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Functional Effects of Soy-Raffinose on the Quality parameters of Yogurt

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Functional Effects of Soy-Raffinose on the Quality parameters of Yogurt

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TABLE OF CONTENTS

CHAPTER THREE: MATERIALS AND METHODS

CHAPTER FOUR: RESULTS AND DISCUSSION

LIST OF TABLES

LIST OF FIGURES

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ABSTRACT

Yogurt is widely known for its nutraceutical properties. Beans are a healthy and easily affordable food containing good amount of raffinose that has been proven to possess prebiotic properties. Recent research shows that consuming oligosaccharides is possibly the best way to balance gut microflora. Raffinose Family Oligosaccharides (RFOs) is the most commonly found sugar belonging to this group and is mostly contained in pulses. However, studies on yogurt and bean-raffinose are limited. The goal of this research was to isolate and characterize raffinose oligosaccharide from soybeans and demonstrate practical utility of raffinose oligosaccharide by studying its effect on the quality of yogurt. Raffinose powder was prepared from soybeans using the methods of soaking, incubation and freeze drying. In this study, 2% low-fat milk was supplemented with 1% and 2% (w/v) Soy-RFO, inoculated with starter culture, *Streptococcus* thermophiles and L. *delbrueckii subsp. Bulgaricus*, fermented and stored at 4 ºC. The fortified yogurts were studied for changes in physiochemical (pH, titrable acidity, color, syneresis, viscosity, water activity, total soluble solids, water holding capacity), fermentation, antioxidant, texture profile and microbiological properties. These changes were compared with the properties of 1% (w/v) Inulin fortified yogurt and plain yogurt as the control. Results demonstrated that RFOs enhanced the growth and viability of probiotics present in yogurt. The fermentation rate of RFO yogurt was higher when compared to Plain or Inulin yogurt; 1% (w/v) RFO fermented the milk in 4 hours, which was 1 hour and 1.5 hours lesser than the time taken by Inulin and plain yogurt respectively. A significant ($p<0.05$) decrease in pH and increase in total titrable acidity was observed during the 21-day storage study with 2% (w/v) RFO-yogurt having the least pH and highest titrable acidity. Total soluble solids were found to be the highest in 2% (w/v) RFO yogurt which gradually decreased throughout the storage period. 1% (w/v) Inulin yogurt had a higher

content of soluble solids when compared to the 1% (w/v) RFO yogurt, which proves that RFO was more preferred for consumption by the probiotics. Syneresis was found to be the highest in plain yogurt followed by 1% (w/v) RFO yogurt, then 1% (w/v) Inulin yogurt and least in 2% (w/v) RFO yogurt. This explains the least amount of water holding capacity by plain yogurt and highest by 2% (w/v) RFO yogurt. Though the difference is not very high, by 2% (w/v) RFO yogurt had the highest viscosity with plain yogurt being the least viscous when compared to the other samples. Water activity of all the 4 samples were at a desired level through the 21-day storage period. There were no significant changes in the color between the 4 yogurt samples and it remained the same during storage as well with not much changes. 2% (w/v) RFO yogurt exhibited greatest antioxidant activity and this can be attributed to the increased microbial growth. Addition of Soy-RFO improves the firmness of yogurt and this increase in turn also increases the chewiness of Soy-RFO yogurt. These observed results clearly suggest that raffinose oligosaccharide enhances the overall quality of yogurt, thereby offering a probiotic yogurt with prebiotics, which can also be called a symbiotic yogurt. Basic changes made to the physical and technological parameters of yogurt dairy food products like yogurt is gaining significant interest resulting in the development of new products. Based on this study we can say that the Raffinose oligosaccharide powder extracted from soybeans is an ingredient that is promising from a view of new product development and fortification. In addition to all the benefits, this natural ingredient gains attention due to its health benefitting properties, and this makes it easy for the consumers to adopt them as a practice.

Keywords: Yogurt, soybean, raffinose oligosaccharide, prebiotic, probiotic, symbiotic, physiochemical, antioxidant, texture profile, *Streptococcus* thermophiles, L. *delbrueckii subsp. Bulgaricus*

CHAPTER ONE: INTRODUCTION

For several centuries now, the biggest threat to humans with respect to disease were contagious. But now, this scenario is changing. Diseases that are chronic and non-infectious are being responsible for a huge 63% of world-wide disease threat, as revealed by the United Nations, 2012. These diseases include, obesity, type 2 diabetes, cardiovascular diseases, and hypertension (Casey Ray Johnson., 2013). The world is consciously aware about the health benefits of dietary fibers and probiotics, leaving out prebiotics to be still underused and underrated. Prebiotics are basically carbohydrates that are resistant to digestion and absorption in the human upper gastro intestinal tract. They are fermented by the beneficial microorganisms present in the colon, thus feeding and maintaining the gut microbial flora.

Legumes such as soybeans are being used for a variety of purpose that increases the nutritional benefits and functional properties in the food system. Legume proteins have a variety of functional properties (solubility, water binding, emulsification, foaming, gelation, and thickening and flavor-binding capacity) that can be exploited in food formulations (Boyel et al., 2010). Dietary fibers and carbohydrate fractions of legume also contribute to different food applications. They can increase dietary fiber content and/or serve as functional ingredients in foods. Non-starch polysaccharides and oligosaccharides in soybeans can act as prebiotics in fermented foods. Potential prebiotics in pulses are the raffinose family of oligosaccharides (RFO) (the α-galacto-oligosaccharides raffinose, stachyose and verbascose), which are soluble carbohydrates and found in appreciable concentrations in pulses (Tosh and Yada, 2010). Therefore, it would be interesting to explore the effect of supplementation of pulses such as soy in fermented foods such as yogurt. It may increase physicochemical, textural, and the overall quality of yogurt. Also, probiotic growth and viability may be enhanced due to this supplementation. Enormous availability, wide variety

and easy affordability, at the same time packed with solid nutritious components are the important characteristics of pulses which makes the human population take them more seriously, which makes to increase pulse consumption and benefit to the maximum possible. The announcement of the year 2016 as the Year of Pulses by Food and Agricultural Organization of United Nations has led to the opening of several parades which focuses on increasing awareness and consumption of lentils, beans, peas and chickpeas.

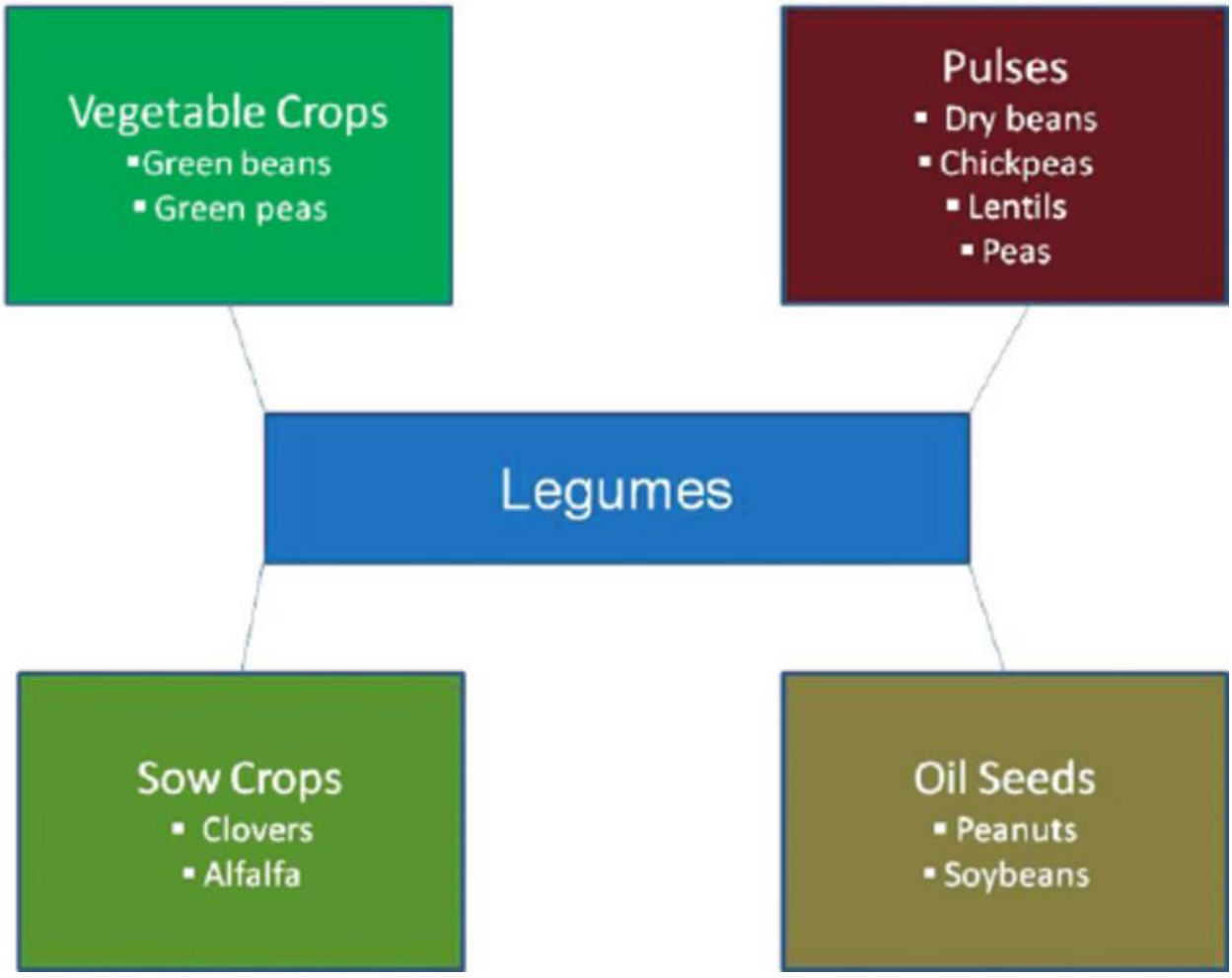

Figure 1. Classification of Legumes (McCrory et al. 2010).

There used to be a time when probiotics were only given all the importance and prebiotics were of lesser significance. But at this point in time, prebiotics are gaining a lot of popularity and importance. Not many naturally-sourced and derived prebiotics are readily available for human consumption. It is of high importance to maintain good quality of the beneficial microorganisms present in the gastrointestinal tract, as these microbes form the base for maintaining several bodily functions in a positive manner.

1.1 **Research Question?** Will Soy-Raffinose oligosaccharide improve the overall quality of yogurt?

1.2 Hypothesis

Soy-Raffinose fortification will result in increasing the overall quality of yogurt, supports probiotic growth and improve shelf life.

1.3 Objectives of this research:

1. Extraction of Raffinose from Soybeans, characterization and quantification of Raffinose from Soybeans using an enzyme assay.
2. Analyze the effect of Raffinose on physio-chemical properties, texture profile, antioxidant activity and microbiological properties on yogurt during a 21-day shelf life study.
3. Evaluate the effect of Soy-Raffinose fortification in yogurt.

CHAPTER TWO: REVIEW OF LITERATURE

2.1 Legumes

Legumes are plants that belong to the Leguminosae family, which are also called as Fabaceae that grows seeds inside a pod (Staniak M, Ksiezak J and Bojarszczuk J, 2014; Kouris-Blazos A and Belski R, 2016). Legumes are becoming more popular and gaining more acceptance due to their numerous health related advantages that relate to them. So, it is of high importance to get to know what they are. Legumes are considered the second most important source of food, next to cereals (Yvonne Maphosa and Victoria A. Jideani, 2017). Legumes are highly nutritional, with a high protein content of about 20–45% along with essential amino acids, complex carbohydrates (±60%) and dietary fiber (5–37%). Hence legumes have also been referred to as "poor man's meat" (Leterme, 2002). Consumption of soybeans has been found to be low in comparison with other food products. These plant seeds have been present for several thousands of years now. They are being raised in practically every part of the world, comparatively more in those parts of countries that does not utilize animal sourced protein. And hence, in these parts of the countries legumes such as soybeans serve as the main source of protein and is a necessary part of their everyday food consumption (Thavarajah, 2012).

2.2 Soybeans

Soybeans can be called a superfood because of their huge list of health benefits and the nutritive ingredients. They are a good source of protein, vitamins, antioxidants, minerals and most importantly they are free of cholesterol providing very little to almost nil fat. Soy also help in reducing cholesterol and the levels of triglycerides, unlike increasing cholesterol like certain proteins from animal sources. Some of the most important benefits of beans include: heart

benefitting, protein-rich, cholesterol-free and less fat, low glycemic index (balances blood sugar levels), cancer inhibition, prevents constipation, provides better satiety in a healthier way and nutrient packed (Abeysekara, S et al., 2012).

Soybeans are healthy and easily affordable food containing good amount of raffinose with prebiotic properties (Swennen, K. et al., 2006). However, studies on bean raffinose are limited. There is no good amount of research available, based on beans as the primary source of prebiotics. Sobeans which are considered a functional food, are again underutilized in the fields of food research and the least expensive source of a significant amount of Raffinose.

Soybeans are a powerhouse that contains bioactive ingredients and possess several functional properties that benefits human health in several ways. They are also a good source of Raffinose family of Oligosaccharides which are considered prebiotics. Prebiotics are food components that act together with probiotics which are the beneficial bacteria, breaking down and digesting certain compounds resulting in the production of even more beneficial microbes, aiding day to-day essential functions in the upper gastro intestinal tract. There are several studies supporting the claim about raffinose as a successful prebiotic. One such study is the research done on lupin seeds as prebiotics and its possible applications in dairy products (Martinez-Villaluenga C et al., 2005). It is closely related to this literature review except for that it is not based on beans, but lupin seeds. Another study evaluates the possible effects of plant originated prebiotics, such as raffinose on intestinal functionality and intestinal bacterial populations (Sarina Pacifici et al., 2016). There is also this study conducted to research about the various functional properties, metabolic activities, regulatory conditions, stabilization and antioxidant properties of Raffinose family of oligosaccharides (Wim Van den Ende., 2013). Based on all these established articles

cited above, justifications can be made regarding beans and raffinose from beans as a potential prebiotic source is a good area for future research.

Soybeans are legumes, which is one of the many commonly and widely available food material. Some other major and important aspects of soybeans include, they are highly nutritious considered a powerhouse of nutrition, they can be consumed by all age groups from babies to older people, and the best of all with these amazing qualities they are less expensive. Easy affordability and easy availability make beans a potential food source. But the researches based on soybeans are very limited in comparison with other food materials possessing the same kinds of qualities. Beans possess several properties that are favorable and affirmative to human population when consumed. Resistant starch that is present in beans functions as a fiber component due to its indigestibility in the upper gastro intestinal tract. It reaches the large intestine undigested where it holds together with fat cells, thereby aiding in bowel movement, weight management issues and prevention of fatal cardiovascular diseases. Soybeans also possess low glycemic index. Glycemic index is something that helps to foretell the impact of any foods on the blood sugar levels. Soybeans when consumed, does not result in instantaneous rise in blood sugar levels in humans, hence they are considered to have low glycemic index (Leterme, 2002). This makes beans an absolute and perfect food material for human population suffering from diabetes. Foods with low glycemic index can also assist in curbing craves for sugar. This is because they give a fuller feeling for a prolonged time and it is attributed to the presence of fiber. Thus, foods with low glycemic index help with preventing the chances of life-threatening heart diseases.

Regardless of all these extremely solid nutritional and nourishing properties of soybeans, the consumption of soybeans has been found to be much lower than the actual dietary recommendation. It has been found that several reasons are behind the decreased consumption of

beans despite their high nutritional properties. The reasons being, varied preference over food and taste, cooking time taken by beans, lack of education with regards to the nutritional effects of beans, and of all the most important, disturbances in the gastrointestinal tract that results due to increased consumption of beans (McCrory et al., 2000). A research carried out by Lucier, Lin, Allshouse and Kantor in the year 2000 to determine the various factors that affect consumption of dry beans has pointed out that after World War II, even though the consumption of dry beans was decreased, there has been an increase in consumption because of the higher growth in Hispanic immigration whose predominant diet includes a higher proportion of soybeans. Other characteristics that affect the consumption of dry soybean can be pointed towards Nationality, ethnicity, beliefs and level of income. A research study found that low-income families, Hispanic and Mexican families have increased consumption of beans when compared to American families. And, it has been found that 27 percentage of cooked beans are being consumed by low income families, Hispanic families and Mexican families (Lucier et al., 2000). Due to all these attributes that decrease and affect bean acceptability along with consumption, educating our population about the significance of beans on human health is of high importance.

