). CIE method indicates L*a*b* color values in 3-dimensional color coordinates, L* is the aperture; L*:0 indicates black and L*:100 indicates white, a* value indicates red/green; +a* indicates red, -a* indicates green, and b* is the yellow/blue coordinate and +b* indicates yellow and -b* indicates blue (Aldosari et al. 2021; Cordaba et al. 2021).

The color off coffee samples is directly affected by the type/origin of bean, the degree of roasting, and the brewing method (Cordaba et al. 2021; Esquivel and Jimenez 2012; Liang and Kitts 2014; Yeager et al. 2022). While the hot-brewed coffees have a darker color cold-brewed coffees had a tendency to be redder (Yeager et al. 2022) and lighter (Cai et al. 2022). The cold brewed coffee sample's that was not heat-treated color values were as follows; L*:16.30, a*:0.69 and b*:3.64. In a study, it was founded that the L*a*b* values for hot brew coffee that was prepared with Arabica beans (blended from Colombia, Costa Rica and Indonesia) were L*: 29.76 ± 1.70, a*: 23.21 ± 0.61, and b*:38.57 ± 2.43 (Cai et al. 2022). The color values of cold-brewed coffee are very low compared to the values of hot-brewed coffee found in the study. In the same study, the color of cold brew coffee was determined by Cai et al. (2022) to be L*:38.79 0.78, a*:19.14 0.91, and b*:50.19 3.95. The values of cold-brewed coffee found were also much higher than those found in the current study. This is because the origin of the coffee beans was mixed and the brewing method was different; coffee samples were brewed at room temperature for only 8 hours (1:10). Also, Yeager et al. (2022) found that the CIE- L*a*b values of cold brewed coffee that was prepared with medium roasted Arabica beans (not Colombian) at +22 degrees for 12 hours were as follows; L*:32.38±1.82, a*:24.35±2.47, b*:15.86±3.79. Again, in all parameters the literature values were very high when compared with the analysis results at t_1. In the compared studies, the brewing time is very short compared to the current study. Brewing time as long as 24 hours may have caused a decrease in color values. Also, the high diversity probably caused by different growth region, the growth areas have effects on color. In addition, filtering has an effect on color. It is thought that because the efficiency of the filter papers was different, it caused coffee

particles to pass into the samples at different rates, leading to serious differences on the color.

There was no information about fermented, cold brewed coffee's prepared with Colombian Arabica beans color in the Literature. No doubt fermentation directly affects the color. The fermentation led to slightly decrease in lightness and a*; b* values thus increased in yellow and blue color slightly. At t_2 FG sample's lightness and redness were the highest. F and FG samples color values were significantly different from each other; for all parameters FG sample had higher values. With time, each cold brewed sample's L* a* and b* values decreased as Asiah et al. (2019) found until end of shelf life. The color values of P, F and FG samples at t_5 respectively were L*:16.06 a*:-1.12 b*:2.74, L*:16.90 a*:-1.21 b*:2.79 and L*:16.33 a*:-1.22 b*:2.26. Only the L* value increased in each sample. At the end of the shelf life, the highest L* and b* value belonged to the F sample, while the highest a* value belonged to the P sample.

The delta E (ΔE) level refers to the variance between the displayed color and the original color standard of the input content. Greater precision is shown by low ΔE values (≤1.0), whereas a large mismatch is indicated by high ΔE values (≥2) (Mokrzycki and Tatol 2011). The effect of pasteurization on the color was calculated with P sample's color values based on the color values of cold brewed coffee (not fermented & pasteurized) at t_1. The ΔE was found as 1.67. This showed that the color difference could be detectable through close observation but pasteurization did not affect the color too much (Mokrzycki and Tatol 2011).

Then, based on the color values of P the effects of fermentation and adding sugar to the fermentation medium on the color difference was calculated with F and FG at t_1. The ΔE of F was 1.69 and of FG was 1.17. Both F and FG color could be detected by close observation (Mokrzycki and Tatol 2011) but F sample ΔE value was higher than FG. Since the F sample contains more probiotic yeast than the FG sample at t_1, it was interpreted that the fermentation of the yeast changes the color more and adding sugar had no significant effect on color. Since the probiotic yeast was not alive after t_1, the ΔE was not calculated at the end of the shelf life.

The results showed that fermentation had a positive impact on the rise in color values at the conclusion of fermentation.

4.2.4. Ethyl Alcohol (Ethanol)

The ethanol level changes with time as shown in Figure 4.6. For P, the ethanol levels were same at t_1 and t_2 and during shelf life the alcohol level decreased significantly until t_5. The primary product of fermentation is ethanol, which can be produced by a wide range of microorganisms, including bacteria and yeasts (Yang, Liu and Zhang 2007). With pasteurization, all samples became safe for 120 days so that no fermentative microorganisms grew. Thus, for P sample no ethyl alcohol generated until t_5. At t_5, the ethanol level of P was 0,23 g/L. The reason for this is thought to be the growth of an alcohol-producing microorganism other than the pathogenic microorganisms that were controlled.

The ethanol production was higher on F and FG than P because of fermentation with *S. boulardii*; the yeast generates fermentation outputs like alcohols, esters and volatile phenols and quantitatively, alcohols are the most abundant, mainly contributed by ethanol (Chan, Liu and Toh 2021c). F sample had 0,07 g/L ethanol at t_1 and 0,05 g/L at t_2. At the end of the 120 days, the ethanol level increased to 0,2 g/L. At the beginning of shelf life, FG sample ethanol level was 0,87 and significantly higher than P and F samples that is because of the yeast broke down glucose into pyruvic acid, which is then decarboxylated to acetaldehyde and CO_2 via glycolysis and finally, acetaldehyde was further converted to alcohol so, simple sugars can be oxidized and fermented by yeast to produce CO_2, H_2O, and ethanol (Lazo-Velez et al. 2018). To sum up, with fermentation ethanol levels increased (Chan and Liu 2022b). It was thought that this increase led to bad taste. If the probiotic yeast continued to live, it would be expected that the ethanol level would follow a sharp increasing graph but the ethanol production slowed down due to yeast death but at t_5 the ethanol level of FG increased to 1,3 g/L.

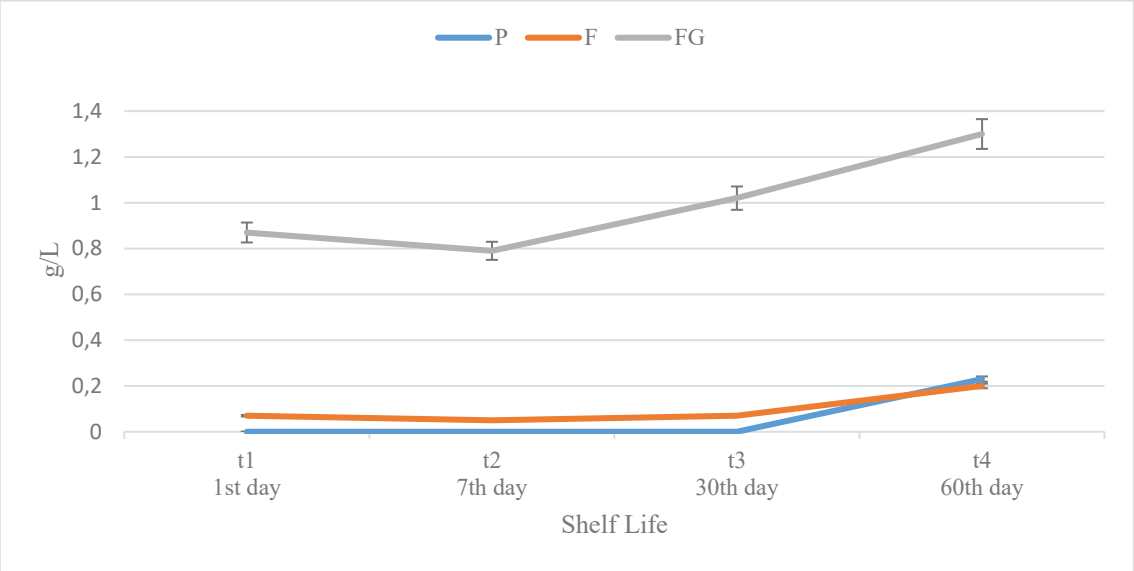

Figure 4. 6. Ethanol levels of pasteurized (P), Fermented (F) and Fermented with glucose (FG) samples during shelf life

According to Chan and Liu's (2022 a) study; *S. boulardii* CNCM-I745 produced ethanol, 2/3-methylbutanol, 3,4-dimethoxystyrene, and decanoic acid with fermentation on coffee brews and these microbial metabolites produce odors such as buttery (diacetyl), smoky (4-ethylphenol), whisky/cognac (2/3-methylbutanol), green/floral (3,4-dimethoxystyrene), and fatty/rancid (decanoic acid) when present in amounts over their thresholds. They reported that although the volatile profiles of coffee headspace were changed by the generation of volatile microbial metabolites, it was unclear whether or not customers would notice or find the change acceptable (Chan and Liu 2022a). However, considering the results of the sensory analysis, it is understood that the alcohol produced directly affects the sensory properties negatively (Fig.4.8). Long-term storage of ethanol, 2/3-methylbutanol, 4-ethylphenol, and 3,4-dimethoxystyrene deposits indicated their durability even after coffee brews lost their nutritional value (Chan and Liu 2022a), which may be the cause of the off-flavor's persistence in fermented coffee samples over the duration of the shelf life. But, the reason why fermented coffee is less liked than unfermented coffee is not only considered as the rate of ethanol produced, but it is thought to be other fermentation outputs.

