

**ABANT İZZET BAYSAL UNIVERSITY
THE GRADUATE SCHOOL OF NATURAL AND APPLIED
SCIENCES**



**THE BEHAVIOR OF SELECTED PREBIOTICS AND
PROBIOTICS IN ULTRASONICATED WHEAT GRASS
JUICE**

MASTER OF SCIENCE

ELİF TECEN

BOLU, JULY 2016

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DEPARTMENT OF BIOLOGY



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APPROVAL OF THE THESIS

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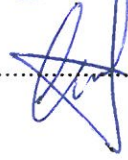
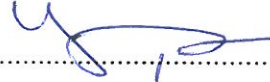
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To my family

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Elif TECEN



ABSTRACT

THE BEHAVIOR OF SELECTED PREBIOTICS AND PROBIOTICS IN ULTRASONICATED WHEAT GRASS JUICE

MSC THESIS

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ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF
NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

(SUPERVISOR: PROF. DR. SEYHUN YURDUGÜL)

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In recent years, wheat (*Triticum aestivum*) grass and its juice become very popular in United States, Europe and other developed countries. Wheat grass juice contains many beneficial compounds and is defined to possess important effects such as anti-inflammatory, cardiovascular protection, anticancer and antiulcer. In this study, selected prebiotics, probiotics and ultrasonication treatments were combined to increase the functionality of wheat grass juice. Ultrasonication is a non-thermal process that uses sound waves with frequencies higher than the limit for human hearing. Leek and persimmon are potential prebiotic sources containing antimicrobial substances. Probiotics, especially, *L. reuteri* has an antimicrobial substance which is “reuterin”. Combination of prebiotics (leek and persimmon) and probiotics (*L. reuteri* and *L. acidophilus*) with a ten minutes of 90 dB amplitude at 24 kHz frequency of ultrasound treatment resulted an effective inhibition against total mesophilic aerobic bacteria, yeast-mold and *E.coli* compared to control. Lactic acid bacteria were increased with treatments of ultrasonication process. The main chemical and physical properties of wheat grass juice were found to be significantly different between prebiotic-probiotic containing and 90 dB amplitude at 24 kHz frequency of ultrasound treatments. The sugar content of wheat grass juice was increased with prebiotic-probiotic containing treatment groups on the other hand no difference was observed in protein with respect to the control.

KEYWORDS: Wheat (*Triticum aestivum*), Wheat grass juice, Ultrasonication, Leek (*Allium porrum*), Persimmon (*Diospyros kaki*), *L. reuteri*, *L. acidophilus*

ÖZET

ULTRASONİKASYON UYGULANMIŞ BUĞDAY ÇİMİ SUYUNDAKİ SEÇİLMİŞ PREBİYOTİK VE PROBİYOTİKLERİN DAVRANIŞLARI

YÜKSEK LİSANS TEZİ

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Günümüzde, buğday çimi ve suyu başta Avrupa ve Amerika olmak üzere birçok gelişmiş ülkelerde oldukça popüler olmuştur. Buğday çimi suyu birçok faydalı bileşen içerir ve kansere, ülser ve iltihaplanmaya karşı etkili olduğu ve kardiyovasküler sistemi koruyucu etkiye sahip olduğu belirtilmiştir. Bu çalışmada buğday çimi suyunun işlevselliğinin artırılması için seçilmiş prebiyotik, probiyotik ve ultrasonikasyon birleştirilerek uygulanmıştır. Ultrasonikasyon insanın duyamayacağı kadar yüksek frekansta ses dalgaları kullanan ısı olmayan bir işlemdir. Trabzon hurması ve pırasa antimikrobiyal maddeler içeren potansiyel prebiyotik kaynaklarıdır. Probiyotikler, özellikle *L. reuteri* “reuterin” adlı antimikrobiyal maddeye sahiptir. Pırasa ve Trabzon hurması ile *L. reuteri* ve *L. acidophilus*’ un buğday çim suyunun birliğinde ve de ultrasonikasyon işlemi uygulandığında toplam mezofilik bakteri, maya-küf ve *E. coli* üzerinde en etkili azalma görülmüştür. Laktik asit bakterileri ultrasonikasyon ile artmıştır. Prebiyotik-probiyotik eklenmiş ve ultrasonikasyon uygulanmış gruplarda buğday çimi suyunun kimyasal ve fiziksel özelliklerinde anlamlı farklar bulunmuştur. Şeker konsantrasyonu prebiyotik-probiyotik içeren gruplarda artmış diğer yandan proteinde kontrole göre önemli bir fark gözlenmemiştir.

ANAHTAR KELİMELELER: Buğday çimi, Buğday çimi suyu, Ultrasonikasyon, Pırasa, Trabzon hurması, *Lactobacillus reuteri*, *Lactobacillus acidophilus*

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LIST OF ABBREVIATIONS AND SYMBOLS

BB-12	: <i>Bifidobacterium animalis</i> subspecies lactis
BGA	: Brilliant Green Agar
°C	: Centigrade degree
CFU	: Colony Forming Unit
dB	: decibel
DNS	: Dinitrosalicylic Acid
EMB	: Eosin Methylene Blue Agar
<i>E.coli</i>	: <i>Escherichia coli</i>
FAO/WHO	: World Health Organization
FDA	: Food and Drug Administration
FOS	: fructooligosaccharides
g	: gram
H₂O₂	: Hydrogen peroxide
HHP	: High hydrostatic pressure
hPa	: Hectopascals
Hz	: Hertz
kHz	: kiloHertz
kPa	: Kilo Pascal
LAB	: Lactic acid-producing bacteria
log	: logarithm
mg	: Miligram
min	: Minute
mL	: Mililiter
mPa	: Mega Pascal
MRS	: de Man Rogosa Sharpe Agar
NaOH	: Sodium Hydroxide
NDOs	: non-digestible oligosaccharides
NEC	: Necrotizing enterocolitis
O.D	: Optical Density
PCA	: Plate Count Agar
PEF	: Pulsed electric field
SCCs	: Short-chain carbohydrates

SD	: Standard Deviation
Std	: Standard
SOD	: Superoxide dismutase
TA	: Titratable Acidity
TMAB	: Total Mesophilic Aerobic Bacteria
TUIK	: Turkish Statistical Institute
XLD	: Xylose lysine desoxycholate Agar
YEA	: Yeast Extract Agar
YM	: Yeast and Mold



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1. INTRODUCTION

1.1 Wheat (*Triticum aestivum*) Grass

Wheat grass is grown up from common wheat (*Triticum aestivum*), a subspecies of the family Poaceae (Padalia et al., 2010). It is thoroughly grown along with temperate regions of North America and Europe, particularly in developing countries. Wheat (*Triticum aestivum*) also is a significant source of energy and micronutrients in the developing world. It is the universal cereal for a wide range of products such as bread, noodles, cakes, biscuits, cookies, etc. so that, wheat is one of the major grain and is an important crop in the world (Kardas and Durucasu, 2014).

There are many cereal crops grown for human and animal utilization and wheat grain that contains lipids, fibers, minerals, vitamins and many micronutrients differ from than the others'(Nadaud et al., 2015). Several researchers have implied that wheat grass is an excellent source of minerals like calcium, cobalt, iron, phosphorus, potassium, sulfur, zinc and antioxidant vitamins such as A, B, C, E, and antioxidant enzymes such as SOD, cytochrome oxidase and other enzymes (Stevenson et al., 2012 ; Leoncini et al., 2012).

Wheat (*Triticum aestivum*) is germinated over a period of 7-10 days and generally named "Wheat Grass". During this germination period, vitamins, minerals, and phenolic compounds containing flavonoids are synthesized in wheat sprouts, and also wheat sprouts achieve the maximum antioxidant potential.

Wheat grass is rich in chlorophyll and enzymes. It contains more than 70% chlorophyll which is an important dietary constitutive. The chlorophyll molecule in wheat grass is almost same as hemoglobin in human blood but, the only difference is which the central element in chlorophyll is magnesium and iron in hemoglobin. The molecular structure of chlorophyll in wheat grass and hemoglobin in the human body is similar, and so wheat grass is named 'Green Blood' (Ferruzia and Blakesleeb, 2007).

In additionally, wheat grass includes many beneficial and significant effects such as anti-inflammatory, cardiovascular protection, anticancer activity, anti-ulcer (Bitra et al., 2014). Indigenously, wheat grass has been used as an herbal medicine since ages. It has been traditionally used as a herbal medicine in a number of serious illnesses like thalassemia and myelodysplastic syndrome.

Moreover, it has also been believed to strengthen the immune system and raise the life span of cancer patients by regressing the spread of cancer cells (Marwaha et al., 2004; Mukhopadhyay et al., 2009). In 1978, Lai et al. indicated an antimutagenic activity of wheat grass extract in the Ames test system and was binded to chlorophyll.

Wheat grass extract also demonstrated high anti-oxidant activity (Kulkarni et al., 2006). A current hypothesis suggested that the antioxidant enzymes present in wheat grass helps rid of free radicals so improving memory (Bitra et al., 2014). And also, the purified compounds and crude extract of *T. aestivum* were determined to have iron chelation potency and antioxidant activity. Therefore, it is necessary to evaluate the toxic properties of any compound isolated from plant extract to prevent any inappropriate side effects (Das et al., 2014). Wheat (*Triticum aestivum*) is also a substantial graminaceous plant species that can acquire iron for physiological need from the soil by release of phytosiderophores (Wiren and Khodr, 2000).

1.1.1 Wheat Grass Juice

Wheat grass juice is a popular drink in Europe, United States and other developed countries. Wheat grass juice is an essence squeezed from the mature sprouts of wheat seeds (*Triticum aestivum*). Dr. Ann Wigmore (1909-1996) developed and made popular of the usage of wheat grass juice for therapeutic purposes, like part of her therapeutic nutritional approach. The qualities of therapeutic effect of wheat grass juice have been imputed to its rich nutritional content, including chlorophyll, vitamins (A, C, and E), bioflavonoids, iron, minerals (calcium and magnesium), and 17 amino acids, 8 of which are essential (BarSela et al., 2007).

Wheat grass juice is high in vitamin K, a blood-clotting agent. BarSela et al (2007) indicated that wheat grass juice might also inhibit hematological toxicity involved to chemotherapy in breast cancer patients. Moreover, it is observed that people undergoing chemotherapy, who are on wheat grass, have sailed through this

treatment, and not required hospital acknowledgment, while side effects such as nausea, vomiting and mouth ulcers have been actually non-present (Cassileth et al.,1991). And also, wheat grass juice is a complete protein source. Proteins are responsible for a sequence of diverse functions along the body ranging from cell reconditioning and building of hormones, to the repairing of organs, blood, and muscles (Wheat and Currie 2008).

Another study on wheat grass juice has been related with ulcerative colitis. Ben Arya et al. suggested that severity of rectal bleeding has reduced and ulcerative colitis activity index has decreased.

1.2 Prebiotics

The healthy food requisition has increased from the consumer's belief on the benefits of functional and natural foods. In these days, food is not only a simple source of nutrients but also a way to improve health and even combat some disorders related with nutrition and food input habits (Day et al., 2009; Niva, 2007; Shah, 2007; Sangwan et al., 2011; Siró et al., 2008). Prebiotics are non-digestible and non-active food components which shift to the colon and after that are selectively fermented. Prebiotics affect the colonic microbiota that is, stimulating the growth of beneficial bacteria (Roberfroid, 2002).

Prebiotics are short-chain carbohydrates (SCCs) that are non-digestible by digestive enzymes in humans and that have been named resistant SCCs. Moreover, they are sometimes referred to as non-digestible oligosaccharides (NDOs). The fructooligosaccharides (FOS), which prebiotics have different physiological functions in the body, such as changes in the intestinal transit, prevention of colon cancer, lowering serum cholesterol and triglycerides, helping mineral absorption, especially calcium, and reduction of the diarrhea associated with antibiotic treatment (Manning and Gibson, 2004).

In humans, prebiotic addition to the diet has brought positive effects to the gut microbial balance (Gaggia et al., 2010). Furthermore, they exert an osmotic effect in the intestinal lumen, and are totally or partially fermented in the colon into short chain fatty acids and gas too (Marteau, 2001).