2.3 Prebiotics and Probiotics

Gut microbiota plays a very significant role in human health. In simpler words, Probiotics are the beneficial microbes and Prebiotics are their energy source. Probiotics play an important role in lactose intolerance, diarrheal diseases (that includes, infectious diarrhea caused by bacteria/viruses and antibiotic-associated diarrhea), lowering of cholesterol, and cancer prevention (Corliss A O'Bryan et al., 2013). Prebiotics and probiotics work together resulting in symbiotic effects. This symbiotic effect is the cause for certain positive changes. Prebiotics help to maintain the overall quality and increase the number of beneficial microorganisms in the lower intestine.

Prebiotics encourage the development and increase activity of probiotics, thereby becoming the perfect food source. Prebiotics are nothing but, basically food compounds. They are found in abundance in foods that contain plenty of dietary fibers.

An experiment conducted by Corliss A O'Bryan et al., in the year 2013 states that, overall health and quality of the gut is of high importance because it is closely associated with several other functions of the body which includes, lowering risk of cardiovascular diseases, maintaining healthy cholesterol levels, improved gut health, better digestive ability, reduced stress response, improved hormone balance, increased immune function, reduced risk for weight gain/obesity, decreased reactions to inflammation and autoimmune responses. Several types of research were done to quantify the intake of prebiotics in an American diet. It was found that the amount of prebiotics consumed on an average is very low. (Alanna J. Moshfegh et al., 1999).

Lactobacilli and bifidobacteria are the common target groups for prebiotics. The number of bifidobacteria residing in the human colon is more when compared to lactobacilli and so bifidobacterial are more likely to be positively affected than lactobacilli. One other factor is that bifidobacteria display a greater liking for oligosaccharides. Fermentation in colon and microbial changes in the gut are the primary action mechanism of prebiotics (Gibson. G. R., 2010). The colon contains around 1000 different bacteria that includes a microbial population of about 10^{11} –10^{12} cfu/g of contents. Slower transit time, ready availability of nutrients, and suitable pH makes the colon a favorable place for the growth of bacteria (Cummings, J. H., 1991). Both lactobacilli and bifidobacteria exhibit saccharolytic metabolism, meaning no proteolytic activity which is considered very beneficial. This interaction and diversity between the human microflora have led to the initiation of Human Gut Microbiome Initiative (HGMI), as a way to learn more about this bacterial ecosystem (Gordon. J. I., 2011). The immune system of the gut, microbes in the mucosal

area and colon together significantly contribute to the prevention of pathogenic bacteria's invasion into the gastrointestinal tract. These microbes get their energy to function effectively and efficiently by the process of carbohydrate fermentation that passes to the large intestine undigested. It has been found out that fermentation of every 100 g carbohydrate produces about 30g of bacteria (Slavin, Joanne, 2013).

2.4 Raffinose Family of Oligosaccharides

The most predominantly studied prebiotics are Inulin and Fructo-oligosaccharides, leaving out legumes (pulses) which contains a good amount of Oligosaccharides, also called as alpha-galactosides or otherwise called the Raffinose family of Oligosaccharides (RFOs). These RFOs are digested and used by the bifidobacteria. The alpha-galactosides are low-molecular-weight, soluble oligosaccharides represented by raffinose, stachyose, verbascose, and other oligosaccharides formed by alpha-(1→6)-galactosides linked to C-6 of the glucose moiety of sucrose (Dey, P. M.; 1985). Raffinose is a bioactive carbohydrate, which is considered to possess prebiotic properties (Martínez-Villaluenga et al., 2005) because they are not hydrolyzed in the upper intestine and favors the development of colon microflora.

2.5 Soybeans as a Prebiotic source

Soybeans contain several components that are highly beneficial to human health. The best out of all the chemical components is Raffinose, also called Raffinose family of Oligosaccharide (RFO). An experiment conducted by Phoung in 2017, to determine the content of raffinose in different pulses and legumes states that Soybeans has the highest content of raffinose amongst the others. Raffinose is a trisaccharide that consists of alpha-(1-6) galactosidic bond which is linked to the glucose unit of sucrose. This specific configuration of oligosaccharide was considered in the

past as opposing due to its characteristic that results in severe flatulence in humans. Flatulence was found to occur due to the presence of alpha-galactosidase bond which stays undigested in the upper gastrointestinal tract and is subjected to fermentation when it enters the large intestine (Tajoddin, Manohar & Lalitha, 2012). Like mentioned before, a prebiotic must be able to withstand gastric acid and be fermented by the intestinal gut microbial flora. As a result, Raffinose family of Oligosaccharide which is found in beans can be stated as a prebiotic. Raffinose family of oligosaccharide is one among the oligosaccharides which have been satisfactorily accepted as Generally Regarded As Safe (GRAS) by the United States Food and Drug Administration (U.S. FDA).

Raffinose is a chemical component that has all the quality, capability and capacity of a prebiotic. Several studies have already been done on Raffinose to assess its prebiotic properties, thereby establishing the Raffinose family of Oligosaccharide as a potential and prospective prebiotic source. One among the many studies is the recent research as performed by McCrory, Hamakaer, Lovejoy, & Eichelsdoerfer, 2010 which proves that oligosaccharides are accounted as prebiotics. Prebiotics can be defined as selective chemical ingredients that are fermented in nature, which allows certain positive modifications in the composition and activity of the microbial flora in the gastrointestinal tract (Charalampopoulos & Rastall, 2012). These modifications that are made on the intestinal gut microflora results in several health improving benefits to the consuming population. Function foods are those food components that possess the capacity to contribute health benefits, thereby sustaining better health. Hence beans can be considered a functional food. Advantages of prebiotics with respect to human health are just immense, which includes prevention and improvement of microbial infections in the gastrointestinal tract, increase the presence of readily available minerals, and assistance with immune responses (Charalampopoulos

& Rastall, 2012). Any food component that possesses the ability to resist gastric acid can be marked as a prebiotic. By withstanding gastric acid, the food component must be able to stay undigested in the upper gastro intestinal tract, alter and develop the constitution of the microbial flora in the gut making it more healthful (Kolida, Tuohy & Gibson, 2002).

2.6 Yogurt

Yogurt is a very popular fermented food product consumed by both old and young in the United States and worldwide (Soccol et al., 2014). It is prepared by allowing milk to ferment at a regulated temperature by the addition of live bacterial cultures such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Yogurt is rich in proteins, minerals, vitamins, and probiotics; hence it is considered nutrient-dense and a superfood (Gahruie, Eskandar, Mesbahi, & Hanifpour, 2015). Several kinds of research are being conducted on the probiotic quality of yogurt and one such research conducted by El-Abbadi, Dao, and Meydani (2014) confirms yogurt as a prebiotic drink due to the live microbes present in the culture that is used to produce yogurt. These microorganisms help with the improvement of gut functions and overall health. Legumes are rich in prebiotics including raffinose-family oligosaccharides (RFO), resistant starches and fibers (Johnson et al., 2013). Pulse ingredients which suffer from under-consumption in the American diet, could serve as prebiotic and nutritional source for yogurt. Functional oligo-saccharide present in pulses also offers health benefits on gastro intestinal health (Guillon and Champ, 2002).

Besides the health benefits of yogurt, its physical properties, appearance and texture are also important for consumer acceptability. Addition of ingredients (e.g. milk solids, stabilizers, prebiotic, milk protein, calcium, fibers) and modifying process conditions are the common practices to increase overall quality and shelf life of yogurt (Zare et al., 2011). In the recent past, a large number of strategies to improve the prebiotic content of yogurt have been investigated.

Common ingredients rich in prebiotics such as gum arabic, wheat bran, dietary fiber, and inulin have been investigated (Charalampopoulos et al., 2002; De Souza Oliveira et al., 2009; Seckin and Ozkilinc, 2011). The addition of lentil flour in yogurt has recently been investigated by a research team in Canada (Zare et al., 2012; Zare et al., 2012).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Materials

Whole soybeans obtained from Walmart were ground by Vitamix dry grinder and passed through a 250-micrometer sieve (Advantech Manufacturing, New Berlin, WI). Pasteurized, homogenized 2% fat milk was collected from local market (Braums, Edmond, OK). Yogurt culture containing *Streptococcus*. *thermophilus* and Lactobacilli. *delbrueckii subsp. Bulgaricus* (YO-MIX 883 LYO 500 DCU) was obtained from Danisco USA Inc. (Madison, WI, USA). The culture was obtained in freeze-dried form, packaged in laminated foils. They were stored at 4°C until use.

3.2 Extraction of Raffinose Oligo Saccharides

The whole soybean were ground using a Vitamix dry blender and filtered through an Advantech tray (U.S.A Standard Testing) into fine powder, as small as the size of 250 micrometer particles using micro-screening (Advantech-U.S.A Standard Testing, metric 250 µm). Samples were stored in sealed bags and placed in dry, cool, room temperature storage. 10g of soybean powder was homogenized in 100mL of nano-pure water using a magnetic stirrer for 15 minutes. This solution was incubated at 70^{o}C for 12 hours and centrifuged at 1500 rpm for 30 minutes. The supernatant (RFOs) was collected in dry clean test tube with a cap. The extracted RFO solution was freeze-dried using a Harvest Freeze Dryer. The drying cycle was up to 6-8 hours. The freeze-dried product was grinded into a fine powder using a clean pestle and mortar. Approximately 50g of concentrated prebiotic powder was prepared for yogurt preparation.

3.3 Quantification of Raffinose in the Freeze-dried powder

Commercially available Raffinose assay kit was ordered from Megazyme and used to determine the concentration in the extract. Content of the kit included a buffer solution containing sodium azide (0.02%w/v), NAD+, D- galactose dehydrogenase plus galactose mutarotase suspension, α-Galactosidase (pH 4.6) lyophilized powder, Galactose standard solution and raffinose control powder. The reagents were prepared and used according to procedure provided in the manual. Absorbance was read at 340nm using a spectrophotometer.

3.3.1 **Sample preparation:** Accurately 0.50 g freeze-dried powder was weighed into glass test-tubes (18 x 150 mm). 5 mL of ethanol (95% v/v) was added to each tube and incubated at 84-88°C for 5 min (this treatment inactivates endogenous enzymes). The contents in the tube were then quantitatively transferred to a 50 mL volumetric flask and the volume was adjusted to the mark with 50 mM sodium acetate buffer (pH 4.5). The sample is allowed to extract over 15 min with occasional swirling. An aliquot of this slurry is transferred (approx. 5 mL) to a glass test tube (16 x 120 mm). 2 mL of chloroform is added, mixed vigorously on a vortex mixer for 15 s and centrifuged at 1,500 g for 10 min [this treatment removes most of the lipids from the aqueous phase into the chloroform (lower phase); insoluble plant material tends to concentrate between the phases]. The upper (aqueous) phase is used for analysis.

3.3.2 Raffinose quantification procedure

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: optimally 40°C in a dry hot-block heater or in the spectrophotometer, but otherwise ~ 25°C
Final volume: 2.62 mL
Sample solution: 3-250 µg of raffinose per cuvette (in 0.10-0.20 mL sample volume)

Read against air (without a cuvette in the light path) or against water

If the sample contains free D-galactose (D-Gal), it must be determined in a separate assay without solution 4 (free D-Gal sample) (see below).

Pipette into cuvettes	Blank raffinose + free D-Gal	Raffinose + free D-Gal sample	Blank free D-Gal	free D-Gal sample
sample solution	-	0.20 mL	-	0.20 mL
distilled water	0.20 mL	-	0.30 mL	0.10 mL
solution 4 (α-GAL)	0.10 mL	0.10 mL	-	-
Mix* and incubate for 20 min (NOTE: before pipetting solution 4, first warm it to 25-30°C). Add:				
solution 1 (buffer)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
distilled water	2.00 mL	2.00 mL	2.00 mL	2.00 mL
solution 2 (NAD^+)	0.10 mL	0.10 mL	0.10 mL	0.10 mL
Mix** and read absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:				
suspen. 3 (β-GalDH)/GalM	0.02 mL	0.02 mL	0.02 mL	0.02 mL
Mix** and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 40 min at 25°C or 20 min at 40°C). If the reaction has not stopped after 20 min (i.e. in incubations at 40°C), continue to read the absorbances at 5 min intervals until the absorbances remain the same over 5 min.				

* pipette sample solution, distilled water and solution 4 into the bottom of the cuvette and mix by gentle swirling.

** for example with a plastic spatula or by gentle inversion after closing the cuvette with a cuvette cap or Parafilm®.

Figure 2: Raffinose quantification procedure as provided by Megazyme
(RAFFINOSE/ D-GALACTOSE ASSAY PROCEDURE. (n.d.). Retrieved from https://www.megazyme.com/documents/Booklet/K-RAFGA_DATA.pdf).

3.4 Supplementation of Raffinose in yogurt preparations

Pasteurized 2% milk (Braums, Tuttle, OK) was heated to 42^{o}C. Milk was then supplemented with RFOs at a concentration of 1-2% w/v. 1% w/v of inulin was used as positive control. A negative control experiment without RFOs was also done. Milk was homogenized thoroughly, and samples were cultured with starter cultures containing *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Danisco, KS). Cultured milk was then incubated at 42^{o} C for 6 hours or until a pH of 4.4 was reached.

3.5 pH and titrable acidity

pH and titrable acidity of each samples were measured at room temperature after every 2 hours for 6-8 hours to monitor fermentation process using a pH meter (HI 9125, Hanna Instruments). To determine titratable acidity, a mixture of yogurt and distilled water (9:1) was titrated with 0.1 N NaOH using 0.1% phenolphthalein indicator. Triplicate readings were recorded, and average was taken to achieve statistical significance.

Titrable acidity = [(Volume of titrant * N * 90)/Weight of sample] *100

Where, N = normality of titrant; 90 = Equivalent weight for lactic acid.

3.6 Water activity

Samples were put onto the sample plate. The sensor of the gauge was covered onto the sample plate and kept aside for about 5 minutes, when the reading was stable, the water activity was obtained. Wellzion WA-160A handheld water activity meter was used. Triplicate readings were recorded, and average was taken to achieve statistical significance.

3.7 Total soluble solids

The total soluble solids of the yogurt samples were analyzed using a portable Hanna instrument refractometer, expressed in degree brix. A portion of the sample was placed on the refractometer and reading were taken in triplicates. The average reading was calculated and recorded. Triplicate readings were recorded, and average was taken to achieve statistical significance.

3.8 Water holding capacity

RFO and Inulin fortified milk (10ml) were incubated in polypropylene centrifuge tubes at 42 ^{o}C until the end of fermentation. After 24 hours of cold storage at 4 ^{o}C, the stored samples were centrifuged at 500 rpm for 10min. The expelled whey was weighed, and the following formula was used to calculate the water holding capacity of the yogurts (Mei Yang and Li Li, 2010).

$$w(WHC)\% = (1-m_1/m_2) *100$$

where, m_1 is the mass of whey after centrifugation and m_2 is the mass of yogurt sample. Triplicate readings were recorded, and the average was taken to achieve statistical significance.

3.9 Color

The color of each sample was measured using Hunter color lab system (Color Flex EZ, Hunter lab). The color was determined as lightness (L*), red/greenness (a*), and yellow/blueness (b*). Triplicate readings were recorded, and average was taken to achieve statistical significance.

3.10 Syneresis index:

Syneresis was determined as the amount of spontaneous whey separation from yogurt as described previously with some modifications (Zare et al., 2011). The volume of whey drained from 50 ml of undisturbed set yogurt samples was measured and expressed in percentage. Triplicate readings were recorded, and average was taken to achieve statistical significance.

3.11 Viscosity:

Viscosity was measured using an NDJ-9S Digital rotary viscometer. Viscosity was measured on the first day of sample preparation to ensure that no form of agitation had occurred. A no. 3 rotator was inserted into the sample and spun for 40 seconds at 30 RPM. Results were expressed in Pascal-second (Pa.s). Triplicate readings were recorded, and average was taken to achieve statistical significance.