The ethanol concentrations of the P and F samples are almost the same after 120 days. This shows that ethanol production is directly increased by the presence of glucose.

In addition, storage time has an effect on its concentration. Although ethanol levels tend to decrease at first, they increase after 120 days with time.

4.2.4.1. Statistical Analysis of Ethanol Levels

The ethanol levels were examined in relation to the factors that the period of storage (t_1 and t_5) (storing at + 4° C) and the presence of a sugar source in the fermentation medium (presence and absence). "DOE" (design of experiment) function was used in Minitab (Version 17). Two factors and two levels were introduced for full factorial design with two-replicates. So, there were four combinations of the levels of the factors and with replications eight coffee samples' data were considered. No block variables were assumed for experiments. Effects of factors and their interactions on response were examined at alpha 0.05. The ethanol levels under factors are given at Appendix C.

After introducing the factors to Minitab, the software gave full factorial design summary (Appendix C.1). The residual plot was examined; the responses were assumed as normal but it was determined that the Normal Probability Graph could be improved. (Appendix C.2). According to Normal Plot; time and sugar were significant but their interactions were insignificant (Appendix C.3).

The regression equation showed; interaction's regression coefficient was too low and assumed as insignificant while factors' regression coefficients high and assumed as significant. According to the ANOVA table, p value of storing time (p:0,023) and glucose addition (p:0,001) factors was small and they were significant at alpha 0.05 so ethanol levels were affected by these factors directly (Appendix C.4). Glucose factor was more significant than storing time. However, storing time and glucose interaction (p:0,678) was insignificant; it did not affect ethanol level and time and glucose fortification were independent from each other. The result was useful to understand effects of factors on ethanol levels, and their interaction should not be considered as significant and must be removed to improve model.

Also, the R-sq(pred) (81.70%) value was high and R-sq (95.42%) and R-sq(adj) (91.99%) values were assumed as close to each other. This result showed that the model was good.

After their interaction was removed storing time's p value decreased from 0,023 to 0,011 and glucose addition factor's p value became 0,000. Factors' p values were decreased and they became more significant so they had too much effect on ethanol levels. Lack-of-fit was insignificant and it showed that the model was good and design was satisfactory and useful.

The R-sq(pred) (87.70%) value was increased by removing interaction. R-sq (95.20%) and R-sq(adj) (93.27%) values become more close to each other. It showed that the model was improved. Normality plot became better after interaction was removed (Appendix C.5).

The experimental design results showed similarity and correct proportion with the information in the literature. It can be interpreted that the experiment was designed correctly. The model showed that the ethanol levels increased proportional with storing time and glucose addition. As Chan et al. (2021c) stated, *S. boulardii* generates fermentation outputs and alcohols were the most abundant, mainly contributed by ethanol. Ethanol levels at t_1 were smaller than at t_5; time affected the concentrations. And, ethanol levels of F were smaller than FG at both t_1 and t_5. It can be concluded that glucose addition on fermentation media supported ethanol production.

The design can be reconstructed with coffee samples, where the probiotic yeast remains viable for 120 days. It can be improved with changing and/or increasing factors and their levels and considering block variables. Using more levels increases outputs' range and provides the better understanding of their effects on response. Also using more factors (such as, different brewing method, storing time, storing temperature, sugar type, prebiotic addition etc.) and understanding effects of these factors' interactions help to determine the best fermentation conditions to reduce ethanol levels.

The numerical summaries showed that high ethanol level was achieved with the following combination of storage period and glucose: t_5, with glucose. The lowest ethanol level was achieved with storage period and glucose: t_1, without glucose. To decrease ethanol levels, instead of glucose different type of sugar can be added to fermentation medium. Or, prebiotics could be added to fermentation media.

4.2.5. Caffeine and Chlorogenic Acids (CGAs)

Caffeine and chlorogenic acids (CGAs) are the two primary bioactive ingredients in coffee beverages (Bellumori et al. 2021).

P sample's caffeine content was found as 997,33 mg/L at t_1. The caffeine content of hot brewed coffee (95 °C, 5 min) was founded 1090 mg/L by Angeloni et al. (2019) and it was similar to levels obtained with cold immersion extraction (22°C, 24 h). Due to the oxidation of the coffee acids and oils and the solubility of caffeine at high temperatures, hot brew contains more caffeine than cold brew (Kyroglou, Thanasouli and Vareltzis 2021). In contrast to hot brewing, where the high extraction temperature is thought to be the driving force behind the extraction, cold brewing requires longer time to make up for the lower processing temperatures (Kyroglou, Thanasouli and Vareltzis 2021) and it's possible that the cold method's extended brewing period (24 h) compensates for the temperature differential (90 °C against 22 °C) (Angeloni et al. 2019). The caffeine concentration of P was comparable to that founded by Angeloni et al. (2019), who discovered that 970 mg/L of caffeine was present in cold brew coffee that was obtained at room temperature after six hours of extraction time. Also, in a different study the caffeine content of the cold brew coffee (15 C, 15 h) ranges were about from 1166 to 1801 mg/L (da Silva Portela et al. 2021). The differences are thought to be due to the change in brewing time & temperature (Angeloni et al. 2019), coffee bean origin, bean growth region, roasting degree etc. According to literature, grind size of coffee does not alter caffeine contents of cold brew samples considerably, and extraction of caffeine is not depending on surface area but longer brewing times for the cold brew samples led to increased caffeine extraction (Fuller and Rao, 2017; Kyroglou, Thanasouli and Vareltzis 2021).

Pasteurization had no significant effect on the caffeine concentration as reported by Bellumori et al. (2021). Additionally, because cold brewing takes a long time, both cold brew and hot brew coffees have roughly the same amount of caffeine.

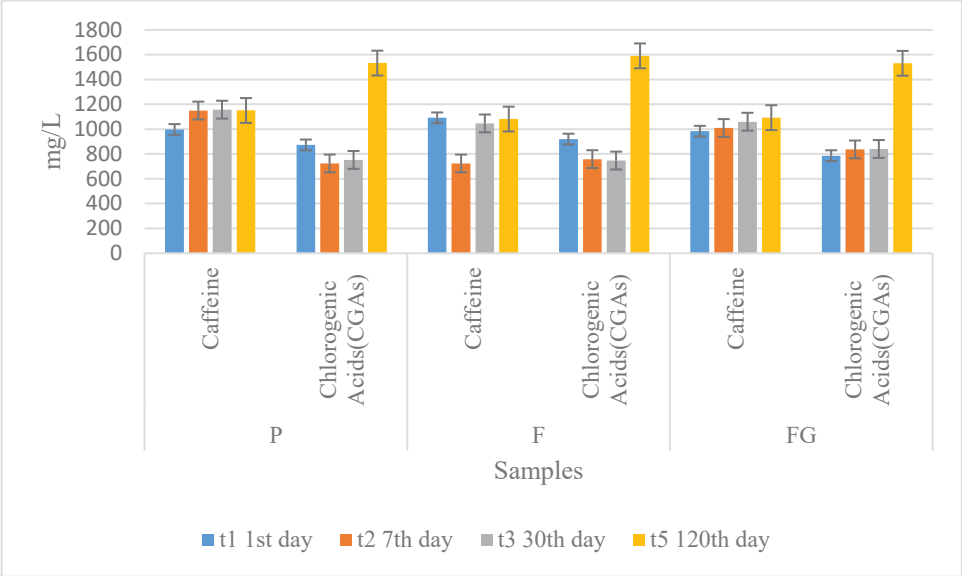

Figure 4. 7. Caffeine and Chlorogenic Acids (CGAs) values of pasteurized (P), Fermented (F) and Fermented with glucose (FG) samples during shelf life

Fermentation with *S. boulardii* did alter caffeine that is principal coffee bioactive compounds (cold storage) as Chan et al. (2021c) founded.

As shown in Figure 4.7, P sample caffeine content increased at t_2 and then did not alter. Caffeine concentrations of F and FG increased during 120 days as opposite of Asiah et al. (2019) (only the F sample's caffeine concentration decreased at t_2; the reason for this is not understood). However, while the caffeine content did not change significantly in the unfermented sample, P, an increase was observed in the fermented samples. This may be because the coffee loses water as it waits and the amount of caffeine per ml increases. Caffeine is stable, does not evaporate or is produced. The reason for the increase may also be the collapse of the suspended molecules to the bottom.

At the end of the shelf life, caffeine concentrations of P, F and FG respectively were 1150.64 mg/L, 1081.36 mg/L and 1092.75 mg/L. The highest caffeine concentration was found in the P sample and the caffeine in the FG sample was slightly higher than the F sample's during the shelf life. The negative effect of fermentation on the caffeine concentration was found promising for low-caffeine product design.

Coffee contains phenolic substances called CGAs, which are known to increase the acidity, astringency, and bitterness of the brew (Angeloni et al. 2019). The chlorogenic acid concentration of FG sample was significantly higher than P and F samples and their

CGAs concentrations were similar during shelf life. Over time, the amount of CGAs in P and FG samples increased and there are studies supporting this in the literature (Asiah et al. 2019) but interestingly F sample's CGAs content decreased with time until t_5.