1.2.1 Persimmon (*Diospyros kaki L.*)

Oriental Persimmon (*Diospyros kaki L.*) is classified under the *Diospyros* genus of Ebenaceae family. Persimmon (*Diospyros kaki L.*) is a subtropic fruit and fundamentally native to China and cultivated in warm regions worldwide. And also, in recent years the popularity of persimmons has grown up except of the traditional production areas (China, Japan, Korea) becoming a promising crop in Brazil and some Mediterranean countries such as Italy and Spain. In Turkey, the production areas of persimmon fruits are mostly in Mediterranean Region and Black Sea, Marmara and Aegean Regions (Karkacier, 1988).

Persimmons generally ripen from late August until early December, depending on climate and region too. The fruit is eaten both fresh and dried (McEachern and Hancock 1997). In generally, oriental persimmons consist of two groups of cultivars with distinct degrees of astringency at harvest; one is an astringent type and the other one is non-astringent (Harima et al., 2003). Astringent fruit must be soft or artificially treated before astringency is removed because they are considered as suitable for eating but non-astringent types lose astringency as still hard and could be eaten hard or soft (Miller and Crocker, 1994).

Persimmon fruits have been used in Chinese traditional medicine for their positive actions for human health (Mallavadhani et al., 1998). Several researchers were reported that persimmon fruits include many medicinal bioactive compounds, such as carotenoids, tannins, flavonoids, terpenoids, amino acids, steroids, naphthoquinones, sugars, minerals, and lipids (Kawase et al., 2003). Gorinstein et al (1994) submitted that persimmon fruits are rich in antioxidant phenolic compounds other than tannins, and also, it has been showed that these compounds might reduce the risk of chronic illnesses by protecting tissues against free radical-mediated damage (Özen et al., 2004).

1.2.2 Leek (*Allium porrum L.*)

Leeks (*Allium porrum L.* = *Allium ampeloprasum L.*), along with onions (*Allium cepa L.*) and garlic (*Allium sativum L.*), are the three most popular cultivated species in the Alliaceae (Amaryllidaceae) family. Leeks (*Allium porrum L.*) also belong to the *Allium* genus.

Plants of the genus *Allium* have been recognized as sources of secondary metabolites richly endowed with interesting biological activities (Augusti, 1990; Block, 1992). In the Mediterranean region, the domesticated species of modern leek is likely originated and grown in tropical, temperate and subtropical regions of the world and also, from an economical point of view, considered as a very significant vegetable in Western Europe.

According to the Turkish Statistical Institute (TUIK) (2013), in Turkey, leek corresponds to 13.5% of the total agricultural production (Canlı Tasar et al., 2015). Leek is available from October to May, so it is a seasonal vegetable (Magra et al., 2006). Leek is a biennial crop, but grown as a short-lived annual in commercial cropping. The leek is grown for its thickened cylindrical stem or shank made up of long leaf bases (Burt, 2008).

Leek (*Allium porrum L.*) is a useful vegetable for human health. Furthermore, it has a great antioxidant potential and many useful phytochemicals as S-alkyl-L-cysteine sulfoxides, polyphenols, and fructans (Canlı Tasar et al., 2015). In recent years, many studies have been put forward with leek and consumption of this causes reduction of colorectal, stomach, and breast cancer (Bianchini et al., 2001) and also, prevention of neural tube defects and other diseases (Stover, 2004).

Leeks are explained to have the similar medicinal values as garlic, which show some anticancer activity. And also, leeks are good source of carbohydrate, protein, vitamin, cellulose and mineral (Chen et al., 2009). Leeks also include important amounts of lutein, β -carotene, vitamin C and vitamin E (Hart and Scott, 1995; Proteggente et al., 2002; Roy, Takenaka and Isobe, 2007).

1.3 Probiotics

Nowadays, food industry develops new products in the area of functional foods and functional food components. Probiotics are considered as significant segments of functional foods (Granato et al., 2010; Bigliardi and Galati, 2013). Many definitions have been suggested for the term of probiotic. Probiotics were first described by Metchnikoff in 1908, based on his observations on the longevity of individuals who lived in a certain part of Bulgaria and which he connected with their

ingestion, on a regular basis, of a fermented milk product (Quigley, 2010). The term “Probiotics” was coined by Kollath in the 1950s and afterwards Lilly and Stillwell operated the term in 1965. In 1989, Fuller redefined a probiotic as “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. The generally accepted one is according to the World Health Organization FAO/WHO (2001), probiotics are defined as “live microorganisms which, when administered in adequate amounts (10^7 CFU g/L), confer health benefits to the host”. These health benefits are anticarcinogenic effect, prevention of diarrhea, immunostimulation, and protection against pathogenic microorganisms (Bianchi et al., 2014).

The beneficial modes of probiotics action include regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function (Salminen et al., 1996), expression of bacteriocins (Mazmanian et al., 2008), enzymatic activity stimulating absorption and nutrition (Hooper et al., 2002; Timmerman et al., 2005), immunomodulatory effects (Salzman et al., 2003), inhibition of procarcinogenic enzymes and parasite with the ability of pathogens by colonize and infect the mucosa (Gill, 2003). And also, probiotics are beneficial for protecting human hosts from contamination on the gastrointestinal tract (Sanders, 2003).

Probiotic activity could be related with genera, species, or strains. *Lactobacillus*, *Enterococcus* and *Bifidobacterium* are the main bacterial genera for probiotic applications. The genus *Lactobacillus* is an extensive and heterogeneous taxonomic unit, belonging to the group of lactic acid-producing Bacteria (LAB) (Gaggia et al., 2010). Probiotic lactobacilli are one of the most numerous probiotics in the mammalian gut and are generally used (Kim et al., 2015).

Lactic acid-producing bacteria (LAB) are thoroughly distributed in natural habitats, surrounding from many fermented foods to intestinal tracts of human and animals. Moreover, LAB play a substantial role in the production of foods such as fermented meat products, milk, cheese and yoghurt (Hammes and Hertel, 2007). The most fermented probiotic products in the market are milk based (Martins et al., 2013). The other genus is *Enterococcus* which applies to the LAB group, found naturally in food products. The other important one is *Bifidobacterium*, considered one of the key genera, in the intestinal tracts of animals and humans and in high numbers incorporated

with good health state of the host. And also, there are many genera for probiotic applications too.

The active ingredients of probiotics, regarded with the biological effects are often unknown but excluded for some enzymes and cell wall components with immunomodulating properties (Marteau, 2001). Several studies have reported that probiotics might be effective in rotavirus-induced diarrhea (Allen et al., 2004). Sartor and Muehlbauer (2007) reported that the therapeutic usage of probiotics in inflammatory bowel diseases and its complications, such as pouchitis and postoperative reiteration of Crohn's disease, is derived from the hypothesis that the endogenous intestinal microbiota acts a crucial role on the pathogenesis for these disorders too.

1.3.1 *Lactobacillus acidophilus*

Lactobacillus acidophilus is a gram-positive, homofermentative and catalase-negative rod. It does not produce spore. *L. acidophilus* is a common habitant with the human intestinal tract, mouth and vagina. *Lactobacillus acidophilus* is a LAB (Lactic acid-producing bacteria) demonstrated by many bacteriocin producing strains. This type of bacteria is a nonpathogenic and also, a member of the normal intestinal microflora.

In recent years, probiotics are popular for functional foods or drinks, so, a significant increase in the interest of *L. acidophilus* as a probiotic agent was observed to contribute its application in functional foods and supplements in a worldwide market. Also, it is widely used for production of fermented dairy products (Naidu et al., 1999; Kitazawa et al., 2002). Mack and Lebel (2004) reported that *Lactobacillus acidophilus* is used for the treatment and prevention of gastrointestinal disorders associated with diarrhea of varying etiology. Another study on *Lactobacillus acidophilus* showed anti-carcinogenic effect on colon cancer development (Baldwin et al., 2010; Lee and Lee, 2000; Urbanska et al., 2009).

1.3.2 *Lactobacillus reuteri*

Lactobacillus reuteri is a Gram-positive bacterium and able to colonize the gastrointestinal tract of humans and other animals especially birds (Casas and Dobrogosz, 2000), and isolated generally out of human feces. It was determined to be a probiotic. *Lactobacillus reuteri* is a hetero-fermentative LAB (Kandler and Weiss 1986) and some are known antibiotics (reuterin, Talarico et al. 1988, reutericyclin, Gänzle et al. 2000) producers. Gerhard Reuter (German Microbiologist) first isolated *L. reuteri* from human fecal and intestinal samples in the 1960s, and this work was later recurred by other researchers (Molin et al., 1993). Moreover, *L. reuteri* was discovered to be present naturally in the intestines of healthy sheep, chickens (Sarra et al., 1985), pigs (Naito et al., 1995) and rodents (Molin et al., 1992). *L. reuteri* is recognized to produce reuterin (Talarico et al., 1988), reutericin 6 (Kabuki et al., 1997), and reutericyclin (Gänzle et al., 2000).

Several researchers showed that *L.reuteri* has beneficial effects on intestinal and oral health of humans. Shornikova et al (1997) suggested that *L. reuteri* effected on rotavirus-induced diarrhea, especially in children and it was stopped diarrhea with intensive consumption. And also, *L.reuteri* fought with *Helicobacter pylori*, which is the gut pathogen and causes peptic ulcers (Imase et al., 2007). Nikawa et al (2004) reported that *L. reuteri* might be capable of supporting dental health, like it has been approved to kill *Streptococcus mutans*, which is a bacterium responsible for tooth cavity.

1.4 Synbiotics

The food industry has developed a wide range of functional products in response to the increasing requisition for healthier food (Annunziata and Vecchio, 2011). Functional food products including probiotic microorganisms, prebiotic ingredients, or both are immensely popular. That is, food containing probiotic microorganisms and prebiotic substances, in sufficient amounts, is named synbiotic (Holzapfel and Schillinger, 2002). Synbiotic is the co-administration of probiotic and prebiotics with the expectancy which the prebiotics increase the survival and growth of the probiotics (Grimoud et al., 2010). Gibson and Roberfroid, (1995) described synbiotics as “a mixture of probiotics and prebiotics that beneficially affects the host

by improving the survival and implantation of live microbial dietary supplements in the gastro- intestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare”.

When probiotics and prebiotics have been used together, a higher advantage to the host is informed owing to their synergistic action (Vitali et al., 2010). Few studies have been performed in humans on the influence of synbiotics. While probiotic bacterial strains, e.g. *Bifidobacterium animalis subsp. lactis* HN019 and *Lactobacillus rhamnosus* HN001, have been studied from in vitro studies entirely to human clinical tests (Dekker et al., 2009; Gopal et al., 2001; Waller et al., 2011; Wickens et al., 2013), few studies have analyzed these probiotic strains in synbiotic combinations. Ringel-Kulka et al, (2015) reported that a synbiotic yogurt with the probiotic bacteria *Bifidobacterium animalis* subspecies lactis (BB-12) and the prebiotic inulin remarkably decreased days of fever, developed social and school functioning and improved frequency of bowel motions in healthy children attending to child care centres. Another study showed that enteral addition with probiotics alone or integrated with prebiotics (synbiotics) appears to be beneficial for preterm infants with respect to several outcomes such as sepsis, all-cause mortality and necrotizing enterocolitis (NEC) (Dilli et al., 2015).

1.5 The juice processing and novel methods

Within increasing living conditions and globalization of the food industry, requisition for quality juice and juice type drinks has significantly enlarged. Consumers have less time to devote to food preparation, also seeking natural products that have been least processed to avoid destruction or partial removal of the health promoting substances present in fruits.

Heat sensitive molecules such as vitamins, proteins and aromatic compounds can be demolish by sterilization and pasteurization (Lado and Yousef, 2002; Piyasena et al., 2003; Chemat et al., 2011). The use of processing methods such as high pressure (Lee et al., 2002; Alpas et al., 2003; Buzrul et al., 2005; Lee et al., 2006), radiation (Nakauma et al., 2004) or PEF (Hamilton, 1967) is the most preferred option to avoid the effectiveness of thermal treatment. High hydrostatic pressure (HHP) can alter the

structure of proteins and polysaccharides. High hydrostatic pressure can also cause changes in the texture, physical appearance and functionality of foods (Ross et al., 2003). And also, the examples of alternative technology are ultrasonication and pulsed electric field (PEF).