3.12 Viability determination of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*:

Yogurt samples were prepared in sterilized buffered peptone water by mixing 1 ml of yogurt with 9 ml of buffered peptone water. Serial dilutions were done using sterilized buffered peptone water (BPW). S. *thermophilus* was enumerated by spreading sample on M17 agar. MRS agar was used for *Lactobacillus* spp. (Shori and Baba, 2012). MRS samples were incubated at 37 ^{o}C using a vacuum-sealed anaerobic chamber. Plates were read after 72 hours. M17 plates were incubated at 42 ^{o}C in an aerobic chamber for 72 hours. Results were recorded in triplicates.

cfu/mL = (no. of colonies * dilution factor) / volume of culture plate

3.13 Antioxidant activity:

3.13.1 Preparation of the sample for analysis:

Portions of 15ml were taken from each sample and the pH was adjusted to 4.6 with 1 M HCl, only for those samples with a pH higher than 4.6 (unfermented milk). The samples were then centrifuged at 5000 rpm for 30 minutes and the supernatant was filtered through a coarse filter paper.

3.13.2 Determination of ability to neutralize free radical:

2mL of DPPH in ethanol solution (500mM) was added to 2ml of whey fraction, the sample was intensively mixed and allowed to stand at room temperature in a dark place for 30 minutes. Absorbance was measured at 517 nm wavelength. Ethanol was used as a calibration solution.

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})/ Abs_{control}] *100$

where, $Abs_{control}$ is the absorbance of DPPH radical in ethanol

Abs_{sample} is the absorbance of DPPH radical solution mixed with the sample

3.14 Texture profile

The texture profile analysis test was performed by using a Perten TVT 6700 texture analyzer. A 25mm cylinder probe was used to measure the TPA of samples at room temperature, in triplicates. 2-cycle compression test was performed. The conditions set in the texture analyzer are as follows: Pre-test speed, 1 mm/s; Post-test speed. 1 mm/s; Test speed, 1 mm/s; Trigger force, 10.0g; Time, 5.0 s. For each evaluation a sample size of 5cm was used and 30% of original depth was compressed during the first stage. The following texture parameters were recorded: firmness; maximum compression force in extrusion

thrust into sample (g), consistency; area within curve during extrusion thrust (g.s), cohesiveness; maximum compression force during withdrawal of probe from sample (g) and index of viscosity; area within negative region of curve during probe withdrawal (g.s).

3.15 21-day shelf life study

All parameters mentioned above were repeated over a period of 21 days, results were recorded in triplicates. This result was used to evaluate the overall quality of yogurt.

3.16 Statistical analysis

All experiments were carried out in triplicates (N = 3). Data were expressed as Mean ± SD. Statistical analysis was conducted using ANOVA on Microsoft Excel. A measure of significance using a P value of 0.05 and was derived using Bonferroni test.

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Quantification of Raffinose in freeze-dried powder

Megazyme Raffinose/ D-Galactose Assay is designated based upon the biochemical reaction of RFOs to produce NADH as the final product, using enzymatic series. The enzymes are extremely reactive; hence the enzyme activities must be run quickly and under specifically controlled reaction conditions. Table 1 below indicates a high enzyme efficiency within the assay, 92.44 ± 0.12%, therefore it is suitable to use in measuring RFOs contents in extracted pulse samples.

Table 1: Enzyme Efficiency (%)

	Actual Concentration of Raffinose Control	Measured Concentration of the Control	Enzyme Efficiency (%)
Mean ± SD	4.1	3.79	92.44 ± 0.12

Table 2: Concentration and content of RFOs in Freeze-dried Soybean extracted powder

	Freeze-dried Soybean powder
Mass of sample (g)	0.5
Average concentration of RFOs (g/L)	1.412 ± 0.14
Actual content of RFO (g/100g)	28.24 ± 0.09

Table 2 above shows the average concentration of RFOs and the actual content of RFOs in the freeze-dried powder extracted from soybeans. According to the enzyme assay procedure it has been found that 28.24 ± 0.09 g of RFO is present in 100g of freeze-dried soybean extracted powder. Raffinose is a water-soluble sugar that is present in soybeans along with other water-soluble sugars namely, fructose, glucose, sucrose, and stachyose (Hou A., Chen P., Shi A., Zhang B., and Wang Y. J., 2009).

Raffinose is hydrolysed to D-galactose and sucrose by α-galactosidase (α-GAL) (1). α-GAL also hydrolyses other α-galactosides such as stachyose, verbascose and galactinol [1-O-(α-D-galactosyl)-myo-inositol], if present. The enzyme does not cleave β-linked galactose, as in lactose. Interconversion of the α- and β-anomeric forms of D-galactose is catalysed by galactose mutarotase (GalM) (2). The β-D-galactose is oxidised by NAD^+ to D-galactonic acid in the presence of β-galactose deyhydrogenase (β-GalDH) at pH 8.6 (3). The amount of NADH formed in this reaction is stoichiometric with the amount of D-galactose released. It is the NADH which is measured by the increase in absorbance at 340 nm. The reactions that take place during this enzyme assay is as depicted below:

(1) Raffinose + Stachyose + Verbascose + H_2O $\xrightarrow{(\alpha\text{-galactosidase})}$ D-galactose + sucrose

(2) α-D-Galactose $\xrightarrow{(galactose\ mutarotase)}$ β-D-galactose

(3) β-D-Galactose + NAD^+ $\xrightarrow{(\beta\text{-galactose dehydrogenase})}$ D-galactonic acid + NADH + H^+

4.2 Effect of fortification on fermentation time of yogurt

Table 3 presents the average time taken by each of the yogurt sample under study for fermentation and reaches the desired pH of 4.4. From the table we can definitely infer that addition of soy-raffinose improved the fermentation rate since the yogurt with 1% and 2% soy-raffinose took about 4.5 hours and 4 hours respectively. The yogurt with 1% inulin recorded a time of 5 hours for fermentation which is 1.5 hours less than the time taken by control yogurt. Several research works done in the past have proved that fortification of pulse ingredients to milk reduced the fermentation time significantly. The process where lactose (which is the milk sugar) present in milk is broken down to produce lactic acid by microorganisms which form the starter culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus* in this study) is called fermentation of yogurt. This increase in lactic acid decreases the pH and results in the clotting of milk, thereby forming a soft-gel structure, which is the yogurt. This fermentation is also responsible for the characteristic flavor of yogurt (Chen Chen, Shanshah Zhao, Guozhong Zhao, Haiyan Yu, Huaixiang Tian, 2017).

Table 3: Effect of Soy-RFO and Inulin fortification on fermentation time of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment **	pH***						
	3hrs	3.5hrs	4hrs	4.5hrs	5hrs	5.5hrs	6.5hrs
Control	6.46± 0.01_{aA}	5.46± 0.01_{aB}	5.19± 0.02_{aC}	5.08± 0.01_{aD}	4.75± 0.01_{aE}	4.51± 0.03_{F}	4.43± 0.02_{G}
Sample 1	6.42± 0.01_{Bb}	5.11± 0.01_{bA}	4.85± 0.02_{bC}	4.63± 0.01_{bE}	4.42± 0.02_{bD}		
Sample 2	5.74± 0.02_{Cc}	5.23± 0.01_{cB}	4.67± 0.01_{cD}	4.39± 0.01_{cA}			
Sample 3	5.02± 0.02_{dD}	4.89± 0.02_{dC}	4.41± 0.01_{dA}				

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

***pH of yogurt fermented at 42°C until pH reached 4.4±0.05.

Means followed by the same uppercase letter (ABCDEFG) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$). Means followed by the same lowercase letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

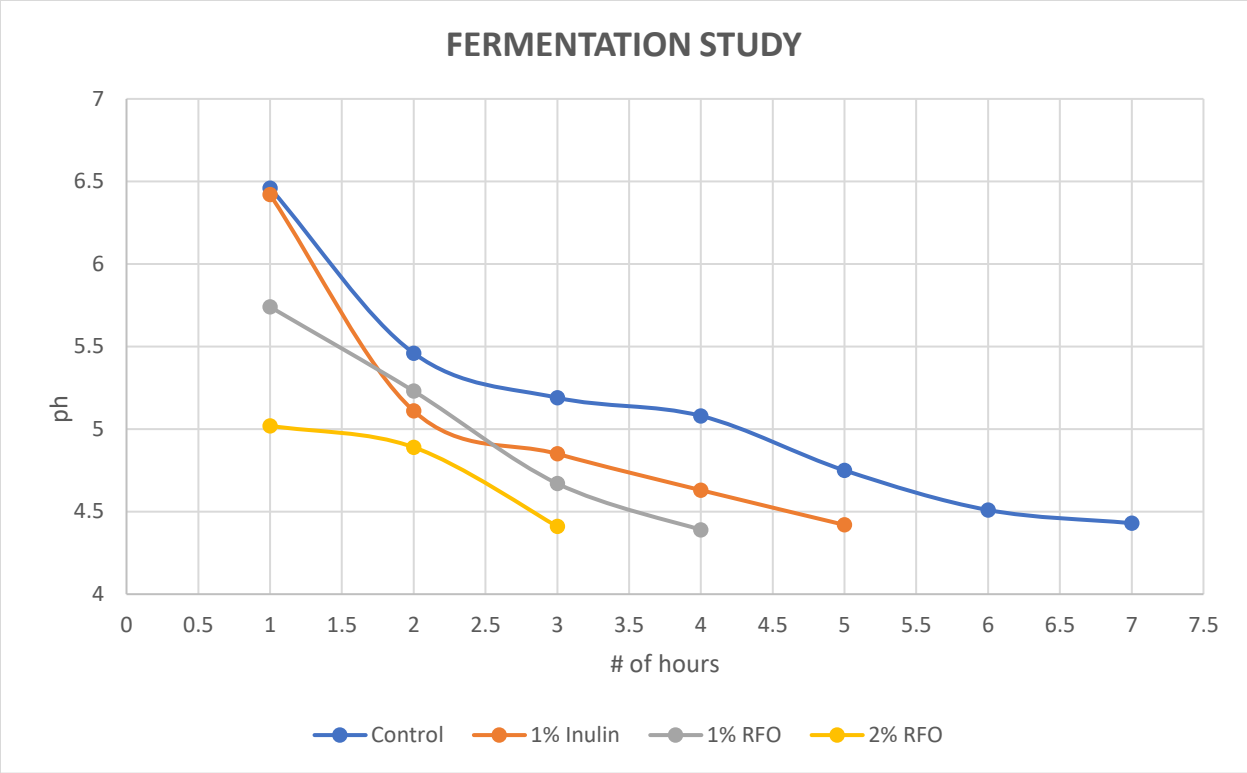

Figure 3: FLine graph of fermentation time taken by the different yogurt samples

One such work was done by Zare et al (Zare et al., 2012) where fermentation time of milk was reduced by almost 4 hours by the addition of different ingredients related to pulses, which include soy flour, lentil flour and chickpea flour. It has been confirmed that this effect is due to the presence of large amounts of dietary fibers present in these pulses. Dietary fibers are nondigestible carbohydrates that cannot be digested by the enzymes present in the human upper intestine, hence they are fermented by the microorganisms present in the lower intestine. One such nondigestible carbohydrate is Raffinose oligosaccharide. Raffinose being a prebiotic helps in increasing the viability of probiotics present in yogurt, thereby increasing the fermentation of lactose which increases the production of lactic acid. This explains the faster fermentation rate in yogurt containing raffinose. From this we can state that the addition of raffinose significantly reduces the fermentation time.

4.3 Effect of fortification on pH and titrable acidity of yogurt

The pH values of yogurt during storage for 21 days at 4^{o} C are presented in table 4 and figure 4. An overall decline in the pH of yogurt occurred during the 21-day storage period. The initial pH values of all the samples were observed to be in the range of 4.43 to 4.45, with plain yogurt which is the control showing higher pH value when compared to the prebiotic fortified yogurts. According to the definition of plain yogurt by FDA (21 C.F.R. § 131.200 2015), the pH must be 4.6 or lower, as recorded before the addition or fortification of any other ingredient. The pH was found to gradually decrease during storage.

Table 4: Effect of Soy-RFO and Inulin fortification on pH of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment**	pH			
	Day 1	Day 7	Day 14	Day 21
Control	$4.45\pm0.01_{Aa}$	$4.40\pm0.01_{Ab}$	$4.35\pm0.01_{Ac}$	$4.22\pm0.01_{Ad}$
Sample 1	$4.44\pm0.01_{Ba}$	$4.39\pm0_{Bb}$	$4.37\pm0.01_{Bc}$	$4.18\pm0.01_{Bd}$
Sample 2	$4.44\pm0.01_{Ba}$	$4.36\pm0.01_{Cb}$	$4.32\pm0_{Cc}$	$4.14\pm0_{Cd}$
Sample 3	$4.43\pm0.01_{Ca}$	$4.33\pm0.01_{Db}$	$4.31\pm0_{Dc}$	$4.07\pm0.01_{Dd}$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$).

Means followed by the same lowercase letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

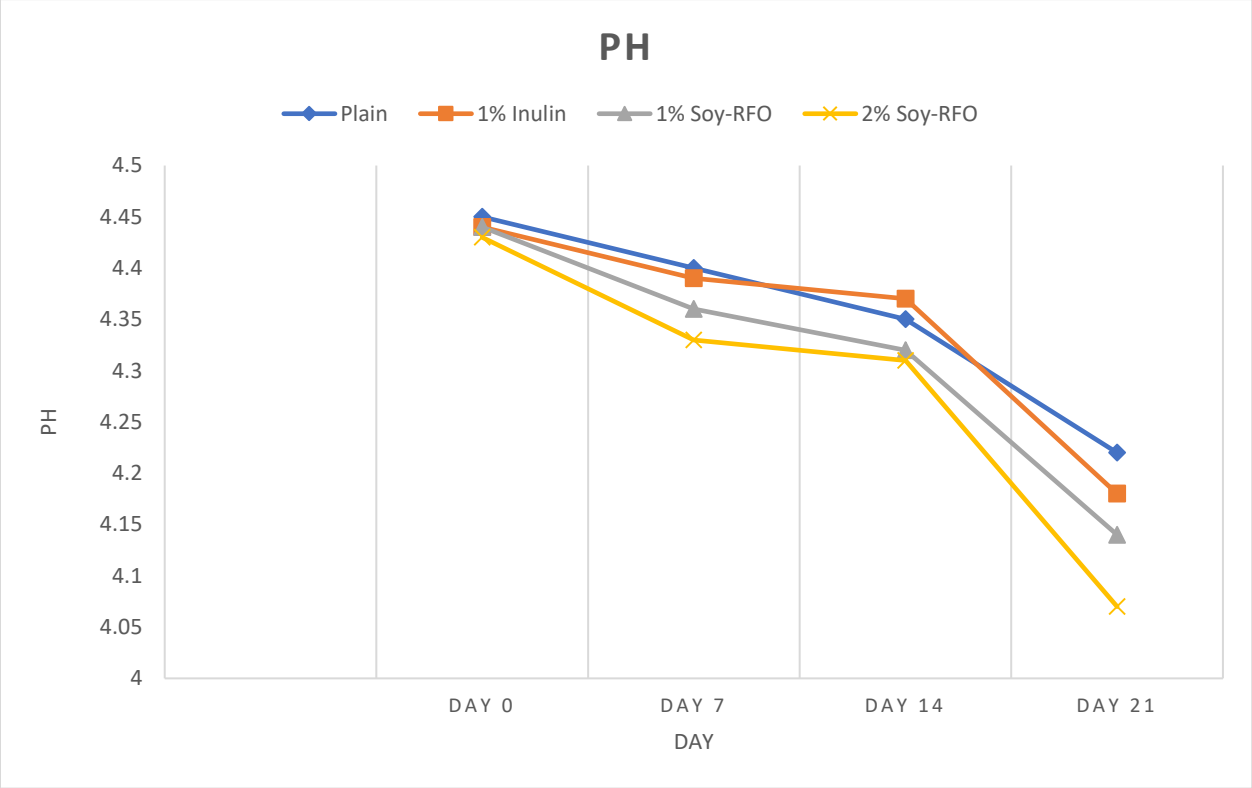

Figure 4: Change in mean values of pH with respect to time

At the end of 21-day storage study, yogurt with 2% (w/v) Soy-Raffinose was found to have the least pH of 4.07 followed by yogurt with 1% (w/v) Soy-Raffinose, yogurt with 1% (w/v) Inulin and control with a pH of 4.14, 4.18 and 4.22 respectively. The rate of increase in the pH of 1% Inulin yogurt on day 14 was found to be slightly less when compared to that of 1% Soy-raffinose yogurt. The changes in pH over time was different depending on the concentration and type of prebiotic ingredient added to yogurt. Increase in the concentration of Soy-raffinose was found to increase the percentage of decrease in pH of the yogurt. The increase in total acidity, which represents the amount of lactic acid present in yogurt during the 21-day storage study is shown in table 5 and figure 5 as follows.