At t_1, CGAs contents of P, F and FG respectively were 872,96 mg/L, 919,88 mg/L and 786,44 mg/L. According to Bellumori et al. (2021) non-heat treated cold brew sample's (Arabica coffee - Ethiopian) CGAs value was 2900 mg/L, and according to da Silva Portela et al. (2021) CGAs content of cold brew coffe sample (Arabica coffee - Mundo Novo) prepared at 15 °C, 15 hours was 4028 mg/L. These results were significantly higher than P, F and FG samples'. But in another study it was founded that commerical cold brewed coffee's CGAs concantration was 361.07 mg/L (Rao, Fuller and Grim 2020) and also Angeloni et al. (2019) founded cold brewed sample's (at 22 °C, 6 h) CGAs concentration as 510 mg/L. The region where the coffee is grown, the brewing method, the degree of roasting of the coffee have a significant effect on the CGAs. Temperature affects CGAs concentrations regardless of the extraction technique, or contact time (Angeloni et al. 2019). This explains the concentration differences.

After t_2, CGAs content of FG samples increased and of P and F samples decreased significantly. Then, P and FG samples' CGAs content increased and F sample's CGAs decreased. At the end of the shelf life, the phenolic content showed a very rapid increase; CGAs concentrations of P, F and FG respectively were 1532.57 mg/L, 1590.60 mg/L and 1530.67 mg/L. Bellumori et al. (2021) stated that the CGAs content of pasteurized cold brewed coffee remained stable over time (for 120 days) but the study showed opposite results. It is thought that this sudden increase is due to the sedimentation of the coffee grounds as the coffee samples stayed and the complete transition of the remaining components in the coffee particles to the liquid phase.

4.3. Sensorial Evaluation

4.3.1. Sensory Analysis with Panellists

The sensory analysis test was carried out with 31 panelists in a panel room (Appendix E), results are given at Appendix F. For samples of P, F and FG the sensory analysis results were illustrated in radar/spider plot in Figure 4.8 which reveal scalar differences in the flavor profiles of the respective methods in terms of color, viscosity, odor, aroma, taste, coffee strength, sweetness, bitterness, and sourness that characterized cold brewed coffee beverage (Cordaba et al. 2021).

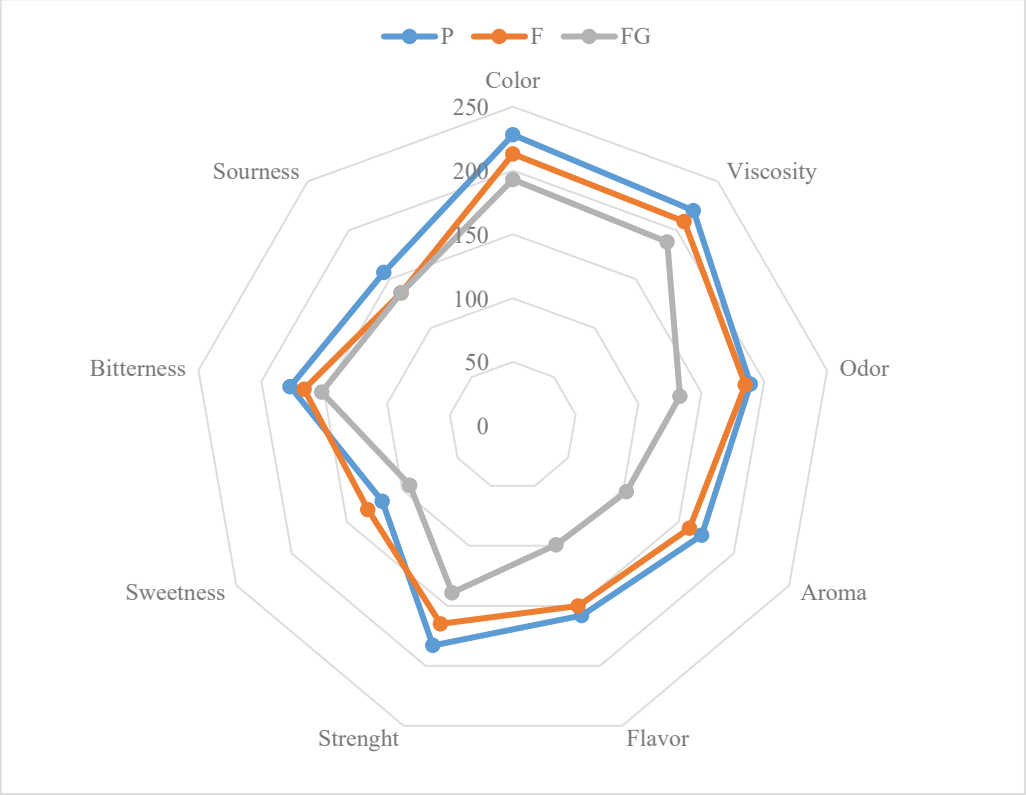

Figure 4. 8. Sensory analysis result of pasteurized (P), Fermented (F) and Fermented with glucose (FG) samples during shelf life

The samples were coded with the following independent 3-digit numbers: P:845, F:563, and FG:237. The panelists then rated the samples using a 9-point scale panel ranging from 1 to 9 (Degirmencioglu et al. 2016). The highest point was 279 (9x31) for each parameter.

Firstly, as Bellumori (2021) found that, sensory properties of the samples were not affected by heat treatment (65 °C, 30 min); there was no foreign or burned taste on all coffee samples.

Because they make up the majority of a coffee drinker's sensory experience, aromatic components are particularly significant in coffee beverage (Angeloni et al. 2019). Regarding the effects of the fermentation of cold brewed coffee samples and effects of glucose addition on fermentation medium on the sensory profile, the results showed that these variables had a significant effect (at alpha 0.05) on aroma (p:0.000, F:13,40), flavor (p:0.00, F:8,31), and sweetness (p:0.025 F:3.86). Each samples' residual plots, normality plot was good and the responses were assumed as normal (Appendix F.2). The lowest scores with regard to all parameters except sourness (Appendix F.2.4) of sample was FG. Prior research on cold-brewed coffee has demonstrated that the brewing technique has an impact on the sweetness of the flavor and other characteristics (Angeloni et al. 2019; Cordoba et al. 2019; Han, Boo, & Chung, 2020) and likewise, a hedonic scale and an overall preference ranking test revealed that the main characteristic of the cold immersion brewing technique was its sweetness (Cordaba et al. 2021). P generally had higher intensities for these characteristics, with the exception of sweetness intensity, which was equivalent to F, while FG had lower intensities for these characteristics (Figure 4.8, Appendix F.2.3). The most preferred sample in terms of sweetness was found to be the F. It shows that in addition to cold brew sweetness, fermentation with *S. Boulardii* increased sweet flavor of the sample. There was no discernible distinction between P and F overall (p: 0,381, F:0,78) (Appendix F.2.6); results were very close to each other (Bellumori et al. 2021) and P, F samples had higher sensory attributes scores than FG.

According to statistical analysis conducted for F and FG; glucose addition had a particularly dominant effect on the aroma, flavor and sweetness (p:0.000, F:7.72) (Appendix F.2.5). The sourness was found insignificant (p:0,409, F:0,90) for F and FG. It was thought that the flavor, aroma, bitterness attributes in FG were scored lower than the other attributes due to yeast's fermentation outputs. Glucose enhanced yeast activities and fermentation products led to unsweet sour taste and undesired aroma. Angeloni et al.

(2019) stated that increased sweetness and sugar caramelization intensity were used to describe cold brew coffees, whereas the fermentation with glucose method was characterized by bad odor, undesirable aroma & flavor and less sweet taste so FG was found as the least preferred sample.

4.3.2. Sensorial Evaluation During Shelf Life

The Figure 4.9 shows the taste, color and odor changes of each sample during their shelf life. The sensory qualities of a sample are significantly affected by changes in chemical constituents (Asiah et al. 2019). The color, odor and taste of the samples were sensorially controlled throughout their shelf life, and the evaluation is given in Appendix G.

At the first, the not heat treated, cold brewed sample showed good and usual sensory properties. The color of each sample was redder than classic hot brewed filtered coffees. Taste of P and F smoother than classic hot brewed filter coffees. The color and smell of P, F and FG were close to each other but taste of FG was disliked. While P's taste was defined as smoother than standard filter coffee, F was a little bit sour but generally same as P. At 7^{th} day, the smell and color were same for each sample. There was no taste alteration on P and F. The FG's taste became sourer and more unfavorable. At 30^{th} day, the samples F and FG became a bit sourer than previous control day. The smell and color parameters were the same for each sample. At 60^{th} day, FG sample was defined as undrinkable in terms of taste, and its smell became strong. The F sample's taste was significantly sourer than P, samples odor was close to each other. Colors of P, F, and FG samples were very similar and darker than previous control step. At the last day of shelf life, each coffee samples taste was altered and sourer. Their color became darker and red color was disappeared. They became thicker and denser. Only P sample was drinkable. Bellumori et al. (2021) stated, the pasteurized sample revealed a flavor profile that was unaffected. It could not be said that the taste was not affected, but P sample's sensory parameters did not change significantly, as much as fermented products.