1.5.1 Ultrasonication

The important issues comparing the functional food industry is detecting how to extract functional ingredients from agricultural and marine food sources in the most economically productive way possible (Song et al., 2015). Sonication is the act of applying generally ultrasound energy to shake particles in a sample to various objectives. The effect of sonication is dependent on a number of factors such as, sample volume, acoustic power density, solution temperature and experimental conditions (Shanmugam et al., 2012). In the laboratory conditions, sonication is generally applied using an ultrasonic bath or an ultrasonic probe, mostly known as a sonicator.

Ultrasonication is a non-thermal procedure which uses sound waves with frequencies higher than the limit for human hearing (i.e., 16 kHz). Usually, ultrasound is applied to sound waves with 20 kHz or higher frequencies (Butz and Tauscher, 2002; Demirdöven and Baysal, 2009; Piyasena et al., 2003). That is ultrasound equipments use frequencies from 20 KHz to 10 MHz (Piyasena et al., 2003). Ultrasound is classified into low intensity ultrasound with a frequency range of 5-10 MHz and high intensity ultrasound with a range of 20-100 kHz (McClements, 1995; Mason, 1998; Lee et al., 2003). Treatments with power-ultrasound (20–100 kHz) are an emerging technology for the conservation of food alone and in combination with other new processing technologies (Christen et al., 2012).

Power-ultrasound produces cavitation that means, the formation, growth and implosive collapse of bubbles in liquids. During the breakdown of these bubbles, localised hot spots comprises with temperatures of roughly 5000 °C, pressures of about 50 MPa and a lifetime of a few microseconds. The pressure changes resulting of these implosions produce shock waves that break the cellular membranes of bacteria resulting in cell lysis (Hartmann et al., 2012).

For the food industry, ultrasonication method is an efficient extraction method because of reducing processing time besides that solvent consumption (Dong et al., 2011). The 2001 FDA report on “Kinetics of microbial inactivation for alternative food processing technologies” suggested that ultrasonication process could be used evenly one of the potential alternative techniques for conventional thermal pasteurization (Salleh-Mack and Roberts, 2007). In recent years, ultrasonication process has been performed to food processing methods which contain filtration, viscosity modification, and emulsification (Chandrapala et al., 2012; Dong et al., 2011; Kentish & Feng, 2014). Additionally, ultrasonication has many applications for food processing, such as dehydration, drying, freezing, thawing and inactivating pathogens in food products too (Bermudez- Aguirre et al., 2008; Czank et al., 2010). Also, Villamiel et al (1999) suggested that ultrasonication has been used in the dairy food industry for equipment cleaning and homogenization with successfully.

During ultrasonication process, an acoustic wave disseminates in the liquid media, and after that cavitation bubbles are composed in the rarefaction region. The hydro-mechanical cut stress localizes the temperature increase up to 5000 K and the OH-radicals produced via the cavitation breaking of lead to the destruction of microorganism cell membranes and walls (Riesz and Kondo, 1992). That is, for microbial inactivation, the principle of ultrasonic mechanism in liquid medium is generally cavitation during that temperature and pressure increase inside the bubble to an estimated to 5500° C and 50 MPa (Bermúdez-Aguirre and Barbosa-Cánovas, 2011; Raso et al., 1998; Villamiel et al., 2009).

For destroying of micro-organisms by ultrasound mechanism has been studied with a view toward in fruit juice to an alternative addition to traditional sterilization methods (Villamiel et al., 2000). Ultrasound has been noticed to be effective against foodborne pathogens found in guava juice (Cheng et al., 2007), orange juice (Valero et al., 2007), milk and apple cider (D’amico et al., 2006). Some researchers informed which pathogens were decreased importantly by ultrasound in a period ranging between 5–60 min. for pathogen inoculated organic fresh lettuce (Mahvi et al., 2005; Seymour et al., 2002; Sagong et al., 2011). Piyasena et al. (2003) reported that the future of ultrasonication in the food industry, for purposes of bacterial inhibition, is

most promising for combined mechanisms involving ultrasounds and pressure or ultrasounds and heat (thermosonication).



2. AIM AND SCOPE OF THE STUDY

The aim of the study is to examine the effects of prebiotics, probiotics, ultrasonication and combination of them on wheat grass juices. Prebiotics and probiotics are important sources for human gastro-intestinal system. When wheat grass is clipped, it allows the growth of certain foodborne pathogens due to its contact with soil so in general, its juice must be processed and consumed immediately. By adding probiotics; fortified with leek and persimmon, working as prebiotics after ultrasonication, this would decrease the load of the pathogens and provide a safe drink, together with its multiple functionality of bearing the anticarcinogenic and antimutagenic property of wheat grass juice. Besides these prebiotics are cheap and easily accessible in mild climates.

In order to avoid destroying or partial elimination of the health promoting substances present in wheat grass juice such as antioxidants, vitamins, aminoacids etc., the behavior of prebiotics and probiotics and the inhibitory effect of ultrasound was studied.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Wheat (*Triticum aestivum L.*) grass

The wheat seeds were obtained from a local herbalist in Bolu. Prior to the experiment, they waited overnight in a glass of tap water. Wheat seeds were sown into turf containing soil and awaited for germination at ambient temperature for a period of approximately ten days up to a grass length of 7-10 cm and clipped.

3.1.2 Wheat grass juice

After clipping, the wheat grass were immediately extracted by a wheat grass juicer (Healthy Grass Juicer, Lexen, China). Subsequently, the juice was filtrated by sterilized filter paper for separating residues of wheat grass before adding prebiotics and probiotics and ultrasonication treatments.

3.1.3 Persimmon (*Diospyros kaki L.*)

Mature persimmon fruits were harvested in November 2014 from Aydın, Turkey, and immediately transported to the laboratory. Prior to the study, the defective and bruised fruits were eliminated before the treatments and washed with tap water.

3.1.4 Leek (*Allium porrum L.*)

Leek was purchased from a local vendor in Bolu, Turkey, in November 2014 and immediately transported to the laboratory. Prior to the study, the defective and bruised fruits were eliminated before the treatments and washed with tap water.

3.1.5 Lactic Acid Bacteria

Lactobacillus acidophilus (Novaflor™) and *Lactobacillus reuteri* (BioGaia®, Sweden) were obtained from the Department of Food Engineering, Abant Izzet Baysal University, in tablet form and kept in the refrigerator at + 4° C before their activation in MRS broth (MERCK).

3.2 Methods

3.2.1 Preparation of microbiological media

A tablet of *Lactobacillus acidophilus* and *Lactobacillus reuteri* was added in MRS broth (MERCK) and incubated at 37° C for 48 hours. After the growth period, 0.5 and 1 mL of bacterial suspensions (approximately 10⁷ cfu/mL) were inoculated to the 9.5 and 9 mL wheat grass juice (ultrasonicated and nonultrasonicated) in order to investigate the behavior of them, either alone or in combination with prebiotics.

3.2.2. Freeze-drying

The freeze-drying process was carried out by a freeze-drier (Christ Alpha 1-2/LD Plus, Germany) for 24 hours (shelf temperature: 30° C and condenser temperature: -45° C) and a pressure of 0.10 hectopascals (hPa) (Litvin et al., 1998), after a deep-freezing process at -80° C at once; for a period of 24 hours. Persimmon fruits were sliced into four and leeks into small parts. Then, they were put into glass plates and subsequently to freeze-drier. No additives or preservatives were added to the persimmon and leek before or after freeze-drying. After 24 hours, the lyophilized persimmons and leeks were removed and kept in sealed vials for further analysis. Before the experiment, lyophilized persimmons and leeks were pulverized with a blender (Moulinex, FRANCE).

3.2.2 The treatment with ultrasonication

10 mL of each of the wheat grass juices were ultrasonicated for a period of 10 minutes at the amplitudes of 30 and 90 dB with a frequency of 24 kiloHertz (kHz) (Hielscher Ultrasound Technology, UP 400S Ultrasonic processor, Germany). Temperature control during ultrasonication treatment was maintained by decreasing excess heat improved during ultrasonication, using ice bath placed to the periphery of

the sample tubes. For each treatment the application temperature was detected. The detected temperatures were ranging between 15 to 24° C.

3.2.3 The treatment with prebiotics

Powdered persimmon and powdered leek was added into wheat grass juices at concentrations of 0.1g/L and 0.5g/L, respectively.



3.2.4 The treatment with prebiotics and probiotics

Table 3.1. Treatment with probiotics and prebiotic.

Treatment	Bacteria (cfu/mL)	Prebiotic(g)	Probiotic(mL)	Wheat grass juice (mL)
T1	<i>L. acidophilus</i> (10 ⁷)	0.1 g freeze-dried leek	0.5 mL	9.4 mL
T2	<i>L. acidophilus</i> (10 ⁷)	0.1 g freeze-dried persimmon	0.5 mL	9.4 mL
T3	<i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried leek	0.5 mL	9 mL
T4	<i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried persimmon	0.5 mL	9 mL
T5	<i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried leek, 0.5 g freeze-dried persimmon	0.5 mL	8.5 mL
T6	<i>L. reuteri</i> (10 ⁷)	0.1 g freeze-dried leek	0.5 mL	9.4 mL
T7	<i>L. reuteri</i> (10 ⁷)	0.1 g freeze-dried persimmon	0.5 mL	9.4 mL
T8	<i>L. reuteri</i> (10 ⁷)	0.5 g freeze-dried leek	0.5 mL	9 mL
T9	<i>L. reuteri</i> (10 ⁷)	0.5 g freeze-dried persimmon	0.5 mL	9 mL
T10	<i>L. reuteri</i> (10 ⁷)	0.5 g freeze-dried leek, 0.5 g freeze-dried persimmon	0.5 mL	8.5 mL
T11	<i>L. reuteri</i> (10 ⁷) <i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried persimmon	0.5 mL <i>L. reuteri</i> , 0.5 mL <i>L. acidophilus</i>	8.5 mL
T12	<i>L. reuteri</i> (10 ⁷) <i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried leek	0.5 mL <i>L. reuteri</i> , 0.5 mL <i>L. acidophilus</i>	8.5 mL
T13	<i>L. reuteri</i> (10 ⁷) <i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried persimmon, 0.5 g freeze-dried leek	0.5 mL <i>L. reuteri</i> , 0.5 mL <i>L. acidophilus</i>	8 mL

Table 3.2. Treatment with prebiotics and probiotics with ultrasonication

Treatment	Ultrasonication	Bacteria (cfu/mL)	Prebiotic(g)	Probiotic(mL)	Wheat grass juice (mL)
T14	10 min. 90 dB	<i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried leek	0.5 mL	9 mL
T15	10 min. 90 dB	<i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried persimmon	0.5 mL	9 mL
T16	10 min. 90 dB	<i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried leek, 0.5 g freeze-dried persimmon	0.5 mL	8.5 mL
T17	10 min. 90 dB	<i>L. reuteri</i> (10 ⁷)	0.5 g freeze-dried leek	0.5 mL	9 mL
T18	10 min. 90 dB	<i>L. reuteri</i> (10 ⁷)	0.5 g freeze-dried persimmon	0.5 mL	9 mL
T19	10 min. 90 dB	<i>L. reuteri</i> (10 ⁷)	0.5 g freeze-dried leek, 0.5 g freeze-dried persimmon	0.5 mL	8.5 mL
T20	10 min. 90 dB	<i>L. reuteri</i> (10 ⁷) <i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried persimmon	0.5 mL <i>L. reuteri</i> , 0.5 mL <i>L. acidophilus</i>	8.5 mL
T21	10 min. 90 dB	<i>L. reuteri</i> (10 ⁷) <i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried leek	0.5 mL <i>L. reuteri</i> , 0.5 mL <i>L. acidophilus</i>	8.5 mL
T22	10 min. 90 dB	<i>L. reuteri</i> (10 ⁷) <i>L. acidophilus</i> (10 ⁷)	0.5 g freeze-dried persimmon, 0.5 g freeze-dried leek	0.5 mL <i>L. reuteri</i> , 0.5 mL <i>L. acidophilus</i>	8 mL

3.2.5 Effects of Combination Treatments on Wheat grass juice

The best effective doses, obtained in Sections 3.2.2, 3.2.3 and 3.2.4 of the combined or alone treatments of prebiotics and probiotics and ultrasonication were investigated to observe their effect on wheat grass juices.