Table 5: Effect of Soy-RFO and Inulin fortification on titrable acidity of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Titratable acidity (% lactic acid)			
	Day 0	Day 7	Day 14	Day 21
Control	1.16 ± 0.12Aa	1.209 ± 0.01Ab	1.313 ± 0.009Ac	1.316 ± 0.006Ad
Sample 1	1.214 ± 0.025Ba	1.281 ± 0.005Bb	1.355 ± 0.01Bc	1.379 ± 0.013Bd
Sample 2	1.296 ± 0.16Ca	1.362 ± 0.01Cb	1.431 ± 0.01Cc	1.445 ± 0Cd
Sample 3	1.326 ± 0.16Da	1.386 ± 0Db	1.438 ± 0.009Dc	1.457 ± 0.001Dd

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$). Means followed by the same lowercase letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

In general, the total acidity increased during the storage period and this upward trend was almost linear compared to the changes in pH. In accordance to FDA, titrable acidity of yogurt must be greater than or equal to 0.9% as expressed in terms of lactic acid that is present. At the end of the 21-day storage study, titrable acidity of yogurt was found to be highest in yogurt with 2% (w/v) Soy-Raffinose and lowest in control yogurt which has only the starter culture. Titrable acidity is an accurate measurement of the total acid content of a solution, an increased titrable acidity could be because of increased number of microorganisms which are the probiotics. The metabolic activity of these probiotics greatly alters the pH and acidity of the yogurt. Higher the activity of probiotics, higher will be the titrable acidity and lower will be the pH.

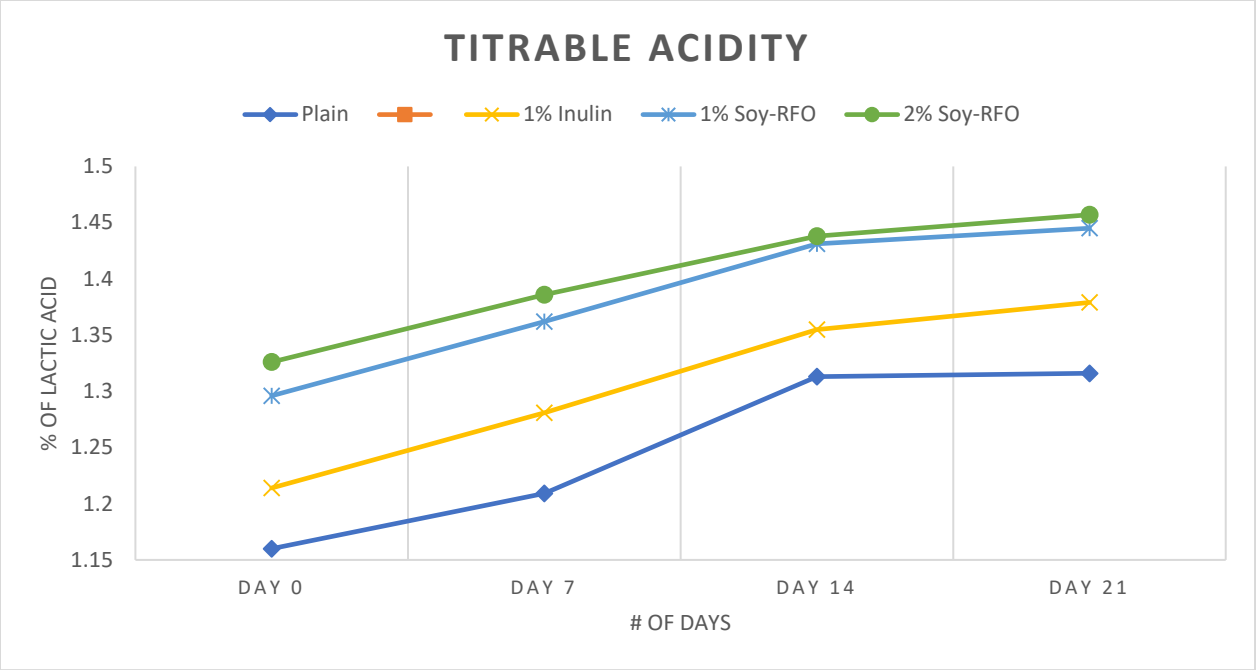

Figure 5: Change in mean values of titrable acidity with respect to time

Yogurt is a produced as a result of the acidic fermentation of milk. The lactose present in milk is converted to lactic acid, which lowers the pH. pH is defined as the measure of strength of the acid present in a solution. Research works done in the past have proved that certain ingredients of the pulse variety that includes pea protein, pea fiber, lentil flour, and germinated soybean possess the potential to increase the amount of acids present in yogurt (Zare et al., 2012). According to the research work done by Mei Yang and Li Li (Mei Yang and Li Li., 2010), the same trend of increased titrable acidity and decreased pH was observed in yogurt prepared with germinated soybeans. Yet another research that was done over a period of 7-day storage study carried out by Oliveira et al (De Souza Oliveira, Perego, Converti, & De Oliveira, 2009) suggest that addition of Inulin had a positive influence on the total amount of lactic acid produced in yogurt that contain different types of probiotic strains. Similar to the findings of Lucey (Lucey, 2004), in this research study continuing to grow the bacteria and also to produce lactic acid through the storage was

responsible for the decrease in pH and increase in titrable acidity. The ideal pH for yogurt may be defined as 4.2 by FDA (21 C.F.R. § 131.200 2015) but in the end it all depends on the preferences of the consumers. Greek style yogurts have a pH that ranges between 3.7 to 3.8 with a high content of fat and total soluble solids which are greatly accepted by consumers (Robinson & Itsaranuwat, 2008).

4.4 Effect of fortification on total soluble solids of yogurt

A significant difference in the level of total soluble solids can be observed between the control yogurt and prebiotic fortified yogurt in table 6 and figure 6. The level gradually decreased during storage until day 14, which did not show any significant change on day 21. From the data that is documented, we can see that the total soluble solids in prebiotic fortified yogurt was higher when compared to the plain yogurt. It was interesting to note that the total soluble solids level of 1% soy-RFO yogurt at 9.43 ±0.32 was lesser than that of 1% Inulin yogurt at 9.47 ±0.58. A steady decrease with time in the level of total soluble solids was noted in all the four yogurt samples. A steady decrease in the levels can be noted until day 14 and this level of decrease in all the yogurt samples after day 14 was significantly lesser when compared to the other days.

Total soluble solids are a measure of the sugar content of a solution using a refraction. It is determined by the index of refraction, measured using a refractometer, and is referred to as the degree Brix. The decrease can be related to the probiotic microorganisms which use up the sugars for fermentation. Between 1% soy-raffinose yogurt and 1% inulin yogurt, inulin yogurt was found to have a higher level of TSS. This could be because Soy-raffinose is more favorable for use by the yogurt probiotic bacteria than inulin and hence its level is much lesser when comparitivey.

Table 6: Effect of Soy-RFO and Inulin fortification on total soluble solids of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Total soluble solids (°Brix)			
	Day 0	Day 7	Day 14	Day 21
Control	8.47 ± 0.58$_{A_a}$	7.6 ± 0.17$_{Ab}$	6.73 ±0.47$_{Cc}$	6.5 ± 0.1$_{Bd}$
Sample 1	9.47 ±0.58$_{Bc}$	8.87 ±0.06$_{Ba}$	7.7 ±0.26$_{Bd}$	7.48 ±0.12$_{Cb}$
Sample 2	9.43 ±0.32$_{Ca}$	8.56 ±0.26$_{Cc}$	7.35 ± 0.1$_{Ab}$	7.27 ± 0.58$_{Ad}$
Sample 3	10 ± 0.1$_{Dd}$	9.2 ± 0.1$_{Da}$	8.77 ± 0.15$_{Dc}$	8.32 ± 0.15$_{Db}$

*Mean ± standard deviation of 3 separate trials. **Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose. Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test. ($p<0.05$). Means followed by the same lowercase letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

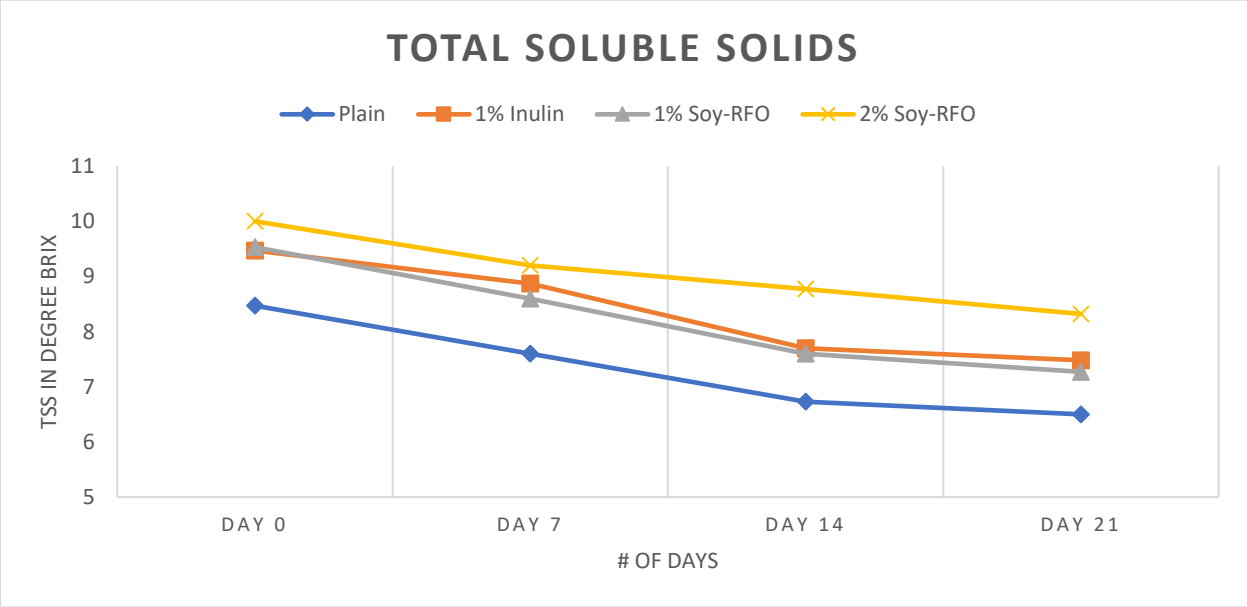

Figure 6: Change in mean values of total soluble solids with respect to time

The lower percentage of decrease in the level of total soluble solids after day 14 can be because of the lower viability of probiotics, due to the increasing acidic environment. According to a previous study *Lactobacillus* bacterium can survive in a more acidic condition (pH of 3.5 to 6.8) when compared to *Streptococcus* bacteria whose growth rate starts to decline at a lower pH (Yu Jin Choi, Hee Sun Yang, Sang Cheon Lee and Chang Ki Huh, 2016).

4.5 Effect of fortification on syneresis of yogurt

Serum release, known as syneresis is considered one of the most important parameters indicating the quality of yogurt during storage. The changes with respect to syneresis during the 21-day storage study is as represented in table 7 and figure 7. Here, the syneresis is expressed as milliliters of serum phase released per 50 mL of yogurt sample. The addition of Soy-Raffinose was found to increase the amount of serum released when compared to the control yogurt. This increase in syneresis was in proportion to the amount of Soy-Raffinose that was added, hence the serum separation was higher when 2% Soy-Raffinose was added.

All the yogurt samples were found to be well intact with no serum release on day 0, and this scenario changed with time. We can see that there was a steady significant increase in serum release until day 14 which dropped to some extent after. On day 7, plain yogurt showed the least amount of syneresis with 3.7 ± 0.1 mL of whey, prebiotic fortified yogurt has a higher amount of syneresis. Between Inulin and Soy-RFO, 1% Inulin was observed to have a lesser syneresis than 1% soy-RFO.

Table 7: Effect of Soy-RFO and Inulin fortification on syneresis of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Syneresis (mL of serum)			
	Day 0	Day 7	Day 14	Day 21
Control	$0 \pm 0_{Ab}$	$3.7 \pm 0.1_{Bc}$	$6.1 \pm 0.06_{Ca}$	$6.2 \pm 0.06_{Ad}$
Sample 1	$0 \pm 0_{Ad}$	$4.4 \pm 0.06_{Aa}$	$7.1 \pm 0.21_{Ab}$	$7.3 \pm 0.17_{Cc}$
Sample 2	$0 \pm 0_{Ad}$	$4.7 \pm 0.20_{Ca}$	$8.7 \pm 0.21_{Bc}$	$8.8 \pm 0.21_{Db}$
Sample 3	$0 \pm 0_{Ac}$	$5.6 \pm 0.14_{Db}$	$9.7 \pm 0.06_{Dd}$	$9.6 \pm 0.46_{Ba}$

*Mean ± standard deviation of 3 separate trials. **Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose. Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$). Means followed by the same lowercase letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

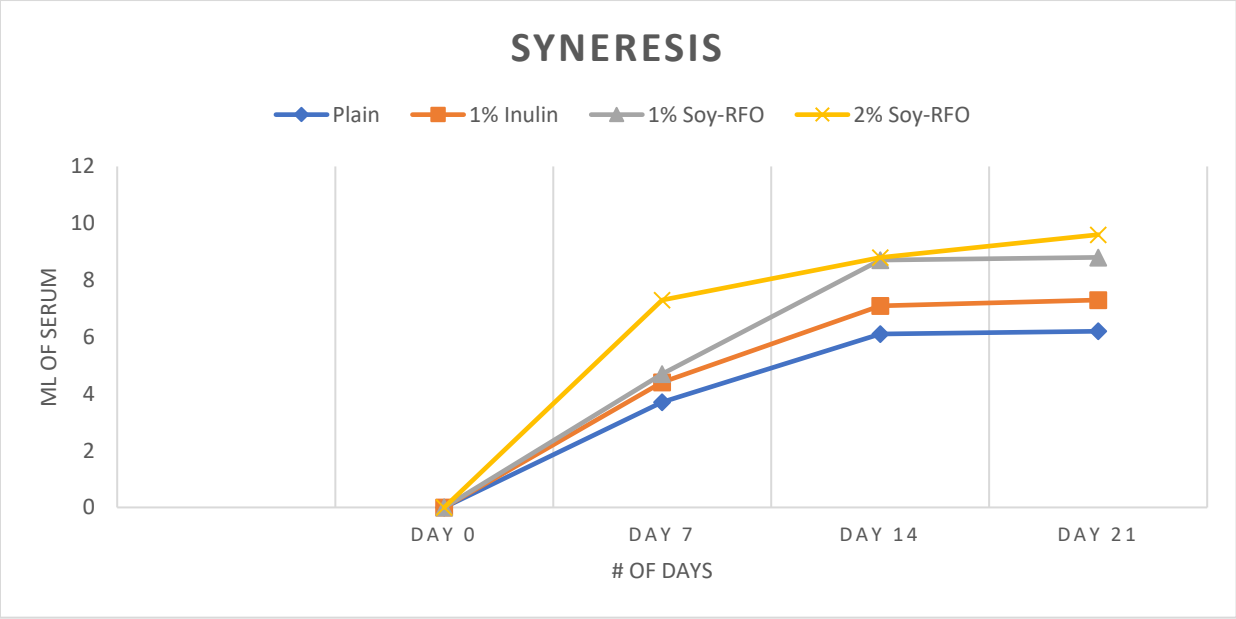

Figure 7: Change in mean values of syneresis with respect to time

Milk has two types of proteins; most of them are casein, and the rest are whey. The higher acidity of yogurt solidifies the casein but on the other hand whey does not get solidified. They separate out from the casein as liquid and this liquid is called whey. Formation of gel matrix in yogurt is performed by casein. (McCann et al., 2011; Cui et al., 2014). Acidification causes several changes which includes the gradual dissolving of calcium and inorganic phosphate. This decreases the net negative electric charge of the casein micelles. The collapse of this layer causes the casein to become insoluble along its isotopic pH of 4.5 resulting in aggregation. Even a small reduction in pH leads to a decreased charge. This in turn weakens the colloidal stability (Walstra et al., 2006). This way, the serum release is enhanced. Also, according to Lucey (2002), one of the main factors that results in increase in the release of whey in yogurts is the continuous production of lactic acid and this phenomenon is called as the post-acidification. One other study done in the past also states that the tendency to exhibit syneresis depends to a greater extent on the changes in pH, which affects the gel structure (Delikanli and Ozcan, 2014).