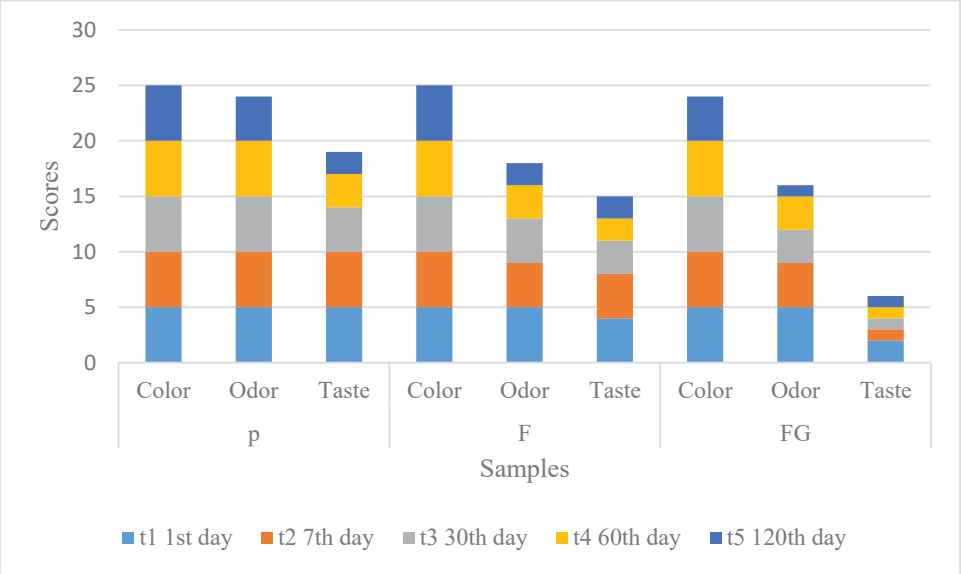

Figure 4. 9. Sensory Evaluation results of pasteurized (P), Fermented (F) and Fermented with glucose (FG) samples During Storage

At the beginning of their shelf life, P and F showed nearly same taste but with time F sample become sourer than P. Because of all parameters and sensory outputs except taste showed F sample desirable and good, and desired aromas could be added.

While using different material for fortification with the yeast, desired aromas like hazelnut, caramel, vanilla etc. may be used to support good taste.

4.4. Consumer Survey Results

In order to understand whether the product can be demanded in the market, a general survey was conducted with google surveys without mentioning the product and 207 people participated in the survey. Coffee drinking habits and awareness of probiotic products were questioned. The Survey results are given at Appendix H with pie-charts.

Most of the interviewers were women with a share of 81.4%. The middle age group was in the majority, people between the ages of 21-30 followed it. Most of the interviewers said that they consume more than 1 cup of coffee every day, while 32.4% stated that they drink 1 cup of coffee every day and 1% of them do not consume coffee;

this result showed that coffee consumption is high in Türkiye. Those who stated that they experience negative effects such as heart palpitations were 16%, the percentage was not small but some part of these people at % 16 share may be under placebo affect because of negative reputation of caffeine. Further studies can be done with people who claimed coffees' negative effects (sleeplessness, heart palpitation etc.) to understand these effects with decaffeinated coffee, standard filter coffee and probiotic cold brewed coffee.

Turkish coffee/ Dibek coffee is the most consumed coffee type with a 53.0% share, then come filtered coffee. 56.3% of the interviewees stated that they do not consume cold/ice coffee. A large part of them stated that they prefer unsweetened/sugarless coffee.

While the ready-to drink cold coffee is not consumed with a share of 58.9%, 24.2% choose to consume it once a month or rarely. Consumers mostly make their own coffee, 23.8% buy it from the market. It is though that the percentages are affected directly by economic conditions. Purchasing power is important issue on marketing and product design. The product price and economic conditions most likely directly increase or decrease the preferences thus with competitive prices consumers may be directing to buy the product.

Most of the participants are familiar with probiotics and they consume its, yoghurt is the most preferred probiotic product, followed by ayran and pickles.16.9% of the participants do not consume dairy products even though they have no allergies and people with %6,3 share have dairy products allergy; these consumers that do not prefer/cannot consume dairy products, probably will demand of the probiotic cold coffee.

It was concluded that consumers who consume probiotic food and mostly drink coffee every day will be interested in the product. High consumption ratio of filtered coffee also supported this. Offered positive health effects, smooth taste and attractive color of the product most probably affect people in a good way and wanted them to consume it.

CHAPTER 5

CONCLUSION

This study demonstrated the effects of *S. boulardii* CNCM-I745 fermentation on cold brew coffee samples in both with glucose and sugar-free fermentation medium.It was observed that *S. boulardii* did not survive after 7 days despite it fermented cold brew coffee samples. Live counts in cultured *S. boulardii* coffee brews dropped below 6 Log CFU/mL over 7 days at 4°C. During its lifetime *S. boulardii* increased the growth of lactic acid bacteria, but after the yeast died, the lactic acid bacteria were also depleted. It was thought that the reason why the probiotic yeast could not survive was the high alcohol concentration.

The pH value of all the samples decreased during the shelf life, but the most decrease was seen in the fermented samples especially on FG due to higher acid production. It was hypothesized that *S. boulardii* decreased post-acidification in the F sample by consuming citric acid or by competing with other organisms for metabolizable substrates, potentially reducing the production of lactic acid by lactic acid bacteia.

Chan et al (2021c) mentioned that more sensory studies are needed to investigate the effects of probiotic metabolites in *S. boulardii* added coffee on consumer acceptance. According to the sensory analyzes performed in the current study the pasteurized samples displayed an unchanged flavor characteristic for the same shelf-life. However, it was not possible to consume fermented samples at the end of their shelf life without applying a different process or adding auxiliary flavors. Due to the fermentation byproducts, the flavor was unpleasant. Since the pasteurized product was drinkable, it was concluded that the sensory problems were caused by fermentation products due to mainly ethanol, not cold brewing. Even in coffee brews with lower nutrient levels, accumulations of ethanol, 2/3-methylbutanol, 4-ethylphenol, and 3,4-dimethoxystyrene continue over the course of long-term storage (Chan and Liu 2022a).

Flavorings can be added after fermentation to balance overall flavors or bring back lost coffee qualities (such as hazelnut, cocoa, chocolate, or vanilla) (Chan and Liu 2022a). If the flavors are not found enough, it is necessary to limit the production of ethanol. Ethanol levels are significantly reduced in the presence of *L. rhamnosus* GG, which may

be dependent on glucose utilization by the probiotic LAB, effectively limiting the pyruvate available for ethanol production. (Chan vd., 2021). Thus, the bitter taste caused by ethanol production can be prevented by adding *L. rhamnosus* GG to the fermentation medium.

Different sugar source could be used. The addition of glucose, a cheap and widely available carbon source that produces energy in the form of ATP from substrate-level phosphorylation, can help coffee brews overcome nutrient shortages (Chan, Liu and Toh 2021c).

Or, slowing substrate transfer rates through microencapsulation may be more effective at maintaining organoleptic qualities.

Caffeine, chlorogenic acid and ethanol content of fermented samples changed during shelf life. It was understood that neither storing time nor glucose fortification affected chemical parameters.

For future studies it has been found that glucose is not a good carbon source for fermentation, and pasteurization at 65 degrees for 30 minutes is sufficient. Further research is needed in order to improve the survival of probiotic yeast in fermented, cold brewed coffee samples. Also, to improve the sensory characteristics of probiotic coffee samples, different production methods that were mentioned above could be applied.

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APPENDIX A

THE COMPARISON OF *S. BOULARDII* AND *S. CEREVISIAE*

Features		***S. Cerevisiae***	***S. Boulardii***
Optimal growth temperature		30 °C	37 °C
High temperature resistance (52 °C)		45% viability	65% viability
Acid pH resistance (pH = 2 for one hour)		No—30% viability	Yes—75% viability
Tolerance to bile acids (>0.3%(*w*/*v*))		No—Survival up to 0.15% (*w*/*v*)	No—Survival up to 0.10% (*w*/*v*)
Basic pH resistance (pH = 8)		Yes	Yes
Assimilation of galactose		Yes	No
Ploidy		Diploid or haploid	Always diploid
Homo or heterothallic		Homothallic	Homothallic
Mating type		Both	Both
Sporulation		Sporogenous	Asporogenous, but produces fertile hybrids with *S. cerevisiae*
Pseudo-hyphal switching		Normal	Increased
Retrotransposon (Ty elements)		Intact Ty elements	No intact Ty1, 3 or 4 elements
Adhesion to epithelial cells	Normal microbiome (mice and human)	No	No
	Gnotobiotic mice	Unknown	Yes
	Humans treated with ampicillin	Unknown	Yes

Appendix A. The comparison of S. *boulardii* and *S. cerevisiae*

(Source: Pais et al. 2020)

APPENDIX B

SURVEY QUESTIONS

1. Gender
2. Age
3. Coffee consumption frequency
4. The most preferred coffee type
5. How do you purchase coffee?
6. How do you consume coffee (with milk, sugar, plain etc.)?
7. Do you consume iced/cold coffee?
8. Do you experience negative effects such as heart palpitations when you consume coffee?
9. Do you consume dairy products/do you have dairy allergy?
10. Do you consume probiotic products?
11. If yes, which prebiotic products do you consume?(several answers)
12. Do you prefer cold coffees, if yes what is the frequency?
13. What is the highest price you would pay for a probiotic cold coffee (250 mL)?