3.2.6 Microbial Identification of Microflora and Microbial Counts

The total mesophilic aerobic bacteria (TMAB) counts were detected by plate count (PCA) (Merck), *E.coli* by Eosin Methylene Blue (EMB) (Merck), the mold and yeast by yeast extract (Merck) and Lactic Acid bacteria by MRS (de Man, Rogosa and Sharpe) agars (Merck). The treated and untreated (control) juice samples were diluted with sterile distilled water to enumerate viable cells of the natural micropopulation. Except yeast and mold, all plates were incubated at 37° C for 24-48 hours in an incubator. Yeast extract agar plates were incubated at 25° C for 5 days. All of the microbial identification was carried out in duplicates.

Salmonella spp. was detected by enrichment method (ISO 6579:2002). Non-selective pre-enrichment medium was prepared. Firstly, 25 mL wheat grass juice was weighed out with sterile spoon and put it into Erlenmeyer flask and added 225 mL buffered peptone water on it (1:9) then incubated at 37 °C for 24 hours. After that, 1 mL of this medium was transferred of the pre- enrichment with a pipette to 10 mL Müller Kaufmann Broth (Liofilchem®, Italy) and 0.1 mL was transferred of the pre-enrichment with a pipette to 10 ml Rappaport- Vassiliadis soy peptone (RVS) broth (Liofilchem®, Italy) and incubated at 37 °C for 24 hours for Müller Kaufmann Broth and incubated at 41.5 °C for 24 hours for Rappaport- Vassiliadis soy peptone (RVS) Broth. Then, a 10 µl loop full from the inoculated and incubated Müller Kaufmann Broth was spreaded on XLD (Merck) and BGA (Merck) Agar plates and a 10 µl loop full from the inoculated and incubated RVS broth was spreaded on XLD (Merck) and BGA (Merck) Agar plates at 37 °C for 24 hours.

Consequently all microbial colonies were counted and reported as log CFU/mL and determined from this formula:

CFU/mL = [(number of colonies) × (dilution factor)]/ Aliquot plated (Mohideen, 2015).

Then, all calculations were converted to logarithmic values.

3.2.7 The shelf life of wheat grass juices

Wheat grass juices were kept in the refrigerator at + 4°C. In order to detect the microbial quality parameters of shelf life, microbial counts of wheat grass juices were done at 20th, 40th and 60th days.

3.2.8 Physical and chemical properties

3.2.8.1 Total soluble solids (°Brix)

A portable refractometer (Miiler, RHB-10/ATC, Germany) was used for the detection of total soluble solid content of the wheat grass juices. Refractive indexes were recorded and converted to °Brix. The measurements were performed at 25 ± 0.5° C in duplicates.

3.2.8.2 pH

The pH of wheat grass juice samples was measured by using a digital pH meter (WTW Inolab pH 720, Germany).

3.2.8.3 Titratable Acidity

In order to detect the titratable acidity (TA), 5 mL wheat grass juice samples and 15 mL distilled water was placed into a beaker. Afterwards, this solution was filtrated by filter paper and 2 mL of this filtrated solution was removed to another beaker. 1 g of phenolphthalein was dissolved with 100 mL of 95 % ethyl alcohol in an Erlenmayer flask. Three drops of this phenolphthalein solution were mixed to the filtrated solution in the beaker. The mixture was titrated with 0.1 N NaOH to the end point of phenolphthalein (pH 8.2 ± 0.1), until pink color was detected. The volume of NaOH was expressed as either citric acid or malic acid per 100 mL of juice (Cemeroğlu, 2007). The total acidity was calculated according to the formula:

$$TA\% = (N \times V / m) \times 100 \times FD \times \text{meq. wt.}$$

Where V is titer volume of NaOH, m is mass of juice (g), meq.wt. is weight of the Standard, N is the normality of NaOH and FD is factor of dilution.

3.2.8.4 Viscosity

The viscosity of the samples was measured by a viscometer (AND Vibro Viscometer, SV-10, Japan).

3.2.8.5 Determination of total sugar content

The total sugar content (in fructose) of the wheat grass juice samples was determined by using dinitrosalicylic acid (DNS) method (Miller, 1959). First of all, DNS solution and 40% sodium potassium tartrate solution were prepared. In order to plot the standard calibration curve, solutions containing 1, 2, 3, 4 % and 5 % D-fructose were prepared. 1 mL of each of these fructose solutions were put into test tubes and 3 mL DNS was added to them. Then these samples were incubated in boiling water bath for 15 - 20 minutes, and subsequently cooled quickly under cool water. After that, 1 mL from each cooled samples were completed to 50 mL with distilled water. The absorbance was determined by using a spectrophotometer at O.D.540 nm (Shimadzu, PharmaSpec UV-1700, UV-Visible Spectrophotometer,Japan).

3.2.8.5.1 The preparation of DNS Solution

10.6 g of DNS (2- hydroxy 3,5 dinitro benzoic acid), 19.8 g of sodium hydroxide, 7.6 g of phenol, 8.3 g of sodium metabisulphide, 306 g of sodium potassium tartrate were dissolved in 1416 g of distilled water.

3.2.8.6 Determination of protein content

The protein content of the wheat grass juice samples was determined by using Biuret method (Kingsley, 1939). First of all, Biuret solution and Bovine serum albumin solution (10 mg/mL) were prepared. In order to plot the standard calibration curve, solutions of bovine serum albumin containing 1, 2, 4, 6, 8 % and 10 % were prepared. Then, 1 mL of each of wheat grass juice samples were put into test tubes and 1.5 mL Biuret reagent was added to them. After that, these samples were incubated at 37 °C for 10 minutes and were completed to 10 mL with distilled water. The absorbance was detected by using a spectrophotometer at O.D. 540 nm (Shimadzu, PharmaSpec UV-1700, UV-Visible Spectrophotometer, Japan).

3.2.8.6.1 The preparation of Biuret solution

3 gram $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (Copper (II) sulfate pentahydrate) and 9 gram Sodium potassium tartrate were dissolved in 500 mL 0.2 N NaOH (Sodium hydroxide) and 5 gram potassium iodide was added then all chemicals were completed to 1000 mL with distilled water.

3.2.8.7 The color determination

In order to detect the color of juice samples, L^* , a^* , b^* values of 2 mL of each sample was determined by a colorimeter (Konica Minolta, CR-400, Japan). The color values were expressed as L^* (whiteness or brightness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness) (Lopez-Nicolas and Garcia-Carmona, 2007). All measurements were performed in duplicates.

3.3 Statistical Evaluation

The data were shown as means and analyzed by the IBM SPSS Statistics 23 program. Anova Scheffe test was used to detect the significance between treatment groups and different quantities and process times of each treatment for wheat grass juice.

In order to detect the significance for variables in the chemical analysis of wheat grass juices, Kruskal-Wallis test; and for the comparison of groups Mann-Whitney U test was used. A P-value of less than 0.05 was considered significant. Pearson correlation was used to correlate related chemical parameters.

General Linear Model Repeated Measures test was used to detect the significance between storage periods during shelf-life determination.

For all analysis, the significant level was set to $p < 0.05$ at the beginning of the study.

4. RESULTS AND DISCUSSIONS

4.1 The microbial analysis of wheat grass juice

The microflora of wheat grass juices was examined and total mesophilic aerobic bacteria, *E.coli*, yeast and mold were observed. No *Salmonella* spp. was detected. All microbial counts were tabulated in Appendix A.3 (log cfu/mL).

Wheat (*Triticum aestivum*) is an important ingredient of the human diet, mostly in developing countries. Many studies have shown that the consumption of whole grain and whole-grain products are protective against chronic diseases such as cardiovascular disease, diabetes, and cancer (Thompson, 1994; Nicodemus et al., 2001). Due to these findings, the possible increase of functionality of wheat grass juice was investigated by probiotics and prebiotics together with ultrasonication during the study. It was demonstrated that no significant difference was found between the microbial counts at 0th, 20th, 40th and 60th days of wheat grass juice (Appendix A.3 and Figure 4.1.) ($p>0.05$).

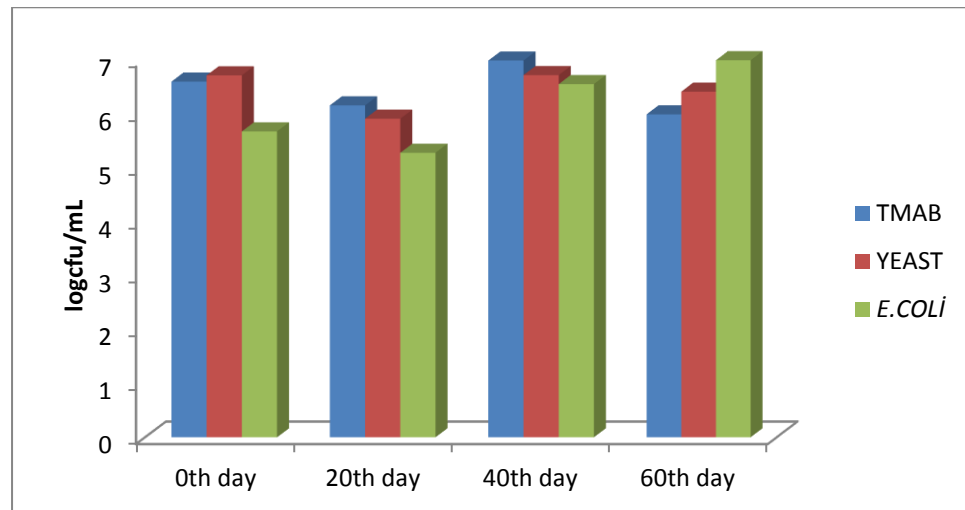


Figure 4.1. TMAB, YM and *E.coli* (logcfu/mL) of control groups.

According to Table 4.1., when control and ultrasonicated groups of wheat grass juice was compared, there was a reduction in the logarithmic counts of the total mesophilic aerobic bacteria and yeast- mold, approximately up to 1.5 log cycle. When

the YM and TMAB were compared, a ten minute treatment of 90 dB 10 min. of ultrasonication was found to be more effective than 30 dB 10 min of ultrasonication ($p < 0.05$). Mohideen (2015) suggested that increasing juice flow rate and sonication amplitude were reduced the microbial counts, especially yeast in blueberry juice. This was found to be in line with our findings of 90 dB 10 min. of ultrasonication treatment for yeast counts. Also, Kırmusaoglu (2013) observed that approximately 3 log reduction of YM counts was found by ultrasonication of 90 dB for ten minutes in blackberry juices and it was found to be similar to our findings (Table 4.1.) ($p < 0.05$).

Table 4.1. TMAB and YM in the ultrasonicated wheat grass juice.

Groups	TMAB	YM
	Mean \pm Std.	Mean \pm Std.
Control	6.59 ^b \pm 0.432	6.71 ^c \pm 0.591
30 dB 10 min.	6.50 ^b \pm 0.220	6.01 ^{ab} \pm 0.368
90 dB 10 min.	5.09 ^a \pm 0.499	5.16 ^a \pm 0.364

Data are means \pm SD from two replicates

Values followed by different letters in the same column are significantly different ($P < 0.05$)

For *E.coli* counts, there was a logarithmic reduction in wheat grass juice, resembling to the TMAB and YM results tabulated in Table 4.1; therefore a ten minute treatment of 90 dB ultrasonication was found to be more effective than 30 dB ultrasonication (Table 4.2.) ($p < 0.05$). Approximately one log cycle reduction between the control and ten minute treatment of 90 dB ultrasonication for *E.coli* was observed.

Table 4.2. *E.coli* in the ultrasonicated wheat grass juice according to doses.

Groups	<i>E.coli</i>
	Mean \pm Std.
Control	5.67 ^b \pm 0.084
30 dB 10 min.	5.18 ^a \pm 0.217
90 dB 10 min.	4.76 ^a \pm 0.270

Data are means \pm SD from two replicates

Values followed by different letters in the same column are significantly different ($p < 0.05$)

4.2 Treatments with probiotics

L. reuteri and *L. acidophilus* were added as probiotic bacteria into wheat grass juice. These are lactic acid bacteria and commonly used in the industry. Activity of probiotic might be related to genera, species, or strains. An initiative in probiotic application could be the use of mixtures of strains to be interested into different genera

or species (Timmerman et al., 2004). In this study, separately, 0.5 and 1 mL *L. reuteri* and similarly, *L. acidophilus* (approximately 10^7 cfu/mL) were inoculated into wheat grass juice (Appendix A.4., Appendix A.5.). When 0.5 and 1 mL *L. reuteri* inoculated groups were compared, no logarithmic reduction was observed between them for total mesophilic aerobic bacteria counts (log cfu/mL) (Appendix A.4) ($p < 0.05$). 0.5 mL *L. reuteri* inoculated group was more effective than 1 mL on the inhibition of yeast and mold and total aerobic mesophilic aerobic bacteria counts (log cfu/mL) due to reduction (Figure 4.3). This was probably due to the inhibitory effect of lactic acid bacteria (*L. reuteri*) on the other microorganisms. The inhibition was possibly related with the release of lactic acid or a proteinaceous substance such as reuterin, hydrogen peroxide or a bacteriocin. When the microbial load of inoculated lactic acid bacteria (*L. reuteri*) was increased, the counts on MRS agar were also increased (Figure 4.2).