4.6 Effect of fortification on water holding capacity of yogurt

In general, water holding capacity of yogurt is defined as the ability to hold its own or added water during the application of force, pressure, centrifugation, or heating. The values recorded over the 21-day storage study for water holding capacity of yogurt are as follows in the table 8 and figure 8 below. Water holding capacity of all the yogurt samples were found to reduce with time, during the 21-day study. From the data recorded, we can see that all the yogurt samples had 100% water retention capacity, which started to decrease with time. At the end of day 7, all the yogurt samples showed a significant decrease with respect to water holding capacity of 81.3 ± 0.58% with plain yogurt having the least capacity and 1% soy-RFO the highest at 87.3 ± 0.58%. After day 7,

there was still a drop in the capacity in all the yogurt samples but the difference between the means of day 7 and day 14 was not as high as the difference between day 0 and day 7. Interestingly at the end of day 21, we can see that the water holding capacity of 1% Inulin yogurt, 1% soy-RFO yogurt and 2% soy-RFO yogurt increased by 4%, 1.5% and 0.6% respectively, while the capacity of plain yogurt dropped by 0.67%. Overall, from the data we can infer that fortification of soy-RFO had a positive effect on water holding capacity of yogurt, more than the effect of Inulin.

Table 8: Effect of Soy-RFO and Inulin fortification on water holding capacity of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Water holding capacity (% of water released)			
	Day 0	Day 7	Day 14	Day 21
Control	$100 \pm 0_{Bc}$	$81.3 \pm 0.58_{Ab}$	$78.67 \pm 0.58_{Ca}$	$78 \pm 1aA$
Sample 1	$100 \pm 0_{Bd}$	$82.67 \pm 1.53_{Ca}$	$80.58 \pm 0.58_{Ab}$	$84.67 \pm 1.53_{cB}$
Sample 2	$100 \pm 0_{Bb}$	$87.3 \pm 0.58_{Ba}$	$85.67 \pm 2.08_{Bd}$	$87 \pm 1_{cD}$
Sample 3	$100 \pm 0_{Ba}$	$90 \pm 1_{Db}$	$87 \pm 1_{Dd}$	$87.6 \pm 1.53_{dD}$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$). Means followed by the same lowercase letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

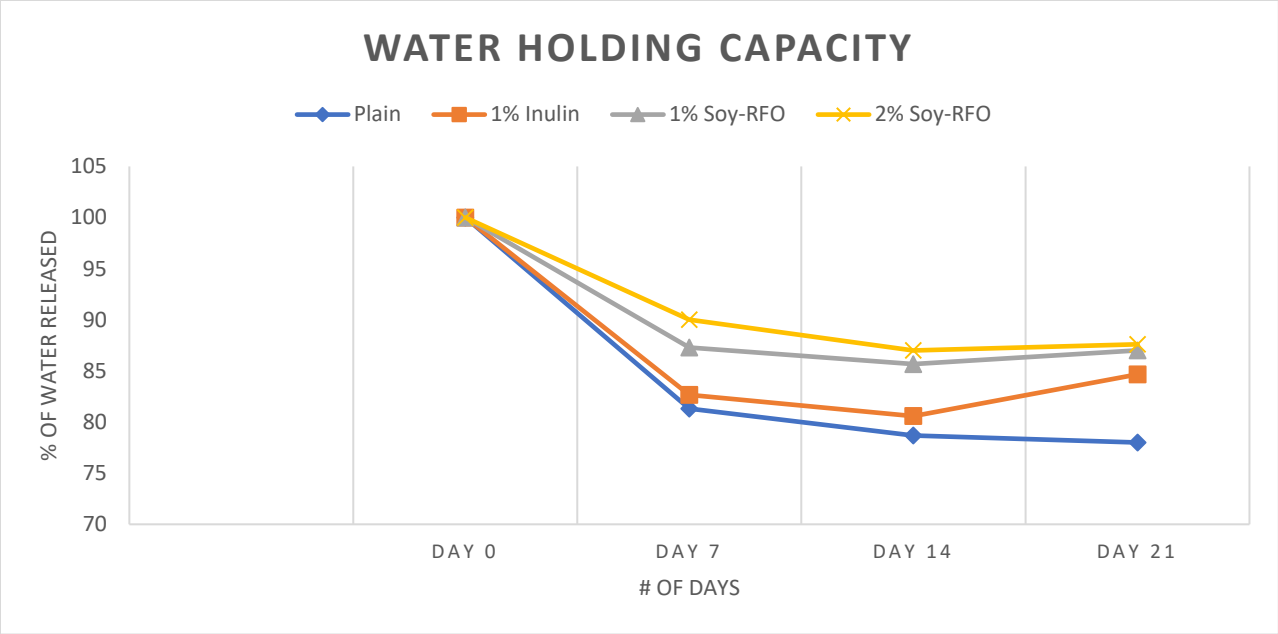

Figure 8: Change in mean values of water holding capacity with respect to time

The water holding capacity of a protein gel is one of the important quality parameters in the process of yogurt manufacturing. It is the ability of proteins to prevent water from being released or expelled from their three-dimensional structure (Hermansson, 1986; Zayas, 1997b). It plays an important role in developing the food's texture. Lower water holding capacity can be related partly due to the unstable gel network of yogurts. This happens because the weak colloidal linkage of protein micelles loses its ability to entrap water in its three-dimensional network (Mei Yang and Li Li., 2010).

4.7 Effect of fortification on water activity

During the entire storage period of 21 days, from table 9 and figure 9 we can observe that the water activity of all the four yogurt samples varied between 0.86 a w and 0.95 a w. From the readings, we can see that soy-raffinose helped in the reduction of water activity. There is not much significant difference between the water activity of 1% soy-raffinose yogurt and 1% inulin yogurt.

But there is a significant difference between the water activity of control yogurt and the prebiotic fortified yogurt. Water activity was also found to decrease with increase in the concentration of soy-raffinose. This difference in water activity between the yogurt samples can be related to the increase in sugar content in the prebiotic fortified yogurts. This follows the result as stated by M. Ruegg (1985) that, added or already present food ingredients such as sugar and salt help in the reduction of water activity by binding to the free water molecules present in it. This finding can also be related to the decrease in water activity level after day 14; this could be because of the decrease in sugar content of the yogurt with increase in viable microorganisms that use up the sugars available for fermentation leaving behind free water molecules.

Table 9: Effect of Soy-RFO and Inulin fortification on water activity of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Water activity (a w)			
	Day 0	Day 7	Day 14	Day 21
Control	$0.94 \pm 0_{Aa}$	$0.89 \pm 0_{Bc}$	$0.95 \pm 0_{Ab}$	$0.90 \pm 0.01_{Cd}$
Sample 1	$0.89 \pm 0.01_{Bc}$	$0.87 \pm 0_{Ac}$	$0.87 \pm 0_{Cc}$	$0.85 \pm 0_{Bc}$
Sample 2	$0.89 \pm 0_{Bd}$	$0.86 \pm 0_{Ad}$	$0.88 \pm 0.01_{dC}$	$0.87 \pm 0.01_{dB}$
Sample 3	$0.88 \pm 0.01_{aB}$	$0.86 \pm 0.01_{Aa}$	$0.89 \pm 0.02_{aC}$	$0.88 \pm 0.01_{Ba}$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$). Means followed by the same lowercase letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

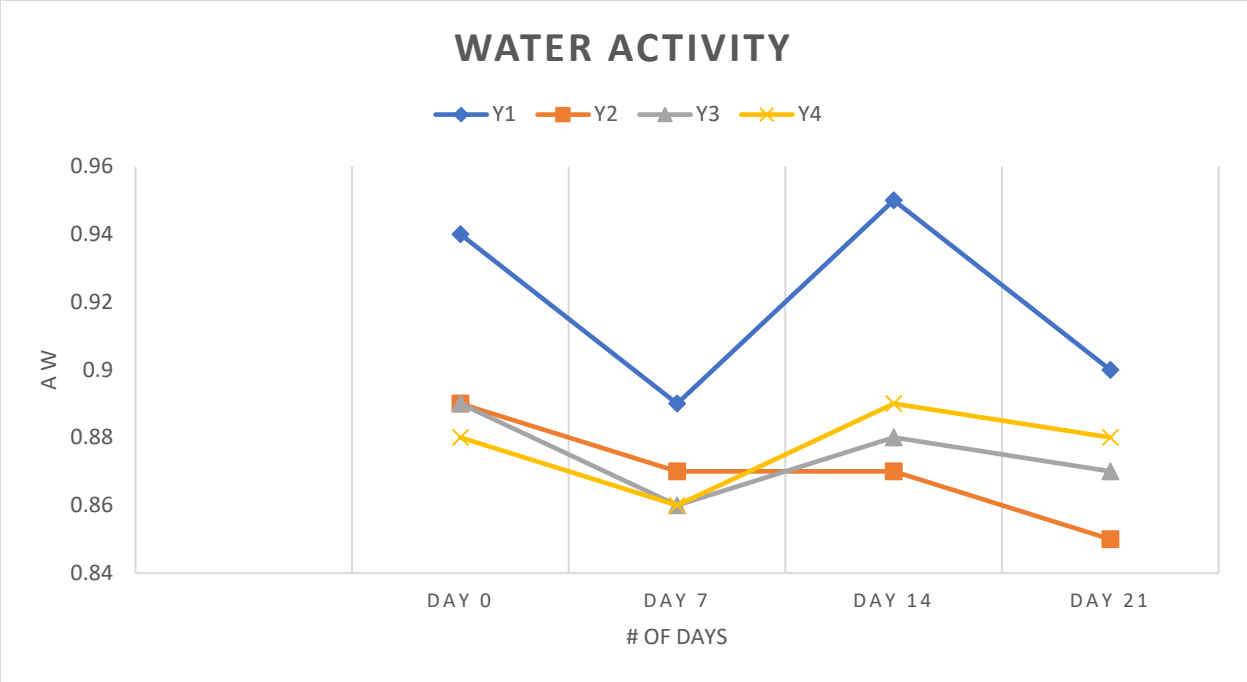

Figure 9: Change in mean values of water activity with respect to time

According to the FDA, water activity in foods can be defined as the ratio between vapor pressure of the food and vapor pressure of distilled water under the same surrounding conditions. It is basically the moisture condition of a food product expressed in decimals. The ideal water activity of yogurt is recorded to be 0.84 or less which is also the optimum water activity level for the growth of good bacteria. At this level of available moisture, the growth of unwanted microorganisms is inhibited.

4.8 Effect of fortification on viscosity of yogurt

Viscosity values for all the yogurt samples documented during the 21-day storage study period are represented in table 10 and figure 10 below. Viscosity is defined as the state of being thick and semifluid in consistency due to internal friction. It is basically the measure of magnitude of internal friction. Viscosity values for all the yogurts taken during the storage period vary between 2.752 ± 0.05 Pa*S and 3.315 ± 0.02 Pa*s. Yogurt with 1% Soy-Raffinose showed highest viscosity when compared to the control and 1% Inulin yogurt. Viscosity was also found to significantly increase with increase in RFO concentration. In all the yogurt samples, viscosity was observed to increase significantly until day 14 and it decreases on day 21. When compared to 1% soy-RFO yogurt, the 2% soy-RFO yogurt had a higher rate of increase on day 14.

Table 10: Effect of Soy-RFO and Inulin fortification on the viscosity of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Viscosity (Pa*s)			
	Day 0	Day 7	Day 14	Day 21
Control	$2.752 \pm 0.05_{Ad}$	$2.805 \pm 0.01_{aC}$	$2.895 \pm 0.01_{cC}$	$2.806 \pm 0.01_{bA}$
Sample 1	$2.889 \pm 0.01_{Cc}$	$2.897 \pm 0.02_{bA}$	$2.959 \pm 0.03_{aB}$	$2.932 \pm 0.02_{dD}$
Sample 2	$2.944 \pm 0.02_{Bb}$	$2.932 \pm 0.04_{dB}$	$2.985 \pm 0.01_{cA}$	$2.977 \pm 0.03_{aB}$
Sample 3	$2.889 \pm 0.01_{Da}$	$3.132 \pm 0.05_{bD}$	$3.315 \pm 0.02_{dD}$	$2.999 \pm 0.05_{cD}$

*Mean ± standard deviation of 3 separate trials. **Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$). Means followed by the same lowercase letter (abcd) in the same row are not significantly different during that particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

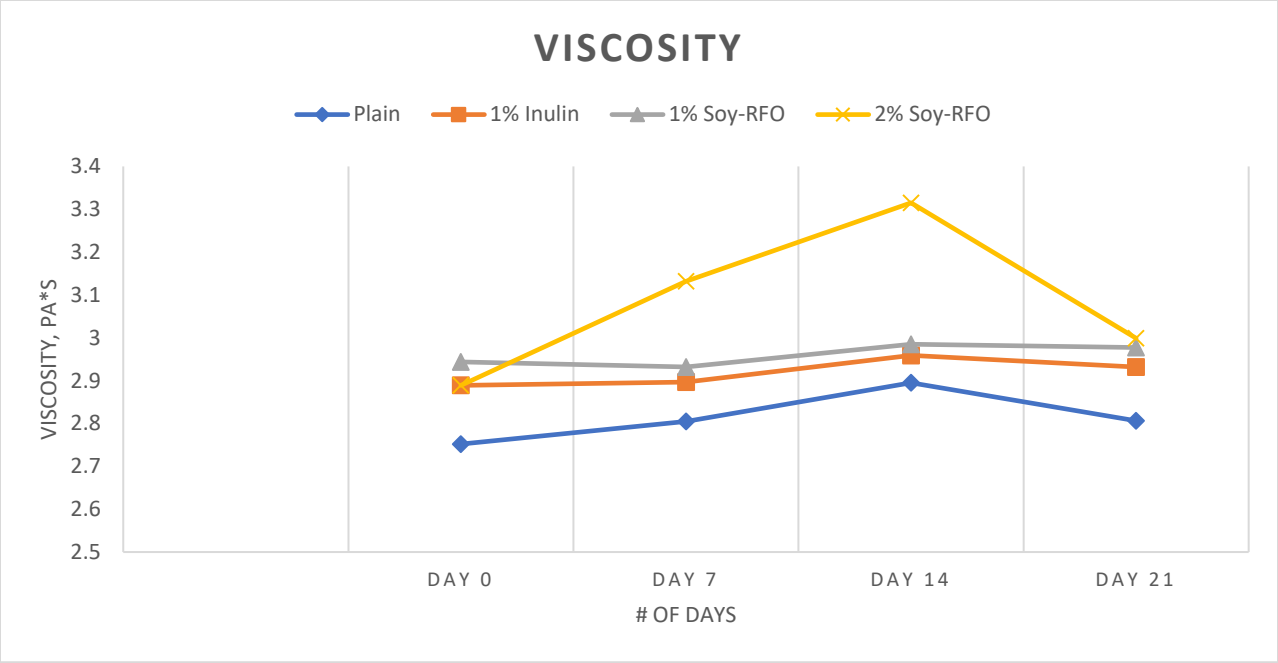

Figure 10: Change in mean values of viscosity with respect to time

Thermophilic bacteria present in the yogurt (*Streptococcus thermophilus*, and *Lactobacillus bulgaricus*) induces the coagulation of milk proteins at high temperatures. With the increase in concentration of lactic acid the protein present in milk forms a gel structure and results in the viscosity of yogurt. The increase in viscosity of yogurt samples can be explained by post-acidification. The lactic acid bacteria continue to produce lactic acid over the storage period and this phenomenon is called as post-acidification. Post-acidification is said to have several effects on yogurt, which includes strong acid taste, increased serum separation and increased consistency (Han et al., 2012). This result is also like another research as carried out by Xu et al., (2015), which states that yogurt samples when fermented by strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*, increasing viscosity was observed during storage. The results obtained from this study is also in accordance with the study conducted by Kip, et al., in 2006, that proved Inulin has a higher ability to retain water and promote a stronger gel behavior (Kip, Meyer, and Jellema, 2006; Meyer, Bayarri, Tarrega and Costell, 2011).