APPENDIX C

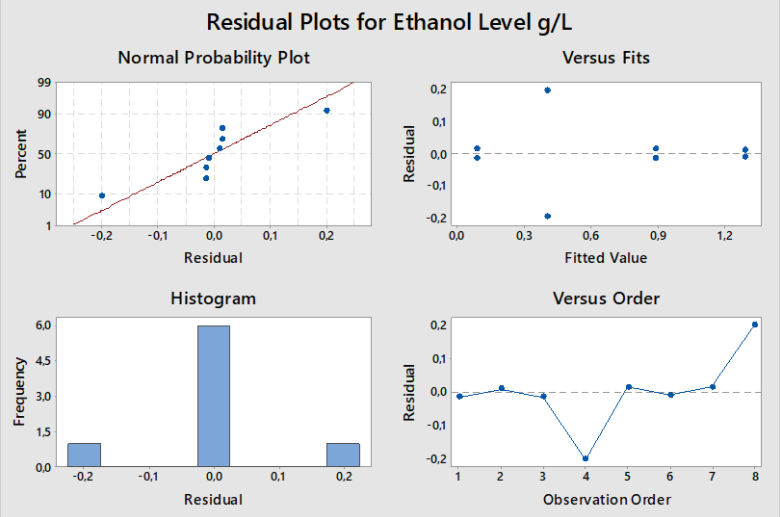
ETHANOL ANALYSIS

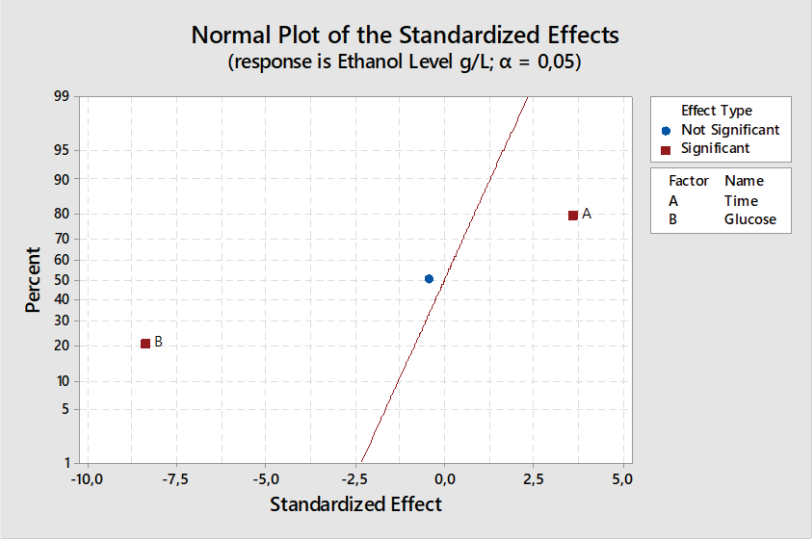
C.1.Full Factorial Design

Factors:	2	Base Design:	2; 4
Runs:	8	Replicates:	2
Blocks:	1	Center pts (total):	0

All terms are free from aliasing.

C.2. Normal and Residual Plots





C.3. Regression Analysis

Ethanol Level g/L = 0,6650 + 0,1800 Time - 0,4225 Glucose - 0,0225 Time*Glucose

C.4. Analysis of Variance

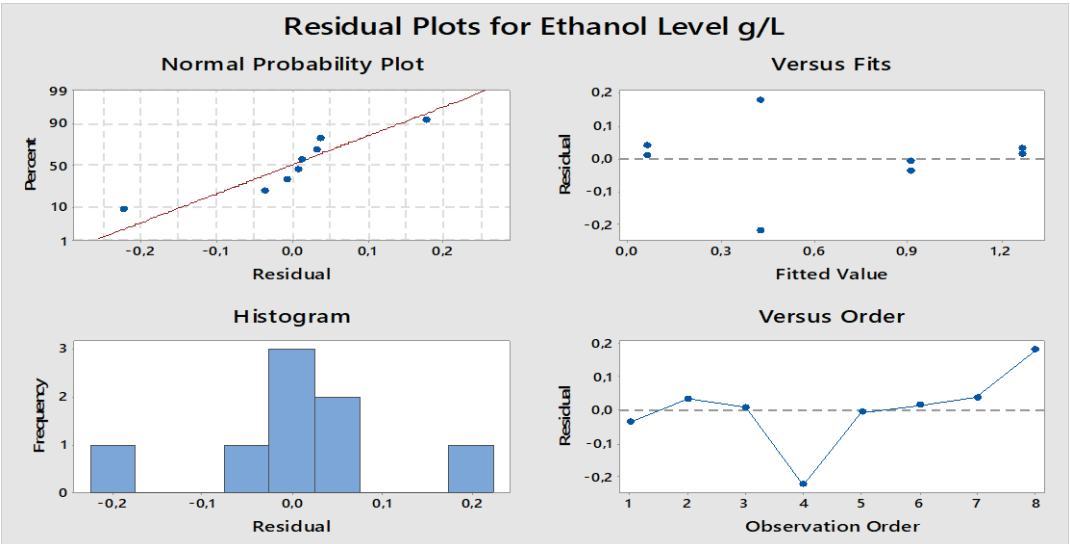
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	3	1,69130	0,56377	27,81	0,004
Linear	2	1,68725	0,84362	41,61	0,002
Time	1	0,25920	0,25920	12,78	0,023
Glucose	1	1,42805	1,42805	70,43	0,001
2-Way Interactions	1	0,00405	0,00405	0,20	0,678
Time*Glucose	1	0,00405	0,00405	0,20	0,678
Error	4	0,08110	0,02028		
Total	7	1,77240			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,142390	95,42%	91,99%	81,70%

C.5. After Removing Interaction

C.5.1. Normal and Residual Plots After



C.5.2. After Remove Interaction

Ethanol Level g/L = 0,6650 + 0,1800 Time – 0,4225 Glucose

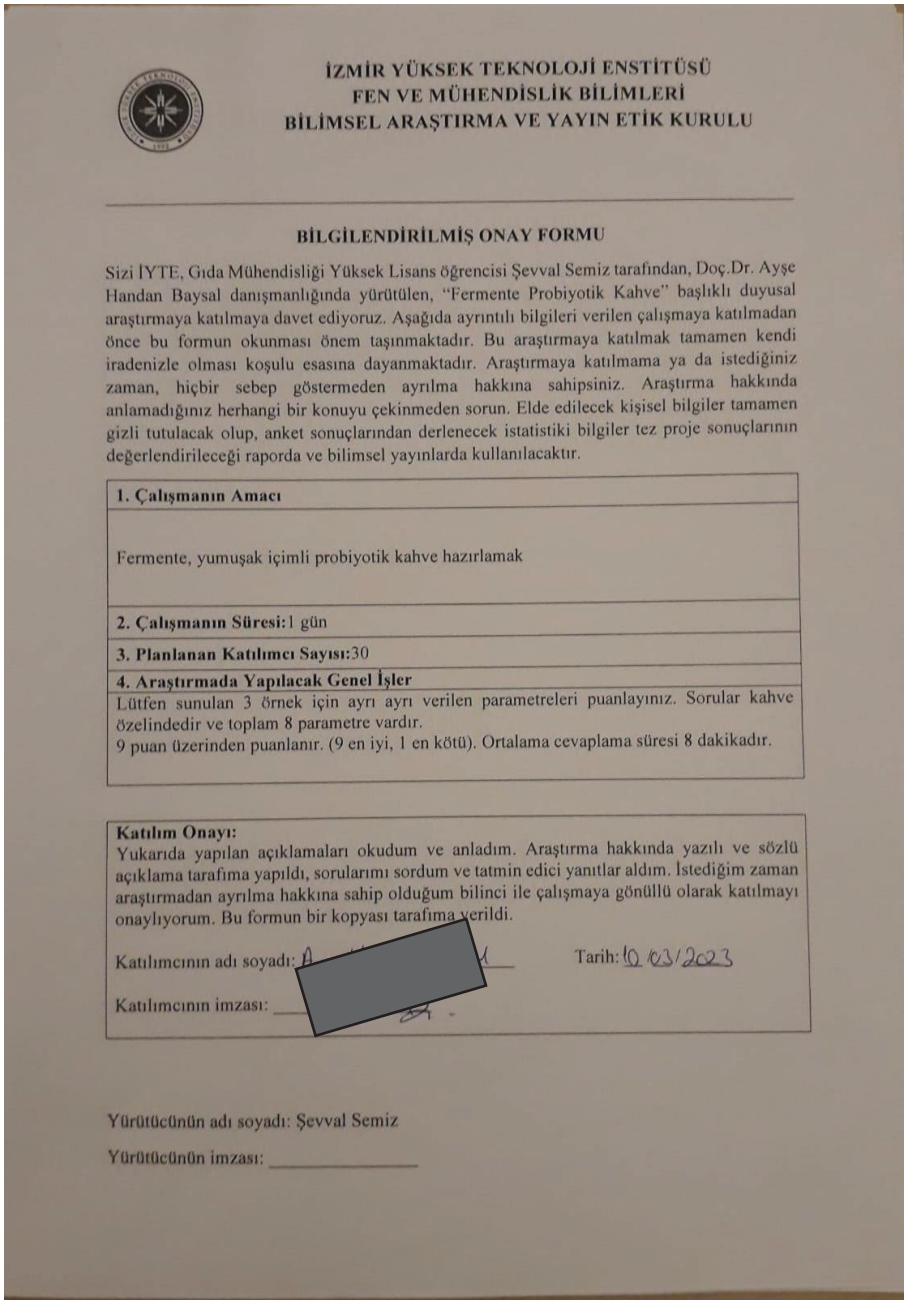
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	2	1,68725	0,84363	49,54	0,001
Linear	2	1,68725	0,84363	49,54	0,001
Time	1	0,25920	0,25920	15,22	0,011
Glucose	1	1,42805	1,42805	83,85	0,000
Error	5	0,08515	0,01703		
Lack-of-Fit	1	0,00405	0,00405	0,20	0,678
Pure Error	4	0,08110	0,02027		
Total	7	1,77240			