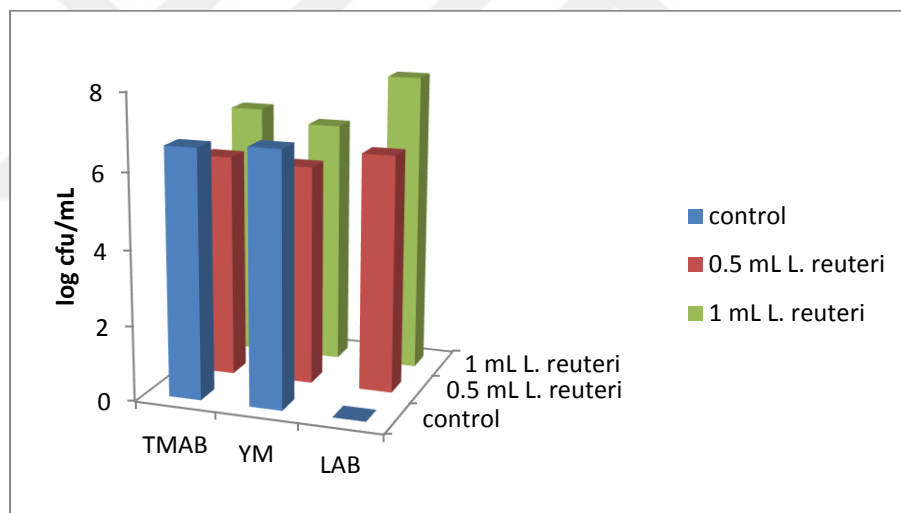


Figure 4.2. *L. reuteri* inoculated (0.5 and 1 mL) into wheat grass juice: TMAB, YM and LAB (log cfu/mL).

Similarly, 0.5 mL *L. acidophilus* inoculated group was more effective than 1 mL on TMAB counts on wheat grass juice, reaching up to an inhibition of two log and one and a half cycles, respectively. When the control was compared with 1 mL *L. acidophilus* inoculated group, there was only 0.5 logarithmic cycle reduction for YM on YEA Agar (Figure 4.3.).

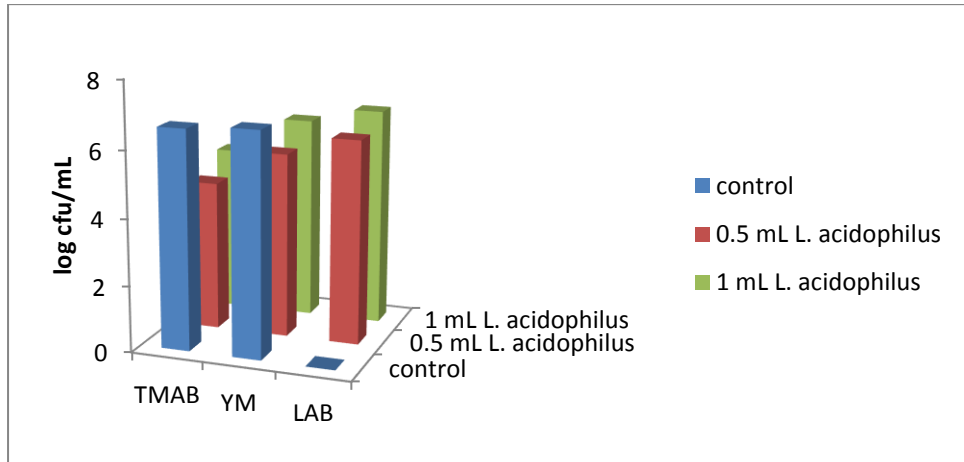


Figure 4.3. *L. acidophilus* inoculated (0.5 and 1 mL) into wheat grass juice: TMAB, YM and LAB (log cfu/mL).

When *E. coli* counts were investigated for the probiotics, both of them showed a slight inhibition. Interestingly, 0.5 mL *L. reuteri* and 0.5 mL *L. acidophilus* added wheat grass juice were more effective than 1 mL inoculated ones on *E. coli* reduction (Table 4.3.). This finding was in line with Krause et al. (2003), demonstrated that, with a high grain diet *E. coli* and MRS counts (containing lactic acid bacteria) followed the same population dynamics; when *E. coli* was high so were MRS counts and vice versa in the digestive tract of animals.

Table 4.3. *E. coli* in *L.reuteri* and *L. acidophilus* containing wheat grass juice.

Groups	<i>E. coli</i> counts
	Mean \pm Std.
Control	5.67 ^a \pm 0.084
0.5 mL <i>L. reuteri</i>	5.18 ^{ab} \pm 0.489
1 mL <i>L. reuteri</i>	5.56 ^a \pm 0.514
0.5 mL <i>L. acidophilus</i>	4.94 ^b \pm 0.440
1 mL <i>L. acidophilus</i>	5.08 ^{ab} \pm 0.891

Data are means \pm SD from two replicates

Values followed by different letters in the same column are significantly different ($p < 0.05$)

4.3 Treatments with probiotics

4.3.1 Persimmon

In the present study, 0.1 and 0.5 g freeze-dried persimmon were added into wheat grass juice as a prebiotic source. Persimmon includes sugars such as glucose and fructose, vitamins such as A and C, and tannin. Tannin is a physiologically active

substance that provide antioxidant, antimicrobial and antitumor activities, lowering cholesterol, and elimination of heavy metals (Seo et al., 2000). When the control was compared to the group containing 0.5 g freeze-dried persimmon, there was a significant reduction of TMAB, YM and this may be related with the possible antimicrobial effect of persimmon fruits. A slight inhibition of *E. coli* was observed when compared to the TMAB and YM counts. Arakawa et al., (2014) suggested that persimmon leaves extract observed antibacterial activity and inhibition against *Escherichia coli* and *Staphylococcus aureus*, also *Campylobacter sputorum*, *Streptococcus mutans* and *Bacteroides thetaiotaomicron*, due to the effect of tannins in the plant. In our findings, approximately similar effect was found on *E. coli* (Table 4.4.). 0.5 g was more effective than 0.1 g freeze-dried persimmon containing group so that the quantity of persimmon might be an important criteria for inhibition (Table 4.4.). And also, if 0.5 g freeze-dried persimmon was added to the wheat grass juice, it was found that an approximate reduction of more than one log cycle was observed in TMAB. On the contrary, no LAB on MRS Agar was found in all treatments including the control (Figure 4.4.).

Table 4.4. TMAB, YM and *E.coli* in persimmon containing treatments.

Groups	TMAB	<i>E.coli</i>	YM
	Mean±Std.	Mean±Std.	Mean±Std.
Control	6.59 ^a ±0.432	5.67 ^a ±0.084	6.71 ^a ±0.591
0.1 g freeze-dried persimmon	5.44 ^b ±0.103	5.37 ^a ±0.013	5.73 ^b ±0.358
0.5 g freeze-dried persimmon	5.21 ^c ±0.495	5.21 ^b ±0.231	5.53 ^{ab} ±0.055

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05).

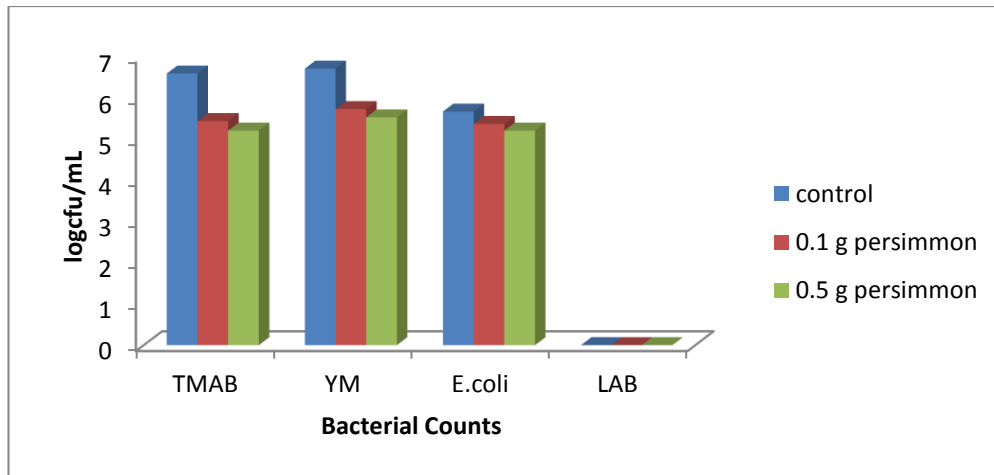


Figure 4.4. TMAB, YM, *E.coli* and LAB for control (wheat grass juice), 0.1 and 0.5 g persimmon containing groups (log cfu/mL).

4.3.2 Leek

Other than persimmon; 0.1 and 0.5 g freeze-dried leek was added to wheat grass juice in powdered form. In recent years, many studies were carried out on leek and it was found that it would be able to reduce many kinds of cancer including colorectal, stomach and breast (Bianchini et al., 2001). This study showed that 0.5 g freeze-dried leek was more effective than 0.1 g in logarithmic reduction of the YM (Figure 4.5.).

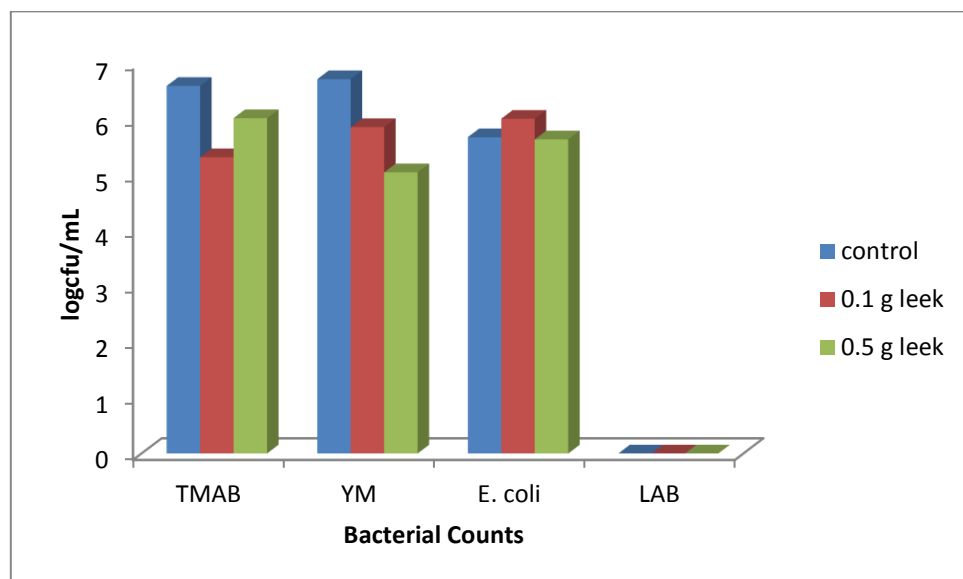


Figure 4.5. TMAB, YM, *E.coli* and LAB for control (wheat grass juice), 0.1 and 0.5 g leek containing groups.

On the other hand, the results in Table 4.5. indicated that when the control and 0.1 g freeze-dried leek treatment was compared, there was more than one log cycle significant reduction in the logarithmic counts of the TMAB (log cfu/mL) ($p < 0.05$). There was a reverse interaction between other microbial counts (YM and *E. coli*); as, 0.5 g freeze-dried leek was found to be more effective on YM; but ineffective on the TMAB. No significant effect of any leek concentration on *E. coli* was observed (log cfu/mL) ($p > 0.05$). It is similar to persimmon that, no LAB on MRS Agar was found in all treatments of leek including the control (Figure 4.5.).