4.9 Color

Color is one of the most important visual quality in any dairy product, especially yogurt. Differences in color affect the storage period, shelf life and deterioration of color in yogurt (Coggins et al., 2010) Color measurements show even the slightest difference of color between the yogurt samples. The average measurement values of L*, a*, b* (a* and b* are the chromatic components) taken over the course of 21-day storage study are represented as below in table 11 (day 0), table 12 (day 7), table 13 (day 14), and table 14 (day 21).

Table 11: Effect of Soy-RFO and Inulin fortification on L*, a* and b* of yogurt on Day 0 during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Color parameters*** on Day 0		
	L*	a*	b*
Control	$95.11 \pm 0.01_{A}$	$-1.34 \pm 0.01_{B}$	$9.78 \pm 0.02_{D}$
Sample 1	$95.29 \pm 0.02_{A}$	$-1.31 \pm 0.01_{B}$	$9.79 \pm 0.01_{D}$
Sample 2	$95.34 \pm 0.02_{A}$	$-1.35 \pm 0.02_{B}$	$9.77 \pm 0.02_{D}$
Sample 3	$95.44 \pm 0.01_{A}$	$-1.36 \pm 0.01_{B}$	$9.73 \pm 0.01_{D}$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

*** L* = luminance or lightness; a* = green to red; b* = blue to yellow.

Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$).

The means values of different color parameters namely, L*, a*, and b* that were observed on day 0 are tabulated in table 11 above. From the table we can observe that the values of all the yogurt samples for L* ranged between 95.11±0.01 and 95.44±0.02; a* ranged between -1.31±0.01 and -1.36±0.02; b* ranged from 9.73±0.01 to 9.79±0.01. 1% Soy-RFO yogurt had the highest brightness of 95.34±0.02 when compared to 1% inulin yogurt at 95.29±0.02 and plain yogurt had the least value of 95.11±0.01. With respect to a*, the value was lowest for 1% Soy-RFO, followed by plain yogurt and highest for 1% Inulin. 1% Soy-RFO yogurt had the least b* value when compared to that of plain yogurt and 1% Inulin with the highest value. The values of L* were found to increase with increase in concentration of soy-RFO while it decreased the value of a* and b* but the difference between them were not statistically significant. Hence, we can say that the fortification had no effect on the color of yogurt samples.

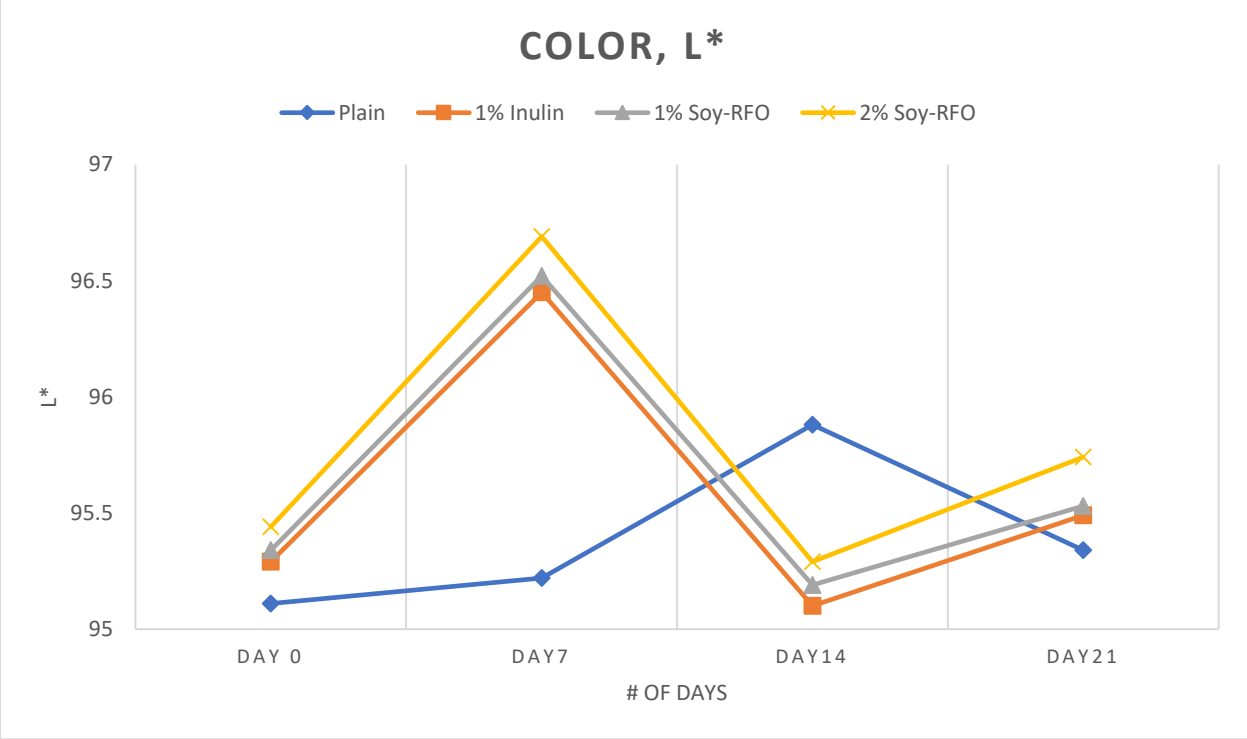

Figure 11: Change in mean values of L with respect to time*

Table 12: Effect of Soy-RFO and Inulin fortification on L*, a* and b* of yogurt on Day 7 during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Color parameters*** on Day 7		
	L*	a*	b*
Control	$95.22\pm0.01_A$	$-1.30\pm0.03_B$	$9.77\pm0.01_D$
Sample 1	$96.45\pm0.02_A$	$-1.36\pm0.01_B$	$9.76\pm0.03_D$
Sample 2	$96.52\pm0.01_A$	$-1.37\pm0.01_B$	$9.76\pm0.01_D$
Sample 3	$96.69\pm0.03_A$	$-1.39\pm0.02_B$	$9.79\pm0.02_D$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

*** L* = luminance or lightness; a* = green to red; b* = blue to yellow.
Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$).

The means values of different color parameters namely, L*, a*, and b* that were observed on day 7 are tabulated in table 12 above. From the table we can observe that the values of all the yogurt samples for L* ranged between 95.22±0.01 and 96.69±0.03; a* ranged between -1.30±0.03 and -1.39±0.02; b* ranged from 9.76±0.01 to 9.79±0.02. Even though the differences between the means of all the 3 parameters of four yogurt samples are not statistically significant, the 1% Soy-RFO yogurt had the highest brightness of 96.52 ±0.01when compared to 1% inulin yogurt at 96.45±0.02 and plain yogurt had the least value of 95.22±0.01. With respect to a*, the value was lowest for 1% Soy-RFO, followed by plain yogurt and highest for 1% Inulin. 1% Soy-RFO and 1% Inulin had the same b* value while plain yogurt had a higher b* value than the former 2

samples. The values of L* increased with increase in concentration of soy-RFO while it decreased the value of a* and b* but the difference between them was not statistically significant. Hence, we can say that the fortification had no effect on the color of yogurt samples.

Table 13: Effect of Soy-RFO and Inulin fortification on L*, a* and b* of yogurt on Day 14 during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Color parameters*** on Day 14		
	L*	a*	b*
Control	$95.88\pm0.04_{A}$	$-1.35\pm0.03_{C}$	$9.77\pm0.02_{B}$
Sample 1	$95.10\pm0.01_{C}$	$-1.37\pm0.02_{C}$	$9.74\pm0.01_{C}$
Sample 2	$95.19\pm0.01_{B}$	$-1.36\pm0.02_{C}$	$9.78\pm0.02_{BBD}$
Sample 3	$95.29\pm0.04_{D}$	$-1.34\pm0.01_{B}$	$9.79\pm0.01_{BD}$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

*** L* = luminance or lightness; a* = green to red; b* = blue to yellow.
Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$).

The means values of different color parameters namely, L*, a*, and b* that were observed on day 14 are tabulated in table 13 above. From the table we can observe that the values of all the yogurt samples for L* ranged between 95.10±0.01 and 95.88±0.04; a* ranged between -1.34±0.01 and -1.37±0.02; b* ranged from 9.74±0.01 to 9.79±0.01. Even though the differences between the means of all the 3 parameters of four yogurt samples were not statistically significant, the plain

yogurt had the highest brightness of 95.88±0.04 when compared to 1% soy-RFO at 95.19±0.01 and followed by 1% inulin yogurt at 95.10±0.0. With respect to a*, the value was lowest for 1% Inulin yogurt, followed by 1% soy-RFO and highest for plain yogurt. 1% Soy-RFO had the highest b* value when compared to plain yogurt and the least for 1% inulin yogurt. The values of L*, a* and b* increased with increase in concentration of soy-RFO. but the difference between them were not statistically significant. Hence, we can say that the fortification had no effect on the color of yogurt samples.

The means values of different color parameters namely, L*, a* and b* that were observed on day 21 are tabulated in table 14 below. From the table we can observe that the values of all the yogurt samples for L* ranged between 95.34±0.02 and 95.74±0.01; a* ranged between -1.33±0.02 and -1.38±0.03; b* ranged from 9.71±0.02 to 9.76±0.01. The 1% soy-RFO yogurt had the highest brightness of 95.53±0.02 when compared to 1% Inulin yogurt at 95.49±0.01 followed by plain yogurt at 95.34±0.02. With respect to a*, the value was lowest for plain yogurt, followed by 1% Inulin yogurt and highest for 1% soy-RFO yogurt. 1% Soy-RFO had the highest b* value when compared to 1% inulin yogurt with plain yogurt the least b*. The values of L*, a* and b* increased with increase in concentration of soy-RFO but the difference between them were not statistically significant. Hence, we can say that the fortification had no effect on the color of yogurt samples

Table 14: Effect of Soy-RFO and Inulin fortification on L*, a* and b* of yogurt on Day 21 during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Color parameters*** on Day 21		
	L*	a*	b*
Control	$95.34 \pm 0.02_{A}$	$-1.38 \pm 0.03_{D}$	$9.71 \pm 0.02_{A}$
Sample 1	$95.49 \pm 0.01_{A}$	$-1.36 \pm 0.02_{D}$	$9.72 \pm 0.03_{A}$
Sample 2	$95.53 \pm 0.02_{A}$	$-1.35 \pm 0.01_{D}$	$9.74 \pm 0.01_{A}$
Sample 3	$95.74 \pm 0.01_{A}$	$-1.33 \pm 0.02_{D}$	$9.76 \pm 0.01_{A}$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

*** L* = luminance or lightness; a* = green to red; b* = blue to yellow.

Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$).