S	R-sq	R-sq(adj)	R-sq(pred)
0,130499	95,20%	93,27%	87,70%

APPENDIX D

SENSORY ANALYSIS ETHICS

D.1. Panellist Informed Consent Form



İZMİR YÜKSEK TEKNOLOJİ ENSTİTÜSÜ
FEN VE MÜHENDİSLİK BİLİMLERİ
BİLİMSEL ARAŞTIRMA VE YAYIN ETİK KURULU

BİLGİLENDİRİLMİŞ ONAY FORMU

Sizi İYTE, Gıda Mühendisliği Yüksek Lisans öğrencisi Şevval Semiz tarafından, Doç.Dr. Ayşe Handan Baysal danışmanlığında yürütülen, "Fermente Probiyotik Kahve" başlıklı duyusal araştırmaya katılmaya davet ediyoruz. Aşağıda ayrıntılı bilgileri verilen çalışmaya katılmadan önce bu formun okunması önem taşınmaktadır. Bu araştırmaya katılmak tamamen kendi iradenizle olması koşulu esasına dayanmaktadır. Araştırmaya katılmama ya da istediğiniz zaman, hiçbir sebep göstermeden ayrılma hakkına sahipsiniz. Araştırma hakkında anlamadığınız herhangi bir konuyu çekinmeden sorun. Elde edilecek kişisel bilgiler tamamen gizli tutulacak olup, anket sonuçlarından derlenecek istatistiki bilgiler tez proje sonuçlarının değerlendirileceği raporda ve bilimsel yayınlarda kullanılacaktır.

1. Çalışmanın Amacı
Fermente, yumuşak içimli probiyotik kahve hazırlamak
2. Çalışmanın Süresi: 1 gün
3. Planlanan Katılımcı Sayısı: 30
4. Araştırmada Yapılacak Genel İşler
Lütfen sunulan 3 örnek için ayrı ayrı verilen parametreleri puanlayınız. Sorular kahve özelindedir ve toplam 8 parametre vardır. 9 puan üzerinden puanlanır. (9 en iyi, 1 en kötü). Ortalama cevaplama süresi 8 dakikadır.

Katılım Onayı:
Yukarıda yapılan açıklamaları okudum ve anladım. Araştırma hakkında yazılı ve sözlü açıklama tarafıma yapıldı, sorularımı sordum ve tatmin edici yanıtlar aldım. İstediğim zaman araştırmadan ayrılma hakkına sahip olduğum bilinci ile çalışmaya gönüllü olarak katılmayı onaylıyorum. Bu formun bir kopyası tarafıma verildi.

Katılımcının adı soyadı: [redacted] Tarih: 10/03/2023

Katılımcının imzası: [redacted]

Yürütücünün adı soyadı: Şevval Semiz

Yürütücünün imzası: _____

D.2. Sensory Analysis Evaluation Form

Scoring Test

Name and Surname:	**Date**:…/…./2022
Product: Fermented Coffee	**Hour**:….:….

Please scoring the codded samples according to quality parameters that given below using a 9-point scale panel ranging from 1 to 9. The point scale instructions are given below.

Quality Criteria's	**Sample Codes**		
	845	563	237
Color			
Viscosity			
Odor			
Aroma			
Flavor			
Sweetness			
Bitterness			
Sourness			

Point Scale according to like	**Desired Parameters**	**Undesired Parameters**
1-Too bad, cannot drink	Light color-turn to red	Not homogeny, separated phases
2-Too bad	Usual texture	Gas formation
3-Bad	Homogeny	Very viscous
4-Bad than medium, good than bad	Normal, slight sweet taste	Suspended/sedimented particles
5-Medium	Usual smell	Sourness/bitterness
6- Bad than good, good than medium		Bad odor
7-Good		
8-Very good		
9-Perfect		

Thank you for your attendance

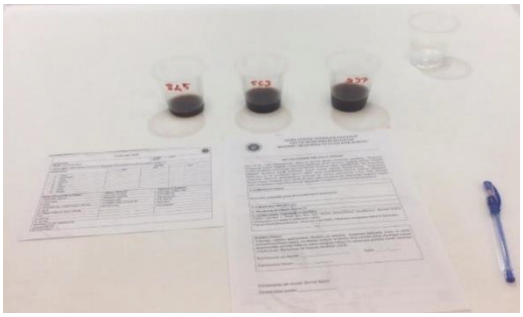
Şevval Semiz
05317224047

APPENDIX E

SENSORY ANALYSIS PANEL ROOM

E.1. Panels

E.2. Samples and Evaluation Form



E.3 Panellists During Sensory Analyses

APPENDIX F

SENSORY ANALYSIS RESULTS

F.1. Answers

Panelist No	Color			Viscosity			Odor			Aroma			Flavor			Coffee Strenght			Sweentness			Bitterness			Sourness		
	845	563	237	845	563	237	845	563	237	845	563	237	845	563	237	845	563	237	845	563	237	845	563	237	845	563	237
1	7	4	6	4	4	4	5	2	2	6	7	2	7	4	4	5	5	5	4	4	4	7	6	6	5	7	7
2	7	7	6	8	8	7	7	5	4	6	4	4	6	4	5	8	6	6	3	3	3	8	7	2	3	5	3
3	7	7	3	7	7	3	7	7	3	7	4	3	7	4	3	7	5	3	3	5	3	7	5	3	7	5	3
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5	8	8	7	6	6	4	7	7	8	6	5	5	4	3	4	7	6	5	4	5	4	8	4	3	8	4	3
6	8	8	8	8	8	8	4	4	3	4	5	1	5	6	1	7	5	5	5	3	3	4	5	5	7	1	2
7	9	9	9	9	9	9	9	9	2	7	8	3	7	8	2	9	6	8	3	4	1	9	6	9	8	5	9
8	7	4	6	6	4	5	3	7	4	5	4	3	6	4	2	4	3	6	5	4	4	5	4	4	5	4	7
9	8	5	5	6	6	6	3	6	5	7	8	2	7	8	2	7	5	2	6	5	2	6	5	7	6	5	2
10	6	7	2	3	6	5	4	7	8	3	7	1	1	6	1	9	7	9	1	3	1	9	6	9	8	5	2
11	9	9	9	9	9	9	6	5	3	5	4	2	2	2	1	8	4	3	2	3	2	7	5	5	2	1	5
12	6	5	6	3	2	2	8	2	3	4	2	1	4	1	1	8	3	5	5	1	1	7	3	7	4	8	5
13	7	9	5	9	9	9	2	4	2	4	3	2	3	8	1	6	9	2	6	9	1	5	5	1	5	5	2
14	9	9	8	8	8	8	6	4	5	3	3	6	3	3	5	4	3	4	4	6	5	5	5	5	3	3	3
15	7	6	7	6	5	4	3	4	2	2	5	1	1	2	1	1	4	2	2	4	3	1	5	2	2	2	5
16	6	5	4	7	7	6	7	6	5	3	6	5	3	6	5	2	7	4	4	7	4	2	7	5	7	6	5
17	8	8	7	8	8	7	7	7	6	8	3	4	5	5	3	6	6	5	7	7	3	7	6	3	9	4	3
18	9	9	9	9	8	9	9	8	9	8	7	5	8	7	5	9	7	7	1	1	1	9	7	7	8	9	5
19	8	6	6	9	7	3	9	7	5	7	2	4	3	1	3	4	5	1	2	1	1	1	4	1	3	1	1
20	7	7	8	9	8	7	6	5	3	7	8	3	5	6	2	4	8	5	4	4	1	6	7	6	7	4	1
21	9	8	3	9	8	3	9	8	3	9	8	3	9	8	3	9	8	3	2	7	5	2	2	3	2	2	3
22	4	5	4	5	4	5	4	7	5	4	4	5	3	5	6	3	5	7	7	6	5	6	7	8	3	3	5
23	8	8	8	8	8	8	4	8	4	4	8	4	7	5	5	4	4	4	4	4	4	4	4	4	4	4	4
24	7	6	5	8	6	3	7	6	3	7	4	4	6	4	3	7	3	2	3	4	2	5	4	3	1	4	7
25	7	7	7	8	8	8	8	6	5	8	6	5	6	5	1	2	2	2	4	4	4	2	2	1	7	3	1
26	8	7	8	7	7	8	7	6	5	6	4	3	7	3	6	7	4	6	3	3	5	7	7	6	7	7	6
27	8	7	6	6	5	7	8	6	7	7	8	6	7	8	6	6	7	5	6	7	5	7	5	6	7	7	7
28	6	7	7	8	8	8	8	3	3	4	3	4	4	2	3	3	4	5	5	5	5	5	5	5	1	3	3
29	9	9	9	9	6	6	9	7	1	7	7	4	7	6	3	6	8	7	2	3	2	8	9	8	6	8	9
30	7	7	7	6	6	7	6	6	5	6	6	4	6	6	4	6	6	4	6	6	5	7	7	6	6	6	4
31	8	6	4	6	8	4	5	8	6	4	4	3	5	5	7	7	2	2	4	2	3	3	4	4	3	3	5
Mean:	7,35	6,870968	6,225806	7,096774	6,741935	6,064516	6,096774	5,967742	4,290323	5,51613	5,16129	3,322581	5,096774	4,83871	3,193548	5,903226	5,322581	4,483871	3,806452	4,225806	3	5,709677	5,354839	4,903226	5,064516	4,387097	4,387097
Mean of total;		6,817204			6,634409			5,451613			4,666667			4,376344			5,236559			3,677419			5,322581			4,612903	
StdDev.		0,566			0,524			1,008			1,177			1,032			0,714			0,623			0,404			0,391	

F.2. Statistical Analysis, OneWay Anova

Null hypothesis	All means are equal
Alternative hypothesis	At least one mean is different
Significance level	α = 0,05

Equal variances were assumed for the analysis.