Table 4.5. TMAB, YM and *E.coli* in leek containing groups.

Groups	TMAB	<i>E.coli</i>	YM
	Mean±Std.	Mean±Std.	Mean±Std.
control	6.59 ^a ±0.432	5.67 ^b ±0.084	6.71 ^a ±0.591
0.1 g leek	5.31 ^c ±0.390	6.00 ^a ±0.442	5.85 ^b ±0.678
0.5 g leek	6.01 ^b ±0.549	5.63 ^b ±0.028	5.04 ^c ±0.464

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different ($p < 0.05$)

4.4 Treatments with prebiotics and probiotics

When prebiotics and probiotics are applied together it is called as synbiotics (Holzapfel and Schillinger, 2002). In order to observe the synbiotic effect, four combination treatments were designed and microbial analysis was carried out and the most effective groups (0.5 g) were selected; as since 0.5 g was found to be more effective than 0.1 for both freeze-dried persimmon and leek (Appendix A.6. , Appendix A.7. , Table 4.2. , Table 4.6., Figure 4.6). When the control (untreated wheat grass juice) and the treatment groups (T9, T4, T8 and T3) (According to Table 3.1.) was compared, there was a significant reduction in the logarithmic counts of the TMAB, *E.coli* and YM, especially in 0.5 g freeze-dried persimmon-0.5 mL *L.reuteri* treatment group (T9) (Table 4.6.) ($p < 0.05$) (log cfu/mL).

Table 4.6. indicated that when the control (untreated) and treatment group of T9 was compared, there was an important and significant reduction reaching up to 2.5 log cycles in the logarithmic counts of the TMAB on PCA agar ($p < 0.05$). When control and the four combination groups without treatment of ultrasonication (Table 4.6.) were compared with the LAB, there were no colonies in the control group (wheat grass juice) but the treatments were ranging between 5.62±0.012-6.39±0.507. *L. reuteri* and

L. acidophilus are very important sources of probiotics for gastro-intestinal system. This study showed that when prebiotics (persimmon and leek) were added and probiotics (*L. reuteri* and *L. acidophilus*) were inoculated into wheat grass juice in combination, there was an important reduction in the logarithmic counts (log cfu/mL) ($p < 0.05$) TMAB, YM and *E.coli* counts (Figure 4.6.).

Table 4.6. TMAB, YM, *E.coli* and LAB in four combination treatment groups (T9, T4, T8 and T3) and their ten minute treatment of 90 dB ultrasonication (T18, T15, T17 and T14).

Groups	TMAB	<i>E.coli</i>	YM	LAB
	Mean \pm .Std	Mean \pm .Std	Mean \pm .Std	Mean \pm .Std
Control	6.59 ^a \pm 0.432	5.67 ^a \pm 0.084	6.71 ^a \pm 0.591	N.D
T9*	4.53 ^c \pm 0.088	4.23 ^c \pm 0.096	5.61 ^d \pm 0.475	5.62 ^{cd} \pm 0.012
T4*	4.73 ^c \pm 0.055	4.56 ^b \pm 0.513	5.43 ^c \pm 0.090	5.98 ^c \pm 0.645
T8*	5.83 ^b \pm 0.423	5.15 ^{ab} \pm 0.167	6.03 ^b \pm 0.457	6.37 ^c \pm 0.507
T3*	5.52 ^b \pm 0.397	5.49 ^a \pm 0.335	5.34 ^c \pm 0.300	6.39 ^c \pm 0.507
T18 [^]	N.D	N.D	N.D	6.09 ^d \pm 0.165
T15 [^]	N.D	5.48 ^{bc} \pm 0.764	4.34 ^e \pm 0.494	7.06 ^a \pm 0.091
T17 [^]	N.D	N.D	5.38 ^{de} \pm 0.357	6.56 ^b \pm 0.505
T14 [^]	5.74 ^f \pm 0.232	5.71 ^c \pm 0.315	6.57 ^{ab} \pm 0.553	7.19 ^a \pm 0.475

Data are means \pm SD from two replicates

Values followed by different letters in the same column are significantly different ($p < 0.05$) N.D:Not determined.

* : According to Table 3.1. and ^ : According to Table 3.2.

Except control among the nine groups presented in Table 4.6., the effective groups were found to be T18 and T17, that is these groups combined with a ten minute treatment of 90 dB ultrasonication, observed in all of the microbiological assays. When the combination treatment group of T14 was examined, it included the maximum LAB counts than the other combination forms of wheat grass juice, considering the ultrasonication treatments (Figure 4.6). This mixture (wheat grass juice and leek) may probably lead the formation of an acidic medium allowing the growth of *L. acidophilus*, an acid lover bacteria.

Some researchers indicated that enhanced amplitude levels of ultrasonication increased inhibition of foodborne pathogens (Hua and Thompson, 2000; Ugarte-Romero et al., 2007). And also, similar observations were reported by Herceg et al (2012) who found that amplitude, time and temperature during ultrasound treatment of milk affected the inactivation of *E.coli*. Our study showed that an independent ten minutes' of 90 dB at ultrasonication treatment of 0.5 g freeze-dried persimmon-0.5 mL *L. reuteri* inoculated wheat grass juice (treatment group of T18) was found to be more

effective than the other groups of ultrasonication treatments, TMAB, YM and *E. coli* were found (Table 4.6.) ($p < 0.05$). When this group was compared with control for LAB on MRS agar, there was a significant increase as well as due to the double combinations since no lactic acid bacteria was found in control (Figure 4.6.) ($p < 0.05$). Surprisingly, ultrasonication may promote the release of an effective microbial metabolite such as reuterin, bacteriocin, lactic acid or H_2O_2 to provide effective inhibition of TMAB, YM and *E. coli*. Bian et al. (2011) observed that reuterin from *L. reuteri* DPC16 showed a significant inhibition in bacterial population against gram-positive (*S. aureus*, *L. monocytogenes*) and gram-negative (*E. coli*, *S. Typhimurium*) bacteria. When combination treatment group of T15 was compared with the other groups, it was observed that there was a significant logarithmic reduction only for TMAB ($p < 0.05$). This may be due to the effect of ultrasonication on *L. acidophilus* to release an effective microbial metabolite such as bacteriocin, lactic acid or H_2O_2 (Table 4.6.) ($p < 0.05$). Interestingly, *E. coli* counts showed that other than *L. reuteri* and the prebiotic combinations no effect of the treatments was observed with respect to the control (Table 4.6., Figure 4.6.). On the other hand, the lactic acid bacteria was significantly increased in all treatment groups ($p < 0.05$). Since the population of lactic acid bacteria was increased, for this reason, ultrasonication may be helpful to be extensively used in different fields of food industry, especially in functional food production. As a consequence, *L. reuteri* was found to be more effective than *L. acidophilus* in synbiotic case; and persimmon containing treatments were found to be more effective than the leek when considering the TMAB, YM and *E. coli* inhibition.

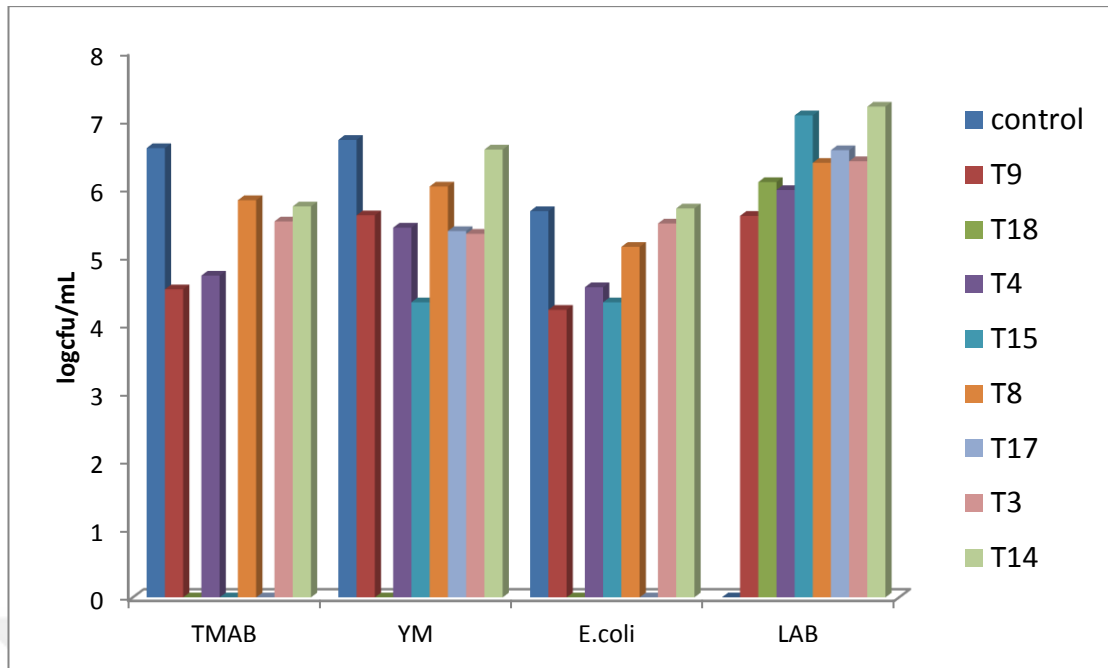


Figure 4.6. TMAB, YM, *E.coli* and LAB in four combination treatment groups (T9, T4, T8 and T3) and their ten minute treatment of 90 dB ultrasonication (T18, T15, T17 and T14).

When combination treatment groups of T8 and T17 were compared with control, not only logarithmic reduction on total mesophilic aerobic bacteria but also on yeast and molds and *E. coli* was observed. The ultrasonicated groups were always found to be significantly more effective than the other combinations (log cfu/mL) ($p < 0.05$), since no TMAB and *E. coli* was determined (Figure 4.6.) ($p < 0.05$). When lactic acid bacteria counts were compared between two groups (T8 and T17), there was a slight logarithmic increase in the ultrasonication treated group. This effect may be related with the population dynamics of *L. reuteri*. Some chemical compounds such as hydrogen peroxide, nitrite and nitrate, which may be produced during the ultrasonication treatment, may also increase the pH (Supeno, 2000). In our findings provide that ultrasonication may provide the degradation of possible potential carbon and nitrogen source polymers into monomers in grass juice, leading to increase the growth and maintenance of the lactic acid bacteria. This would therefore increase the functionality of wheat grass juice (Figure 4.6.) ($p < 0.05$). On the other hand, when the combination group of T14 was compared with the other ultrasonicated groups, there was a logarithmic increase of all microbial counts (TMAB, YM and *E. coli*). It can be concluded that *L. reuteri* containing groups showed better compatibility with the prebiotics (persimmon and leek) and/or the wheat grass juice than *L. acidophilus*,

therefore the inhibition potential of *L. reuteri* was found to be better also (Table 4.6.) ($p < 0.05$).

4.5 Treatment with triple and quadruple combination results

When triple and quadruple combination forms of wheat grass juice with prebiotics and probiotics were compared with the control of, there were significant differences in TMAB and YM counts (Table 4.7) ($p < 0.05$). The triple treatment group of T11 was more effective than the other triple combination forms of wheat grass juice due to the inhibition on TMAB and YM. Table 4.7 showed that when *L. reuteri* and *L. acidophilus* were used in consortia with persimmon or leek, there was a significant logarithmic reduction on TMAB and YM counts. This might be related with the synergistic effect of probiotics (*L. reuteri* and *L. acidophilus*). The two combination treatment groups of T5 and T10 had more bacterial counts such as TMAB and yeast YM than the other two combination treatment groups of T11 and T12 (Figure 4.7) ($p < 0.05$). This may be due to the interaction with wheat grass juice and probiotics (*L. reuteri* and *L. acidophilus*) (Table 4.7) ($p < 0.05$).