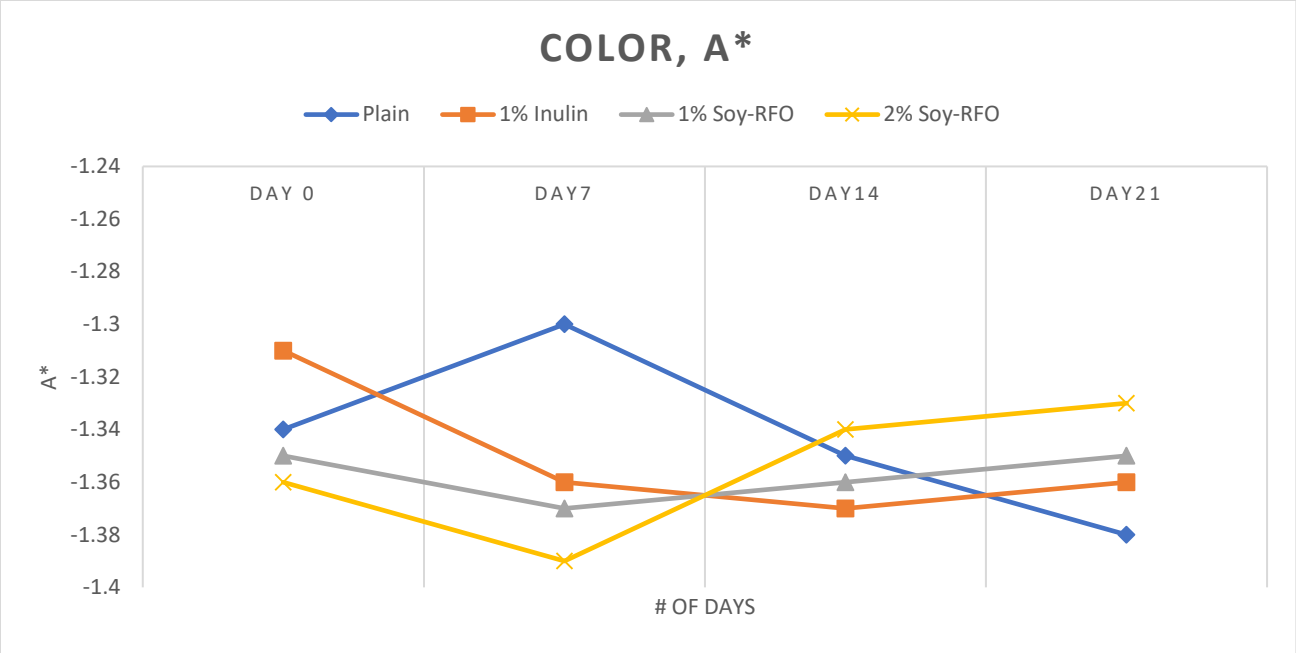

Figure 12: Change in mean values of a with respect to time*

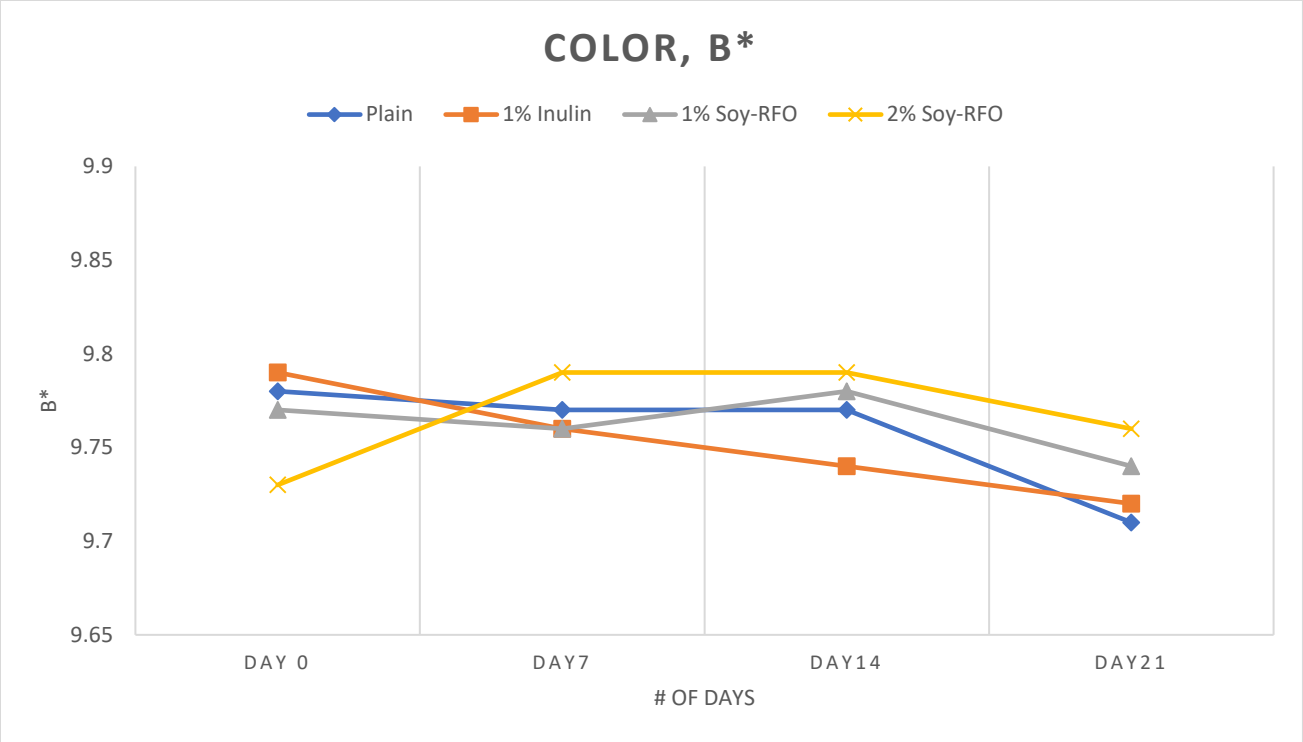

Figure 13: Change in mean values of b with respect to time*

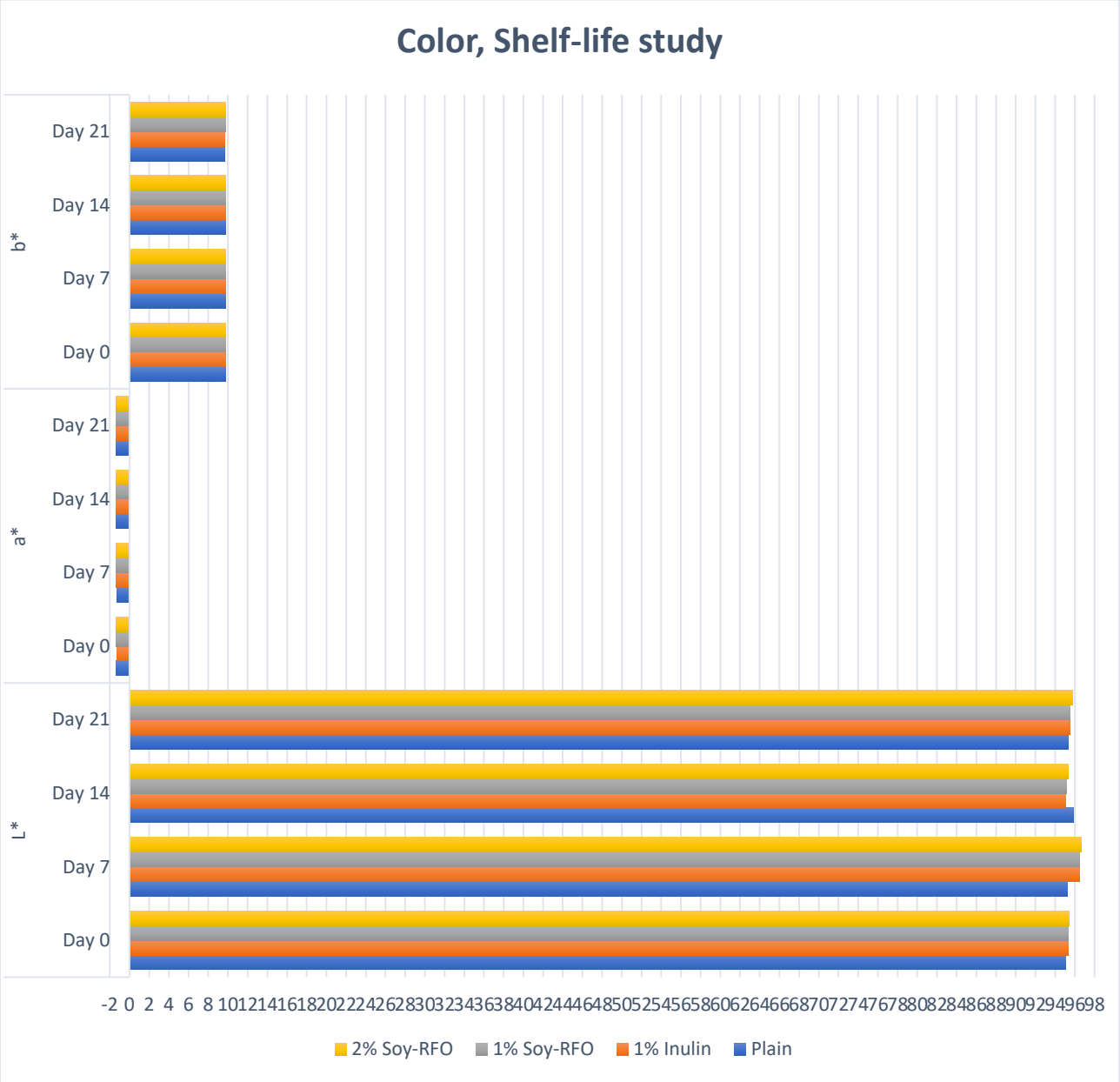

Figure 14: Clustered bar chart of mean values of L, a* and b* with respect to time*

4.10 Texture Profile Analysis

The average values of various parameters of texture profile analysis of yogurt namely, firmness, cohesiveness, gumminess, adhesiveness, elasticity and springiness are presented in table 15.

Firmness, also called as hardness is defined as the first peak that occurred on the graph during the first compression cycle. It is basically the amount of maximum force that was applied during the first compression cycle. The average values of firmness for the different yogurt samples are as presented in table 15. Prebiotic fortified yogurt was found to have a higher hardness when compared to the plain control yogurt. Soy-Raffinose was found to have significantly increased the firmness of yogurt at a little more level at 118±0.02 g when compared to the Inulin yogurt, 114±0.02 g.

Cohesiveness is defined as the quality of forming a united and whole product. The difference between the cohesiveness of yogurt samples was significantly good, which can be noted in table 14. The effect of Soy-Raffinose and Inulin on the cohesiveness of yogurt had no significant difference between them. But we can see a significant increase between the control and fortified yogurts. Soy-raffinose was found to significantly increase the cohesiveness of yogurt and the cohesiveness of yogurt has been observed to increase with increased concentration of Soy-Raffinose.

The ability to stick together is called as adhesiveness. According to the average values of adhesiveness recorded in table 14, it can be observed that there was no significant increase or decrease with the adhesiveness between different yogurt samples with the fortification. From this data we can infer that the force needed inside the mouth to remove the different yogurts that were under study were almost the same.

The elasticity of a food product which makes it to be stretched and its ability to return to its original length is defined as the springiness. No significant difference was seen between the springiness of the four yogurt samples. This means that the fortification of Inulin or Soy-RFO did not have any effect on the springiness of the yogurt.

The mouthfeel sensation that is felt during chewing a food product, caused by the sustained, elastic resistance from the food is called as chewiness. The prebiotic fortification was observed to significantly increase the chewiness of yogurt. Comparatively, Soy-RFO had an increased effect on the chewiness when compared to Inulin.

Table 15: Effect of Soy-RFO and Inulin fortification on firmness, cohesiveness, adhesiveness, springiness, chewiness and gumminess of yogurt on Day 21 during the 21-day shelf life study (mean ± standard deviation)

	Firmness (g)	Cohesivene--ss	Adhesivene--ss (g.s)	Springiness	Chewiness	Gumminess
Control	$110\pm0.01_a$	$0.29\pm0.03_a$	$-31.92\pm0.02_c$	$0.94\pm0.04_a$	$30.4\pm0.02_a$	$32.34\pm0.01_a$
Sample 1	$114\pm0.03_b$	$0.36\pm0.01_c$	$-32.33\pm0.03_c$	$0.95\pm0.02_a$	$39.2\pm0.02_b$	$41.26\pm0.03_c$
Sample 2	$118\pm0.02_c$	$0.37\pm0.01_b$	$-32.67\pm0.02_c$	$0.96\pm0.05_a$	$42.3\pm0.01_d$	$44.01\pm0.04_b$
Sample 3	$184\pm0.01_d$	$0.389\pm0.02_d$	$-32.22\pm0.01_c$	$0.99\pm0.03_b$	$70.7\pm0.02_c$	$71.39\pm0.02_d$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

Means followed by the same lowercase letter (abcd) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$).

The ability to be both cohesive and sticky is defined as gumminess of a food product. We can see a significant difference between the yogurt samples with respect to cohesiveness, the fortification did increase the hardness of yogurt samples. So, a significant difference in the gumminess between yogurt samples were observed. These observations were similar to that of firmness; gumminess of Soy-RFO yogurt was the highest at 44.014±0.01 when compared to that of Inulin yogurt at 41.268±0.03 being the second highest and the least was found in the plain yogurt at 32.34±0.01 and this increase was also found with increased concentration of Soy-Raffinose.

Table 16: Texture profile analysis – parameters of yogurt - firmness, cohesiveness, chewiness adhesiveness, springiness, and gumminess

Hardness/Firmness	Peak force during the 1st compression cycle
Cohesiveness	The ratio of positive force area during 2nd compression cycle to that during the 1st compression cycle
Adhesiveness	Negative force area of the 1st compression cycle
Springiness	Length to which the sample recovers in height during the time that elapses between the end of the 1st compression cycle and start of 2nd compression cycle
Chewiness	Product of hardness, cohesiveness and springiness
Gumminess	Product of hardness and cohesiveness

Texture profile analysis of yogurt is performed by compressing a food product two times with a very short interval between them. This imitates the actual condition that the food undergoes in the mouth (M. Bourne, 1978; A. S. Sczcesniak, 1963). Texture is one of the most important

parameters to assess the quality of yogurt. It is in relation to the sensory perception of foods (Tulay Ozcan, 2013). Chewiness, gumminess, cohesiveness, adhesiveness and firmness are the parameters that are measured in a texture profile analysis (Bourne 1978; Chen and Stokes, 2012). The textural properties of yogurt depend to a maximum extent on the internal structure. It results in a firm protein gel which is also brittle due to the tight structure of molecules. Low firmness can be attributed to looser network architecture. Overall, addition of Soy-RFO only improves the firmness of yogurt which is considered important to assess the quality of yogurt. This increase in turn also increases the chewiness of Soy-RFO yogurt. More research on this area is required to conclude if this increase in chewiness is truly beneficial or how it affects the overall acceptability of Soy-RFO yogurts.

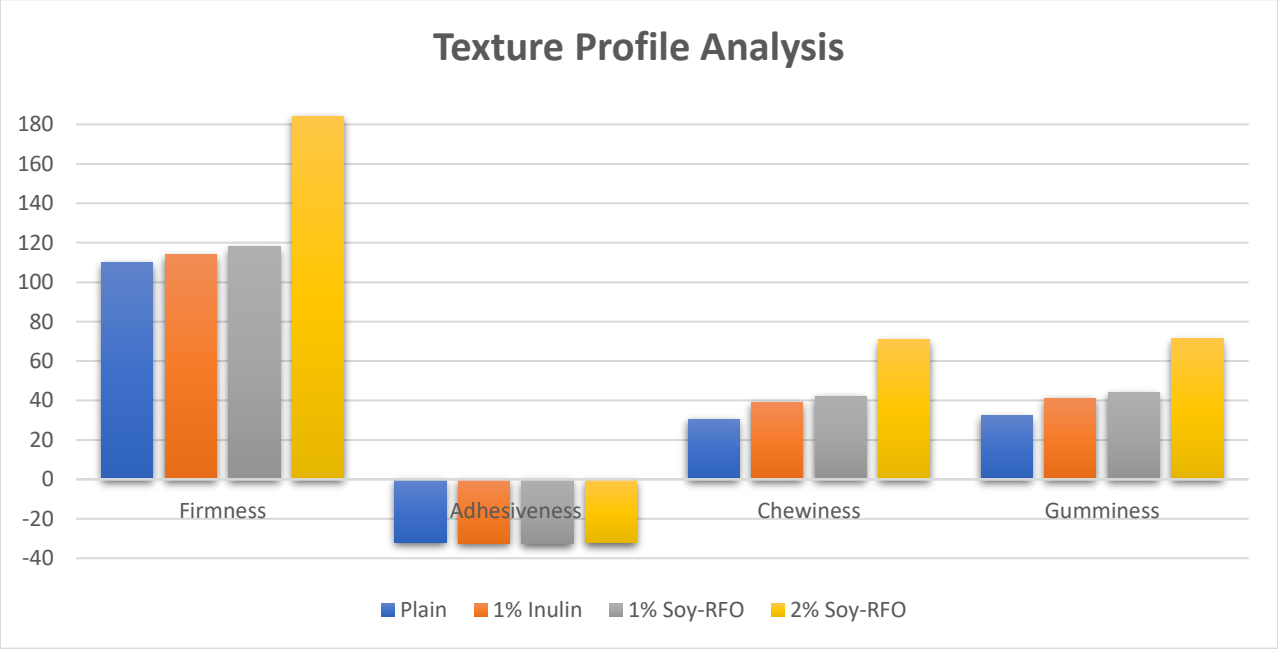

Figure 15: Clustered column chart of mean values firmness, adhesiveness, chewiness and gumminess with respect to time

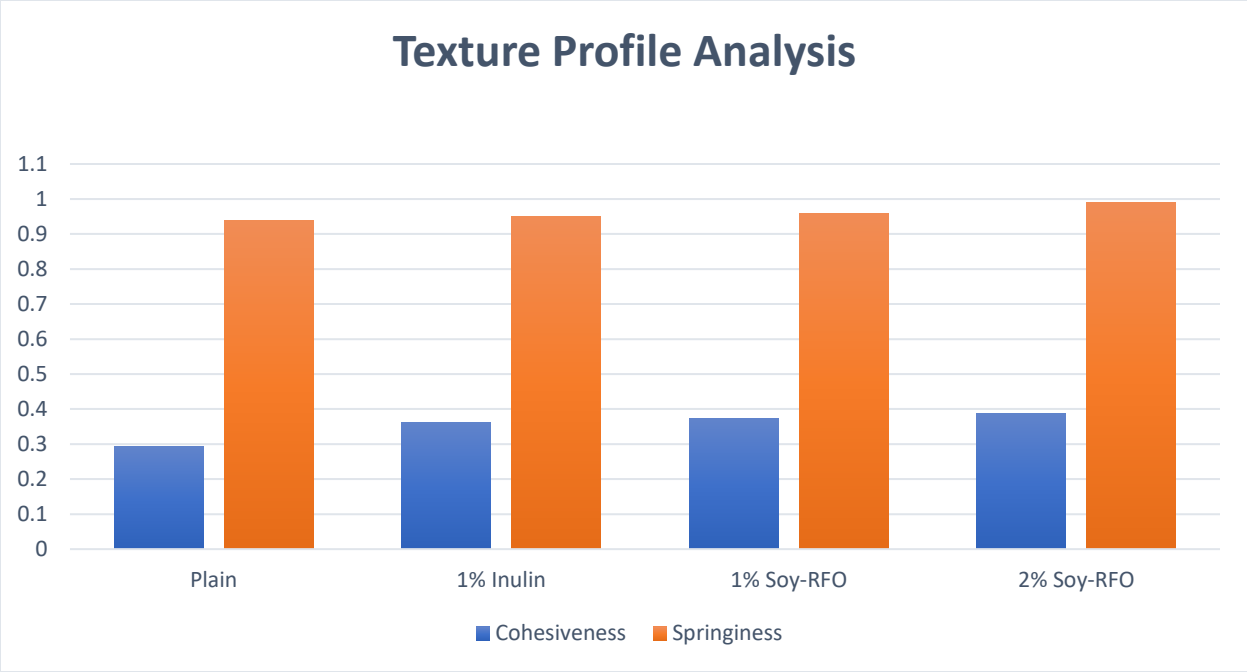

Figure 16: Clustered column chart of mean cohesiveness and springiness with respect to time

4.11 Antioxidant activity

In this study based on the readings obtained, we can observe that the antioxidant capacity of 1% Soy-raffinose yogurt was significantly higher at 36.04 ± 0.57% on day 1 when compared to that of 1% inulin yogurt and control yogurt which had an inhibition percentage of 32.78 ± 1.01% and 29.42 ± 0.61% respectively. The antioxidant activity was found to have significantly increased during storage on day 7 and day 14 which started to decrease after day 14. On comparing 1% Soy-raffinose yogurt and 1% inulin yogurt, we can see that the 1% soy-raffinose showed a significant higher inhibition capacity than the other. We can also relate this to the increased number of viable probiotic bacteria, *Lactobacillus bulgaricus* in 1% soy-raffinose yogurt in comparison with 1% inulin yogurt. It can also be inferred from the data table 17 that inhibition percentage increased with increase in soy-raffinose concentration.