F.2.1. Aroma

Factor	Levels	Values
Factor	3	845 ar; 563 ar; 237 ar

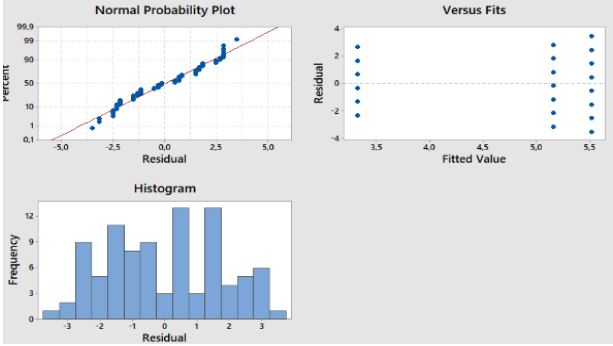
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	85,96	42,978	13,40	0,000
Error	90	288,71	3,208		
Total	92	374,67			

S	R-sq	R-sq(adj)	R-sq(pred)
1,79106	22,94%	21,23%	17,72%

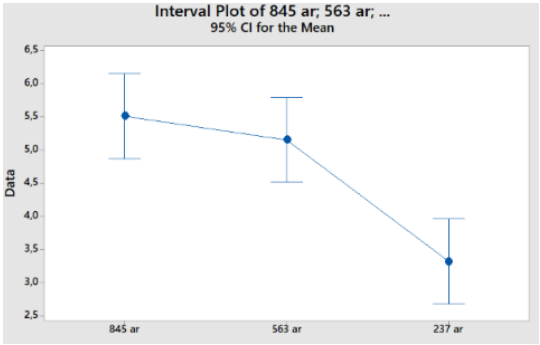
Factor	N	Mean	StDev	95% CI
845 ar	31	5,516	1,860	(4,877; 6,155)
563 ar	31	5,161	1,985	(4,522; 5,800)
237 ar	31	3,323	1,492	(2,684; 3,962)

Pooled StDev = 1,79106

Residual Plot of 845, 563, 237 on Aroma Parameter



Interval Plot of 845, 563, 237 on Aroma Parameter



F.2.2. Flavor

Factor	Levels	Values
Factor	3	845 flav; 563 flav; 237 flav

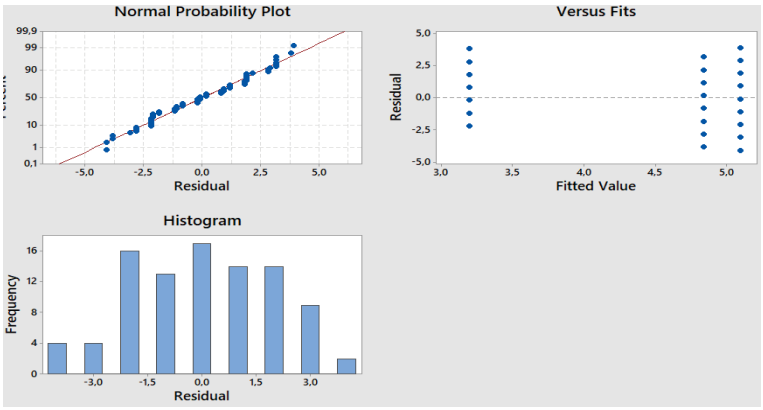
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	66,09	33,043	8,31	0,000
Error	90	357,74	3,975		
Total	92	423,83			

S	R-sq	R-sq(adj)	R-sq(pred)
1,99372	15,59%	13,72%	9,87%

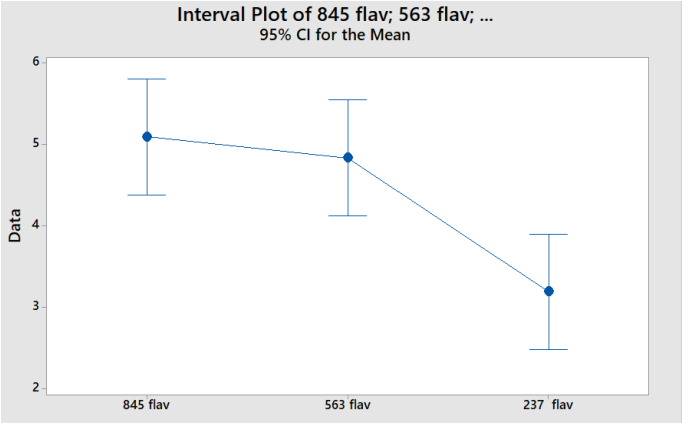
Factor	N	Mean	StDev	95% CI
845 flav	31	5,097	2,055	(4,385; 5,808)
563 flav	31	4,839	2,083	(4,127; 5,550)
237 flav	31	3,194	1,833	(2,482; 3,905)

Pooled StDev = 1,99372

Residual Plot of 845, 563, 237 on Flavor Parameter



Interval Plot of 845, 563, 237 on Flavor Parameter



F.2.3. Sweetness

Factor	Levels	Values
Factor	3	845 swe; 563 swe; 237 swe

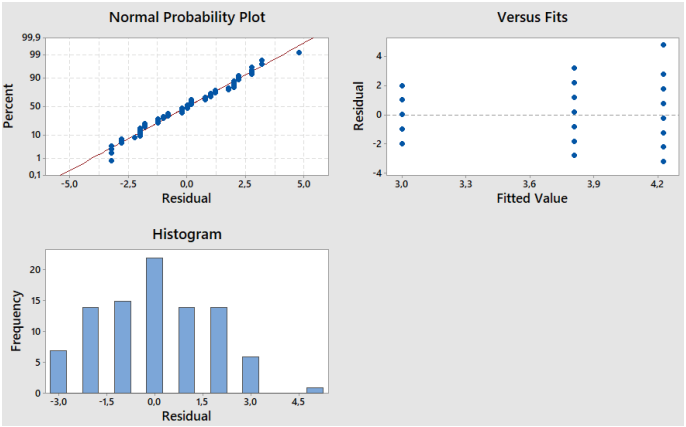
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	24,06	12,032	3,86	0,025
Error	90	280,26	3,114		
Total	92	304,32			

S	R-sq	R-sq(adj)	R-sq(pred)
1,76465	7,91%	5,86%	1,67%

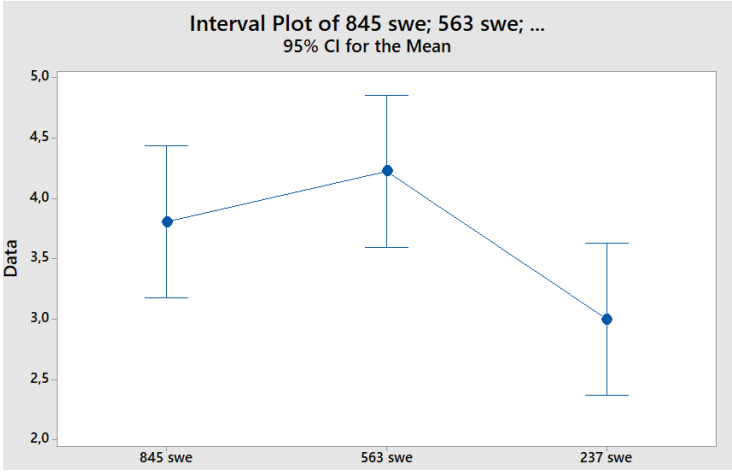
Factor	N	Mean	StDev	95% CI
845 swe	31	3,806	1,721	(3,177; 4,436)
563 swe	31	4,226	2,012	(3,596; 4,855)
237 swe	31	3,000	1,528	(2,370; 3,630)

Pooled StDev = 1,76465

Residual Plot of 845, 563, 237 on Sweetness Parameter



Interval Plot of 845, 563, 237 on Sweetness Parameter



F.2.4. Sourness

Factor	Levels	Values
Factor	3	845 sour; 563 sour; 237 sour

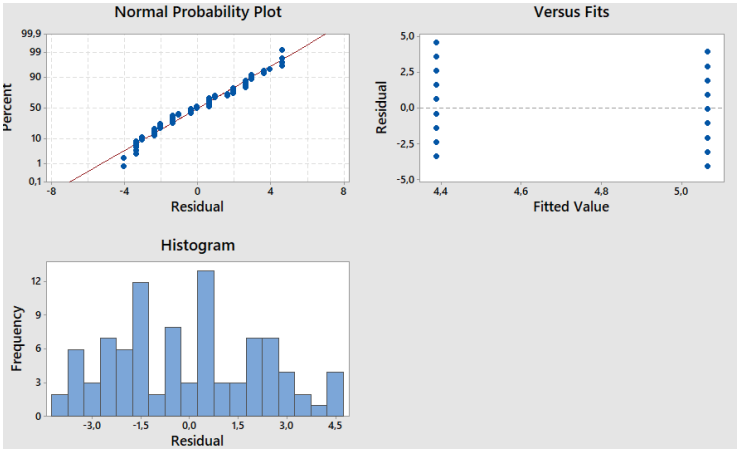
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	2	9,484	4,742	0,90	0,409
Error	90	472,581	5,251		
Total	92	482,065			