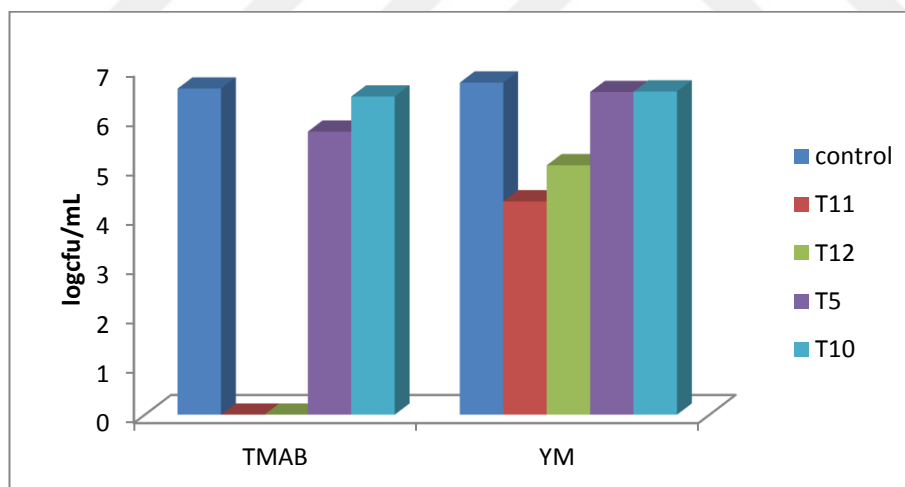


Figure 4.7. TMAB and YM in triple combination treatment groups (T11, T12, T5 and T10) (log cfu/mL).

Then, when *E. coli* counts of triple combination treatment group of wheat grass juices (T5, T10, T11 and T12) were examined, there was a logarithmic reduction of *E. coli* in *L. reuteri* and *L. acidophilus* consortia groups (Figure 4.8) ($p < 0.05$). Similar effect was found on the TMAB and YM counts (Figure 4.8).

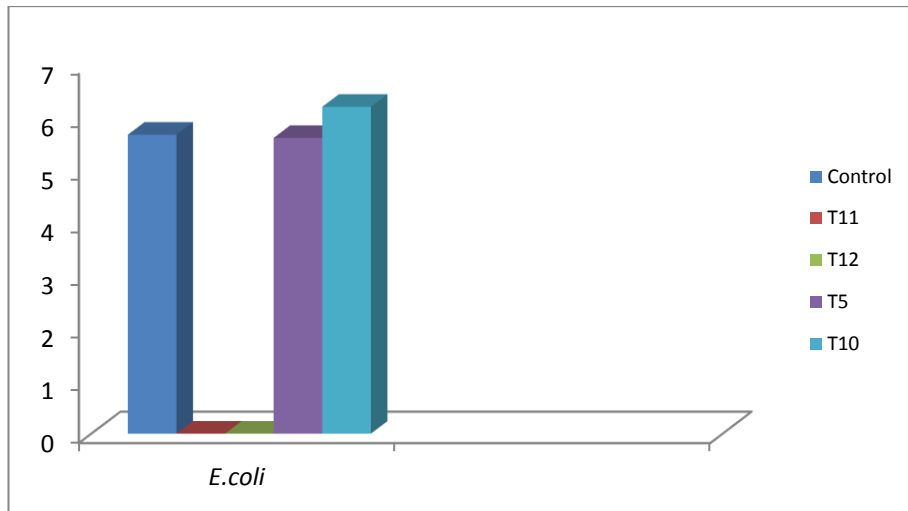


Figure 4.8. *E. coli* in triple groups (T11, T12, T5 and T10) (log cfu/mL).

If the control and the triple combination treatment groups (T5, T10, T11 and T12) were compared, there was a significant increase of lactic acid bacteria on MRS agar. The treatment group of T11 had more lactic acid bacteria than the other group of treatments (Table 4.7.). This might be related with the synergistic effect between *L. reuteri* and *L. acidophilus* and the compatibility of persimmon as a prebiotic source, since it contains high amount of carbohydrates and vitamins (Kawase et al., 2003), as well as other minerals promoting the growth of these symbiotic cultures. On the other hand the inulin, as a prebiotic source content, of leek was reported to be high, but the inulin content may differ in leek due to its stalk and green part (Shoaib et al., 2016) while processing, if it is low then it may lower the effectivity.

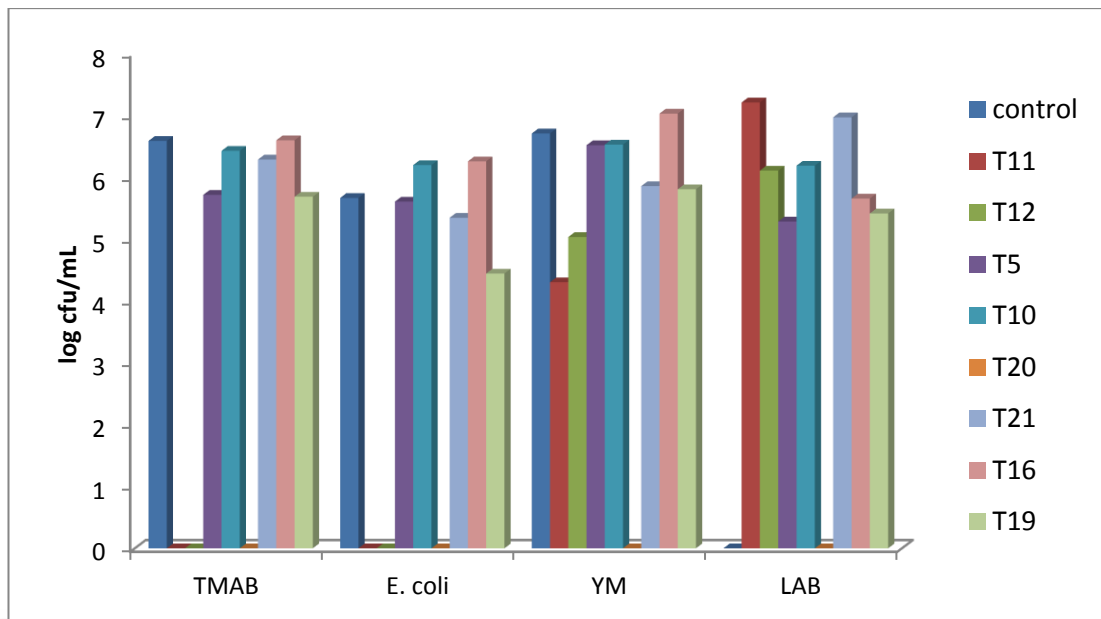


Figure 4.9. TMAB, YM, LAB and *E.coli* for treatments groups (T11, T12, T5, T10, T20, T21, T16 and T19) (log cfu/mL).

Figure 4.9 indicated that there was a logarithmic increase in ultrasonication treated groups excluding the combination treatment group of T20; as since the TMAB, YM, LAB and *E.coli* were not determined and it was found to be significantly different than the others ($p < 0.05$). Interestingly, LAB was compared with control and treated with ultrasonication, there was an increase with respect to control (Figure 4.9) ($p < 0.05$).

Table 4.7. TMAB, YM, *E.coli* and LAB in triple combination groups (T11, T12, T5 and T10) and the groups treated 90 dB ultrasonication (T20, T21, T16 and T19) (log cfu/mL).

Groups	TMAB	<i>E.coli</i>	YM	LAB
	Mean±.Std	Mean±.Std	Mean±.Std	Mean±.Std
Control	6.59 ^{cd} ±0.432	5.67 ^{bc} ±0.084	6.71 ^{cd} ±0.591	N.D
T11*	N.D	N.D	4.31 ^a ±0.405	7.21 ^a ±0.531
T12*	N.D	N.D	5.04 ^b ±0.460	6.11 ^c ±0.507
T5*	5.72 ^a ±0.405	5.61 ^b ±0.310	6.52 ^c ±0.320	5.29 ^{cd} ±0.216
T10*	6.43 ^d ±0.334	6.20 ^c ±0.223	6.53 ^c ±0.372	6.19 ^c ±0.019
T20^	N.D	N.D	N.D	N.D
T21^	6.29 ^{ab} ±0.185	5.35 ^b ±0.390	5.86 ^b ±0.224	6.97 ^b ±0.422
T16^	6.60 ^e ±0.421	6.26 ^d ±0.512	7.03 ^d ±0.131	5.66 ^d ±0.552
T19^	5.69 ^a ±0.523	4.45 ^a ±0.212	5.81 ^{bc} ±0.369	5.42 ^e ±0.106

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05).N.D: Not determined.

*: According to Table 3.1. and ^ : According to Table 3.2.

When quadruple combinations form (*L. reuteri*, *L. acidophilus*, persimmon, leek) and their 10 min. 90 dB ultrasonication treatments were compared with control, there was a significant logarithmic reduction on bacterial counts (TMAB, YM and *E. coli*) (Table 4.8) (p<0.05). And also, when treatments were compared with control, there was a significant increase of lactic acid bacteria since no growth was found in control on MRS agar (p<0.05). Ultrasonication is not only effective on powerful inhibition of TMAB, YM and *E.coli* but also due to an increase in LAB population; the functionality of the wheat grass juice is increased.

Table 4.8. TMAB, YM, *E.coli* and LAB in quadruple combination group T13 and 90 dB ultrasonication treatment group of T22.

Groups	TMAB	<i>E.coli</i>	YM	LAB
	Mean±.Std	Mean±.Std	Mean±.Std	Mean±.Std
Control	6.59 ^b ±0.432	5.67 ^a ±0.084	6.71 ^c ±0.591	N.D
T13*	N.D	N.D	4.45 ^a ±0.638	6.03 ^a ±0.482
T22^	N.D	N.D	N.D	6.87 ^c ±0.428

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05) N.D :Not determined.

*: According to Table 3.1. and ^ : According to Table 3.2.

The powerful inhibition might be related with ultrasonication which may promote the release of an effective microbial metabolite such as reuterin, bacteriocin, lactic acid or H₂O₂ (Figure 4.10).

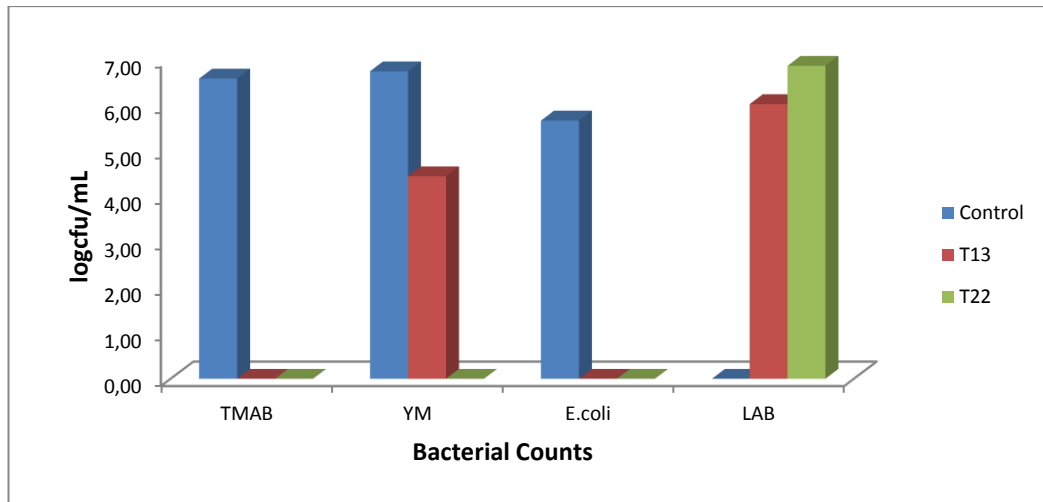


Figure 4.10 TMAB, YM, *E.coli*, LAB in quadruple combination group T13 and 90 dB ultrasonication treatment group T22.

4.6 The shelf life results

The twentieth, fortieth and the sixtieth day results of control and all of treatment groups were examined. According to Appendix 10, Table 4.9 and Figure 4.11 when shelf life counts of *E.coli* were compared with the control, there was a significant reduction and no bacterial growth were observed in 40th and 60th days (logcfu/mL) ($p < 0.05$). Especially, quadruple combination form of treatments (leek, persimmon, *L. reuteri* and *L. acidophilus*) and treated with 90 dB ultrasonication showed no growth of *E.coli* in 20th, 40th and 60th days. This result might be related with symbiotic interaction between leek, persimmon, *L. reuteri* and *L. acidophilus*; and ultrasonication treatment. Dennis et al (2006) have suggested that ultrasonic processing can inactivate the TMAB and *E. coli* in milk and apple cider. Our findings were in line with Dennis et al (2006) (Table 4.9).

Table 4.9. The treatment of *E.coli* in the twentieth, fortieth and the sixtieth day with respect to control.