Table 17: Effect of Soy-RFO and Inulin fortification on antioxidant activity of yogurt during the 21-day shelf life study (mean ± standard deviation)

Treatment**	Antioxidant activity (%)			
	Day 0	Day 7	Day 14	Day 21
Control	$29.42 \pm 0.61_{Aa}$	$31.06 \pm 0.80_{Ab}$	$39.41 \pm 0.67_{Ac}$	$35.5 \pm 0.88_{Ad}$
Sample 1	$32.78 \pm 1.01_{Ba}$	$55.95 \pm 1.49_{Bb}$	$61.19 \pm 0.85_{Bc}$	$49.72 \pm 0.51_{Bd}$
Sample 2	$36.04 \pm 0.57_{Ca}$	$59.95 \pm 0.37_{Cb}$	$64.89 \pm 0.71_{Cc}$	$55.67 \pm 0.51_{Cd}$
Sample 3	$40.22 \pm 0.58_{Da}$	$66.15 \pm 1.01_{Db}$	$69.82 \pm 1.40_{Dc}$	$69.35 \pm 1.54_{Dd}$

*Mean ± standard deviation of 3 separate trials.

**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

Means followed by the same uppercase letter (ABCD) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$). Means followed by the same lowercase letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

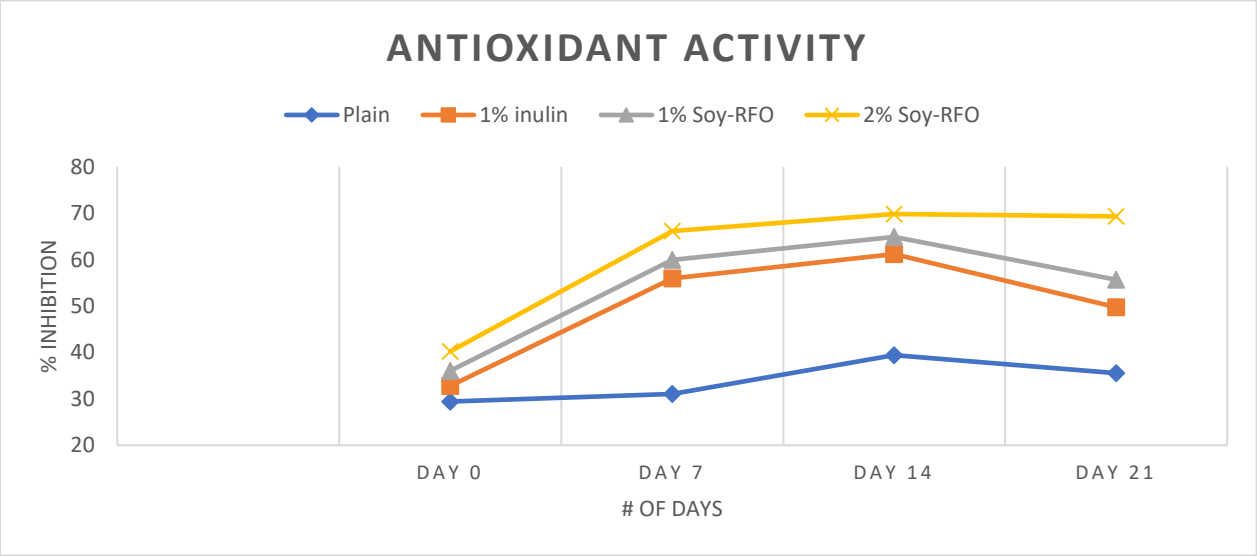

Figure 17: Change in mean values of % inhibition (antioxidant activity) with respect to time

Antioxidants help to prevent cell damage by eliminating the waste products present in human cells, which are called free radicals. Food that are consumed release antioxidants through digestion, which travels in the bloodstream and gets into the cells where it works on cleaning the free radicals. Yogurt by nature has a high antioxidant capacity. Research works done in the past prove that the antioxidant activity of yogurt is very beneficial to human health (Pattron et al., 2012). This is related to the presence of different bioactive peptides from milk proteins through proteolysis by lactic acid bacteria (Kudoh et al., 2001; Virtanen et al., 2007; Gomez Ruiz et al., 2008). The heat treatment that is subjected to milk during the preparation of yogurt is the factor that conditions the antioxidant capacity of yogurt (Galleher et al., 2005). Other reasons include, fermentation and post-acidification during storage that results in the production of organic acids (Correai et al., 2004). According to a study conducted by V. Mishra et al in 2015, *Lactobacillus bulgaricus* has been proved to be a potential antioxidant (Vijendra Mishra, Chandni Shah, Narendra Mokashe, Rupesh Chavan, Hariom Yadav, and Jashbhai Prajapati, 2015).

4.12 Microbial Analysis

The two organisms that were used in the starter culture are, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The average concentration of probiotic microorganisms and total viability count are presented in table 18 and figure 18, as above. The difference between the yogurt samples in supporting the growth of probiotics is clearly significantly different from each other. Control yogurt showed the lowest number, whereas the soy-raffinose fortified yogurt showed the highest amount with respect to all the three categories. Comparing the 1% inulin yogurt and 1% soy-raffinose yogurt, soy-raffinose had a significantly more positive effect on the growth of probiotics. From this observation, we can infer that Raffinose is more favorable to be used by

beneficial microorganisms when compared to that of Inulin.

Table 18: *Lactobacillus bulgaricus* using MRS, *Streptococcus thermophilus* using M17 and Total viability count using Nutrient agar.

Treatment**	Log CFU/mL***		
	Lactobacillus bulgaricus	*Streptococcus thermophilus*	Total viability count
Control	$0.24*10^{11}\pm0.35_a$	$0.3*10^{11}\pm0.22_a$	$0.36*10^{11}\pm0.54_a$
Sample 1	$0.78*10^{11}\pm0.12_b$	$0.76*10^{11}\pm0.39_b$	$1.44*10^{11}\pm0.38_b$
Sample 2	$1.02*10^{11}\pm0.39_c$	$1.62*10^{11}\pm0.52c$	$2.34*10^{11}\pm0.42_c$
Sample 3	$1.68*10^{11}\pm0.26_d$	$2.36*10^{11}\pm0.45_d$	$3.28*10^{11}\pm0.67_d$

*Means ± standard deviations of 3 separate trials.
**Control = starter culture only; Sample 1 = Yogurt with 1% (w/v) Inulin; Sample 2 = Yogurt with 1% (w/v) Soy-Raffinose; Sample 3 = Yogurt with 2% (w/v) Soy-Raffinose.

***Log colony forming units/ml

Means followed by the same letter (ABC) in the same column are not significantly different between each yogurt sample on the same storage day, according to Bonferroni test ($p<0.05$).

Means followed by the same letter (abcd) in the same row are not significantly different for a particular day of storage for each parameter, according to Bonferroni test ($p<0.05$).

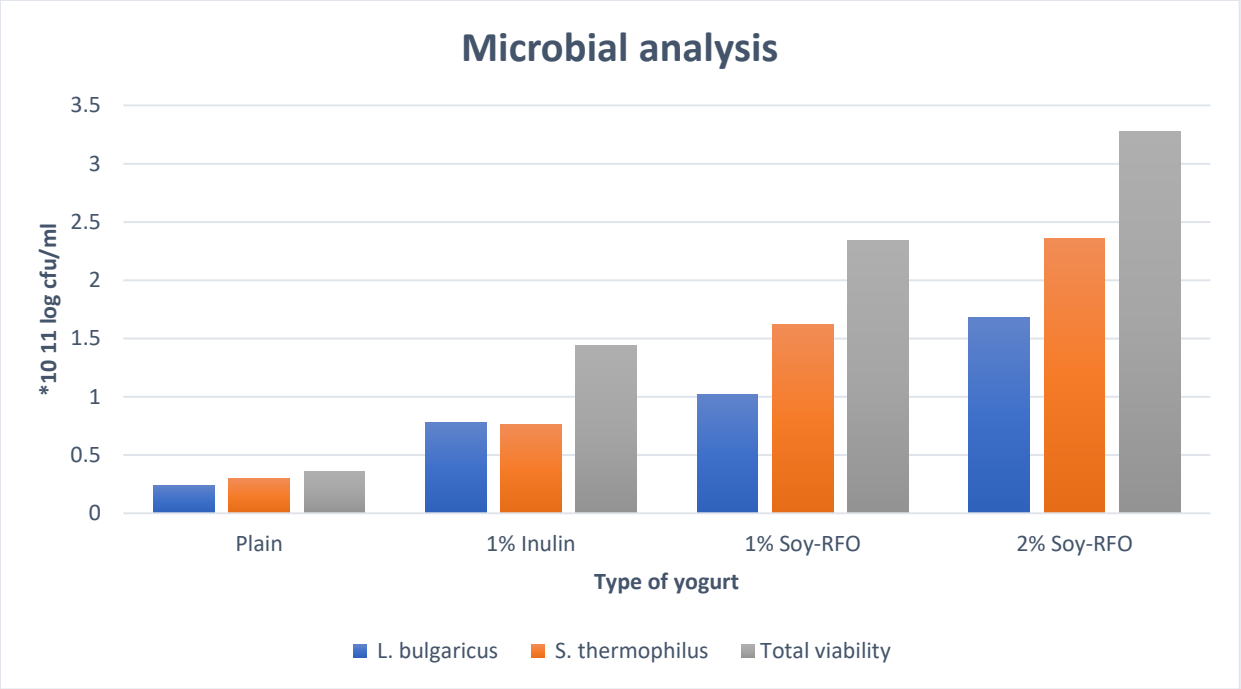

Figure 18: Clustered column chart of mean values of microbial growth and viability

The viability of probiotic bacteria is of most importance to deliver health benefitting effects to human health. Viability gives a quantitative amount of the concentration of microorganisms present in the media. It represents the total number of colony-forming units (cfu) per gram or milli-liter of the sample. Several methods have been adopted to increase the viability in yogurt which includes, the use of oxygen impermeable containers, adopting the method of 2-step fermentation, micro-encapsulation, and incorporation of nutrients such as amino acids and peptides. (N.P. Shah, 1999). Probiotic food is defined as "food that contains beneficial microorganisms that have a health-enhancing positive effect on the human digestive system and balance the microflora surviving in the gut" (Fuller. R, 1989; Fuller. R, 1992). Robinson. R. K, 1987, states that in order to provide the required health benefits the suggested concentration must be at least 10^6 cfu/g in any food product.

Several studies that were carried out in the past states that the viability of probiotics in market available products is low (Iwana. H. M. Masuda, T. Fulisawa, H. Suzuki, and T. Mitsuoka, 1993; Shah. N. P., and L. Ly., 1999). Several factors have been pointed out that affects the viability of probiotics; presence of acid and hydrogen peroxide produced by the bacteria, content of oxygen in the product and oxygen permeation through the package/container (Hull. R. R., A. V. Roberts, and J. J. Mayes, 1984; Ishibashi. N, and S. Shimamura, 1993; Iwana. H, H. Masuda, T. Fujisawa, H. Suzuki, and T. Mitsuoka, 1993). The results obtained from this study are also opposed to a previous study conducted on the ability of fortification of oligofructose, where no significant effect was found on the growth of L. *bulgaricus* and S. *thermophilus* (Cruz et al., 2013). Yet another study conducted by Agil et al recorded the positive effects of lentils on the growth of probiotic bacteria (Agil et al., 2013).

CHAPTER FIVE – CONCLUSION

Food is one of the most important contributors to human health, and also to disease. Soybeans have been used for human consumption for more than 10,000 years. They form an important part of a balanced and healthy diet. For a long time now, they have been associated with several roles that include management of cholesterol, regulation of energy levels, and prevention of severe diseases – cancer, diabetes, and heart diseases. They are a low fat and high protein source of dietary fiber with a low glycemic index. Despite these several beneficial attributes, it has been found that the human consumption of soybeans is very low, and their nutritional benefits are very much underestimated.

The present research study was performed to isolate, quantify, characterize and analyze the various functional effects of Raffinose oligosaccharides from Soybeans on the overall quality of yogurt. Data from the different physico-chemical parameters, texture profile analysis, antioxidant and microbiological analysis carried out over a 21-day shelf life study, show that addition of Raffinose oligosaccharides extracted from soybeans improved the overall quality of yogurt. This fortification decreased the fermentation time taken for the production of yogurt from pasteurized milk; this will be revolutionary and greatly appreciated by dairy industries as this decrease in production time will increase the overall yield and also benefit economically. According to the enzyme assay procedure it has been found that 27.626 ± 0.01 g of RFO is present in 100g of freeze-dried soybean extracted powder. Soy-Raffinose decreased the fermentation time taken for production of yogurt from pasteurized milk; this will be revolutionary and greatly appreciated by dairy industries as this decrease in production time will increase the overall yield and also benefit economically. Increased probiotic growth, hence we can say that Soy-Raffinose promotes the growth of beneficial bacteria. Soy-Raffinose increased the titrable acidity of yogurt; higher the

viability and activity of probiotics, higher will be the titrable acidity and lower will be the pH. Soy-Raffinose also increased the AO activity of yogurt. Between soy-raffinose yogurt and inulin yogurt, inulin yogurt was found to have a higher level of total soluble solids. This could be because Soy-raffinose is more favorable for use by the yogurt probiotic bacteria than inulin and hence its level is much lesser when comparative. Addition of Soy-Raffinose was found to increase the amount of serum released when compared to the control yogurt. This increase in syneresis was in proportion to the amount of Soy-Raffinose that was added, hence the serum separation was higher when 2% Soy-Raffinose was added. Between Inulin and Soy-RFO, 1% Inulin was observed to have a lesser syneresis than 1% soy-RFO. More research on this front is required to find the actual reason behind increase in syneresis despite higher viscosity, firmness and water holding capacity. Yogurt with 1% Soy-Raffinose showed highest viscosity when compared to the control and 1% Inulin yogurt. Viscosity was also found to significantly increase with increase in RFO concentration. With respect to color, the values of L*, a* and b* increased with increase in concentration of soy-RFO but the difference between them were not statistically significant. Hence, we can say that the fortification had no effect on the color of yogurt samples. Soy-raffinose helped in the reduction of water activity of yogurt. There is no significant difference between the water activity of 1% soy-raffinose yogurt and 1% inulin yogurt. Soy-Raffinose significantly increased the firmness of yogurt at a little more level at when compared to the Inulin yogurt. The effect of Soy-Raffinose and Inulin on the cohesiveness of yogurt had no significant difference between them, but we can see a significant increase between the control and fortified yogurts. Soy-raffinose was found to significantly increase the cohesiveness of yogurt and this increased with increased concentration of Soy-Raffinose. Soy-Raffinose had no significant effect on the adhesiveness and springiness of different yogurt. Prebiotic fortification was observed to

significantly increase the chewiness of yogurt. Comparatively, Soy-RFO had an increased effect on the chewiness when compared to Inulin.

Based on the various comparisons that were performed between Inulin and Soy-Raffinose, Soy-RFO proved to be significantly better than the other. So, this makes Soy-RFO to be an eligible product on the shelves of the market just like the commercially available Inulin.

More extensive research is required to study the effects of Soy-RFO on yogurt at higher concentrations. Based on the results obtained from this study, we can infer that fortification of 2% soy-RFO in yogurt does improve the overall quality with respect to most of the parameters. But the increase in this concentration of Soy-RFO may or may not be beneficial because it will also decrease the pH considerably. So further study conducted on this front will be able to show whether an increase in concentration of Soy-RFO greater than 2% affected the quality parameters of yogurt and in what way. Sensory evaluation that includes various parameters like Texture, Color, Flavor, Overall taste, Overall acceptance is one another development that has to be carried out for the Soy-Raffinose fortified yogurt to learn about consumer's opinion on the final end product.

Basic changes made to the physical and technological parameters of dairy food products like yogurt is gaining significant interest resulting in the development of new products. Based on this study we can say that the Raffinose oligosaccharide powder extracted from soybeans is an ingredient that is promising from a view of new product development and fortification. In addition to all the benefits, this natural ingredient gains attention due to its health benefitting properties, and this makes it easy for the consumers to adopt them as a practice.

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