S	R-sq	R-sq(adj)	R-sq(pred)
2,29148	1,97%	0,00%	0,00%

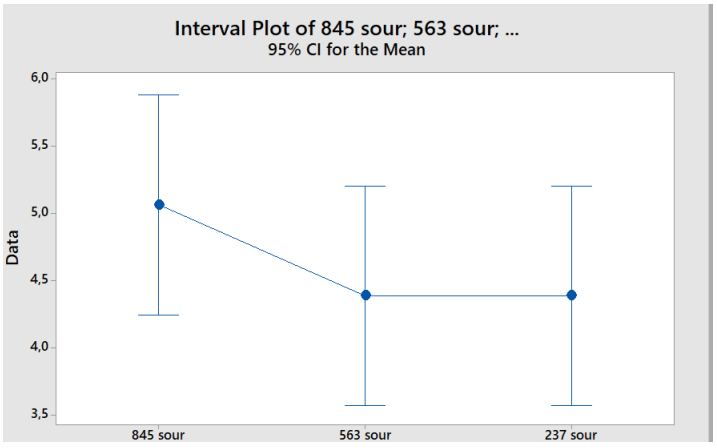
Factor	N	Mean	StDev	95% CI
845 sour	31	5,065	2,366	(4,247; 5,882)
563 sour	31	4,387	2,140	(3,569; 5,205)
237 sour	31	4,387	2,362	(3,569; 5,205)

Pooled StDev = 2,29148

Residual Plot of 845, 563, 237 on Sourness Parameter



Interval Plot of 845, 563, 237 on Sourness Parameter



F.2.5. Aroma, Flavor and Sweetness on F and FG

Factor	Levels	Values
Factor	6	563 ar; 237 ar; 563 flav; 237 flav; 563 swe; 237 swe

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	130,2	26,047	7,72	0,000
Error	180	607,4	3,375		
Total	185	737,7			

S	R-sq	R-sq(adj)	R-sq(pred)
1,83700	17,66%	15,37%	12,07%

Factor	N	Mean	StDev	95% CI
563 ar	31	5,161	1,985	(4,510; 5,812)
237 ar	31	3,323	1,492	(2,672; 3,974)
563 flav	31	4,839	2,083	(4,188; 5,490)
237 flav	31	3,194	1,833	(2,543; 3,845)
563 swe	31	4,226	2,012	(3,575; 4,877)
237 swe	31	3,000	1,528	(2,349; 3,651)

Pooled StDev = 1,83700

F.2.6. Sweetness on F and P

Factor	Levels	Values
Factor	2	845 swe; 563 swe

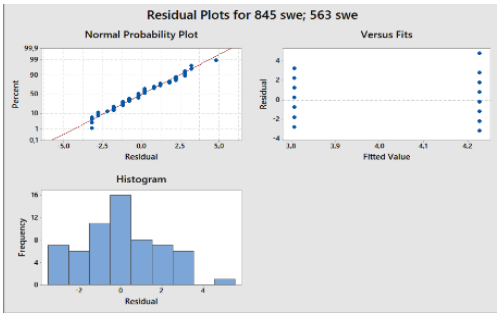
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	1	2,726	2,726	0,78	0,381
Error	60	210,258	3,504		
Total	61	212,984			

S	R-sq	R-sq(adj)	R-sq(pred)
1,87198	1,28%	0,00%	0,00%

Factor	N	Mean	StDev	95% CI
845 swe	31	3,806	1,721	(3,134; 4,479)
563 swe	31	4,226	2,012	(3,553; 4,898)

Pooled StDev = 1,87198

Residual Plot of 845, 563 on Sweetness Parameter



APPENDIX G

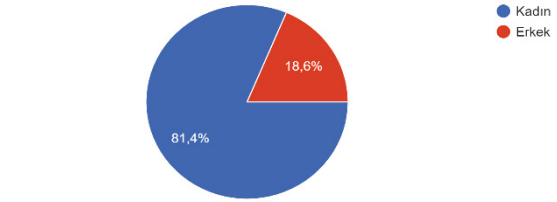
SENSORY EVALUATION DURING STORAGE

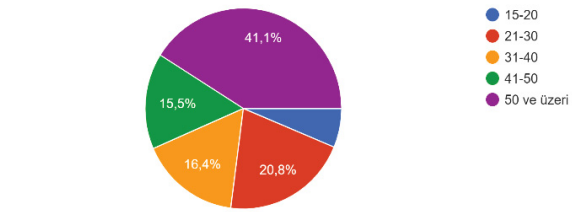
Used 5-point scale (where 1 is equal to “dislike extremely”, and 5 is equal to “like extremely”) at control days.

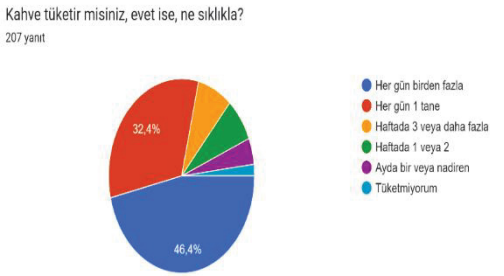
	P			F			FG		
	Color	**Odor**	**Taste**	**Color**	**Odor**	**Taste**	**Color**	**Odor**	**Taste**
t1	5	5	5	5	5	4	5	5	2
t2	5	5	5	5	4	4	5	4	1
t3	5	5	4	5	4	3	5	3	1
t4	5	5	3	5	3	2	5	3	1
t5	5	4	2	5	2	2	4	1	1

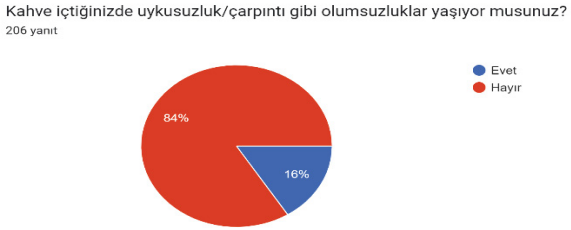
APPENDIX H

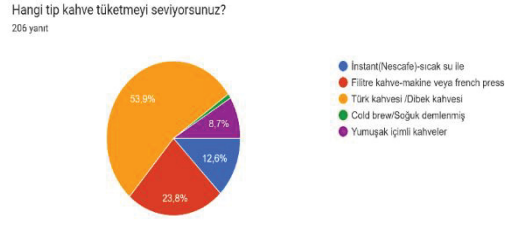
SURVEY RESULTS





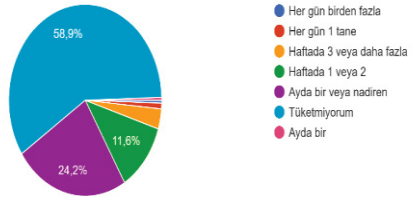




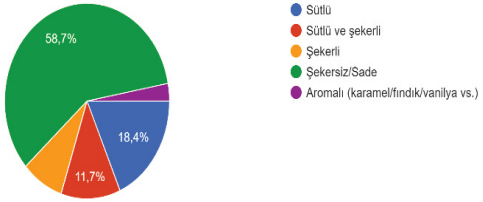


(cont. on next page)

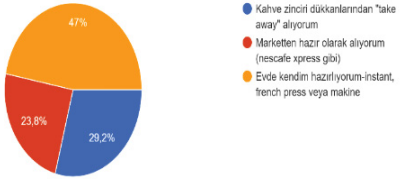
Hazır satılan soğuk kahve ürünlerini tüketiyor musunuz, evet ise ne sıklıkla tüketiyorsunuz?
207 yanıt



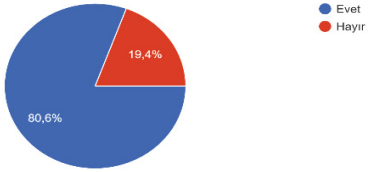
Kahvenizi nasıl tercih edersiniz?
206 yanıt



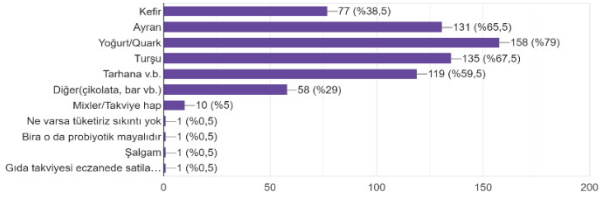
Kahvenizi çoğunlukla nereden alıyorsunuz?
202 yanıt



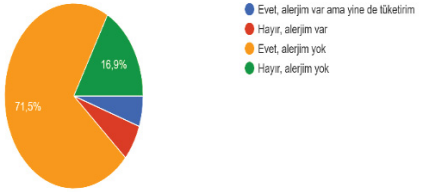
Probiyotik gıda tüketir misiniz/aşına mısınız?
206 yanıt



Probiyotik olarak hangi ürünleri kullanıyorsunuz?
200 yanıt



Süt ürünü tüketir misiniz/alerjiniz var mı?
207 yanıt



Probiyotik soğuk kahve ürünü için ödeyebileceğiniz en fazla ücret nedir?-250 ml ambalajda-
192 yanıt