Groups	0 th day	20 th day	40 th day	60 th day
	Mean±.Std	Mean±.Std	Mean±.Std	Mean±.Std
Control	5.67 ^c ±0.084	5.28 ^{bc} ±0.325	6.54 ^d ±0.388	6.99 ^d ±0.228
10 min. 90 dB	4.76 ^a ±0.270	5.70 ^c ±0.835	N.D	N.D
T3*	4.56 ^a ±0.513	N.D	N.D	N.D
T11*	N.D	N.D	N.D	N.D
T20 [^]	N.D	N.D	N.D	N.D
T13*	N.D	N.D	N.D	N.D
T22 [^]	N.D	N.D	N.D	N.D

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05) N.D: Not determined

*: According to Table 3.1. and ^ : According to Table 3.2.

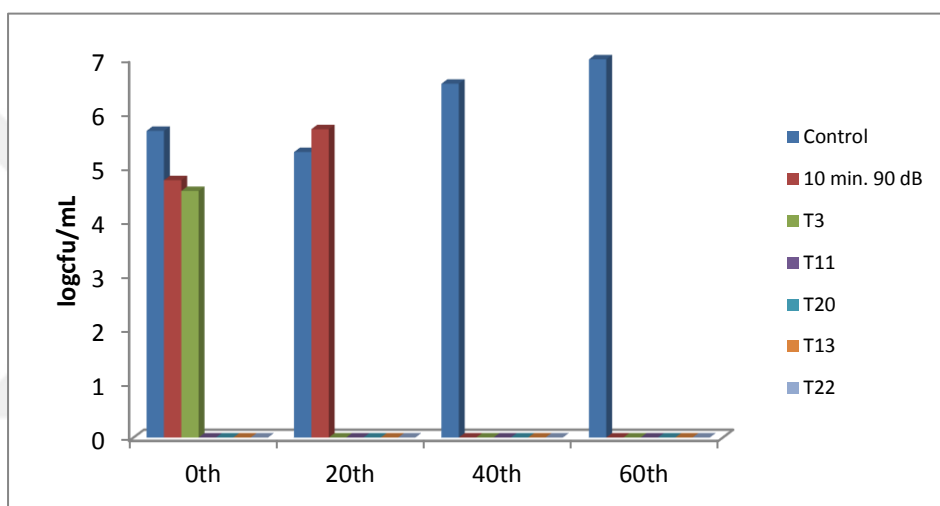


Figure 4.11. The treatments of *E.coli* in the twentieth, fortieth and the sixtieth day with respect to control (logcfu/mL).

Table 4.10 indicated that there was a significant reduction and approximately no bacterial growth were observed in 0th, 20th, 40th and 60th days for quadruple combination form composed of persimmon, leek, *L. reuteri* and *L. acidophilus* and treated with 90 dB ultrasonication(T13 and T22) (p<0.05). YM counts of shelf life were found to be significantly different than *E.coli* and TMAB counts (Appendix 9) (p<0.05).

Only one treatment group of T22 (leek, persimmon, *L. reuteri* and *L.acidophilus* treated 90 dB ultrasonication) had no YM counts on 0th, 20th and 60th days (Appendix 9).

When the LAB counts were compared with the control and treatment groups, surprisingly there was a logarithmic increase with ultrasonication process (Table 4.11.). Especially, T15, T8, T14 and also T5 treatment groups had high LAB counts

Table 4.10. The treatments of the TMAB in the twentieth, fortieth and the sixtieth day with respect to control.

Groups	0 th day	20 th day	40 th day	60 th day
	Mean±.Std	Mean±.Std	Mean±.Std	Mean±.Std
Control	6.59 ^a ±0.432	6.15 ^{ab} ±0.484	6.98 ^a ±0.563	5.99 ^c ±0.164
10 min. 90 dB	5.09 ^{dc} ±0.499	6.69 ^a ±0.087	6.00 ^c ±0.437	4.73 ^d ±0.369
T17[^]	N.D	N.D	N.D	N.D
T11[*]	N.D	N.D	N.D	N.D
T20[^]	N.D	N.D	N.D	N.D
T13[*]	N.D	N.D	N.D	N.D
T22[^]	N.D	N.D	N.D	N.D

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05) N.D:Not determined.

*: According to Table 3.1. and ^ : According to Table 3.2.

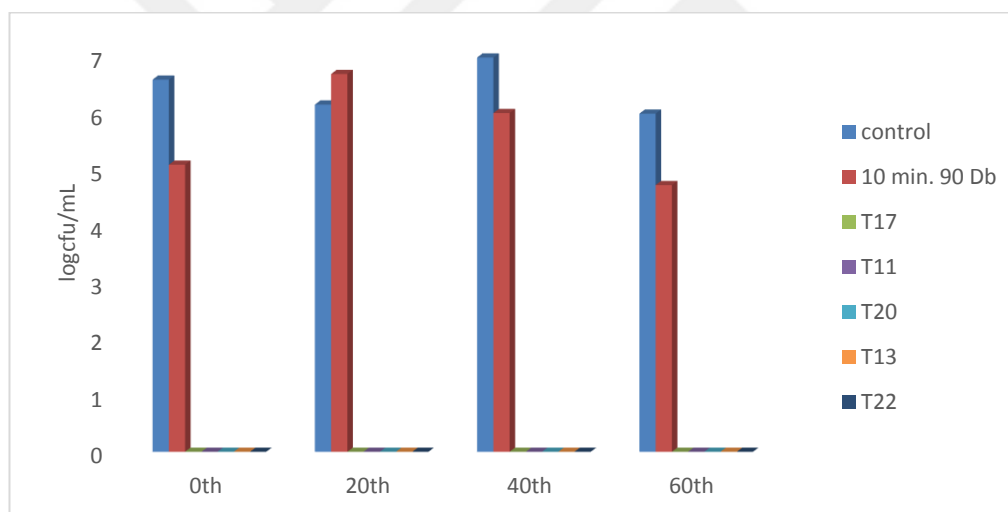


Figure 4.12. The treatments of (TMAB) in the twentieth, fortieth and the sixtieth day with respect to control (logcfu/mL).

in twentieth, fortieth and sixtieth days (Table 4.11.). It can be considered to be a good result, since probiotics can stay alive and continue to multiply in wheat grass juice with prebiotics and withstand to the ultrasonication process.

Table 4.11. The treatments of LAB counts in the twentieth, fortieth and the sixtieth day (logcfu/mL) with respect to control

Groups	0 th day	20 th day	40 th day	60 th day
	Mean±.Std	Mean±.Std	Mean±.Std	Mean±.Std
Control	N.D	N.D	N.D	N.D
10 min. 90 dB	N.D	N.D	N.D	N.D
T15[^]	7.06 ^{ab} ±0.091	6.98 ^{ab} ±0.318	7.28 ^a ±0.110	6.39 ^b ±0.310
T8[*]	6.37 ^a ±0.507	6.31 ^b ±0.261	6.98 ^{ab} ±0.042	6.59 ^b ±0.436
T14[^]	7.19 ^b ±0.475	7.25 ^a ±0.144	6.75 ^b ±0.301	7.05 ^a ±0.379
T5[*]	5.29 ^c ±0.216	6.21 ^b ±0.346	6.16 ^d ±0.234	6.08 ^c ±0.642

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05) N.D:Not determined.

*: According to Table 3.1. and ^ : According to Table 3.2.

4.7 The results of physical and chemical analysis

4.7.1 The physical analysis

The groups indicating a high inhibition capacity of TMAB, yeast-mold and *E.coli* was considered for the physical and chemical analysis. The pH of the wheat grass juice was found to be 6.12, 6.17, 6.16, 5.87, 5.67, 5.85 and 5.90 for the control, the ten minute 30 dB ultrasonication treated, ten minute 90 dB ultrasonication treated, the combination treatment groups of T9, T4, T8, T3, respectively (Table 4.12)(p<0.05). It was found that acidity was slightly increased with respect to control, but it was not found to be significant (Table 4.12.) (p<0.05).

Table 4.12. The physical analysis of treated and nontreated wheat grass juices

	pH	viscosity	acidity	°Brix	L	a*	b*
Control	6.12 ^c ±0.014	1.46 ^a ±0.007	0.71 ^{ab} ±0.014	4.00 ^{ab} ±0.000	24.08 ^a ±0.028	-7.58 ^c ±0.106	8.18 ^b ±0.035
30 dB 10 min.	6.17 ^c ±0.007	1.83 ^b ±0.007	0.52 ^a ±0.099	4.25 ^{ab} ±0.354	26.39 ^c ±0.007	-9.92 ^b ±0.120	11.27 ^d ±0.021
90 dB 10 min.	6.16 ^c ±0.007	2.08 ^c ±0.007	0.59 ^{ab} ±0.007	3.25 ^a ±0.354	27.44 ^f ±0.007	-10.92 ^a ±0.049	11.92 ^c ±0.035
T9*	5.87 ^b ±0.099	3.1 ^e ±0.007	0.70 ^{ab} ±0.028	5.00 ^{ab} ±0.000	24.85 ^c ±0.000	-7.58 ^c ±0.028	8.34 ^c ±0.021
T4*	5.67 ^a ±0.014	3.05 ^d ±0.021	0.66 ^{ab} ±0.035	4.75 ^{ab} ±0.354	23.83 ^a ±0.007	-6.4 ^d ±0.007	6.99 ^b ±0.007
T8*	5.85 ^b ±0.007	3.14 ^f ±0.014	0.68 ^{ab} ±0.000	4.50 ^{ab} ±0.000	25.65 ^d ±0.014	-7.54 ^c ±0.014	8.22 ^b ±0.014
T3*	5.90 ^b ±0.007	3.05 ^d ±0.007	0.73 ^b ±0.064	4.75 ^{ab} ±0.354	25.63 ^d ±0.000	-7.5 ^c ±0.014	8.17 ^b ±0.014

Data are means ± SD from two replicates (Acidity: titratable acidity, Viscosity: mPa.s, °Brix: total soluble solids (%), L: lightness/ darkness, a: red/ green, b: blue/yellow). *: According to Table 3.1.

When the °Brix (total soluble solids %) values of the combination treatment group of T9 was compared to the control, it had the highest value among all of the other treatments (Table 4.12.). Interestingly, when the ultrasonicated groups were examined, they showed the lowest value among the other treatment (Table 4.12.). The viscosity was found to be significantly increased due to treatment of prebiotics and probiotics ($p < 0.05$) when compared to the control and all of the ultrasonicated groups. This might be due to the symbiotic interactions between *L. reuteri* and *L. acidophilus* and biofilm and slime formation capacity of *L. reuteri* (Slížová et al., 2015) and *L. acidophilus* (Akolkar et al., 2005) respectively (Table 4.12.). While the acidity was examined, no significant difference was found between the control and the treatment groups of wheat grass juice ($p > 0.05$). A difference was observed between the lightness / darkness (L*), a* (red/green) and b* (blue/yellow) values with respect to the control (Table 4.12.). It was not great but significant ($p < 0.05$). It was reported that a decrease of the L* value indicates browning in fruit juices (Tiwari, O'Donnell, Patras, et al., 2009).

4.7.2 The chemical analysis

Table 4.13. The chemical analysis of treated and nontreated wheat grass juices

	Total sugar content (mg/mL)	Protein content (mg/mL)
Control	0.78 ^a ±0.177	11.59 ^a ±0.410
30 dB 10 min.	0.20 ^a ±0.021	13.30 ^{ab} ±1.075
90 dB 10 min.	0.16 ^a ±0.071	13.94 ^{ab} ±0.898
T9*	10.32 ^b ±0.071	16.25 ^b ±0.297
T4*	10.58 ^b ±0.467	17.47 ^c ±1.909
T8*	17.82 ^d ±0.764	17.15 ^b ±0.912
T3*	15.84 ^c ±0.099	15.06 ^{ab} ±0.502

Data are means ± SD from two replicates. *: According to Table 3.1.

There was a significant increase of total sugar and protein content of the prebiotic-probiotic treatments and control of wheat grass juice (Table 4.13.). With respect to the control, the ultrasonication reduced the sugar content but at the same time, a slight increase of protein content was observed. The bacteria needs sugar and proteins for its growth and maintenance, so the microorganism fortified treatment (synbiotic) groups help prebiotic sources release the sugars by the activity of probiotics (Akolkar et al., 2005), and as well as the enzymes are in protein nature, the presence of carbohydrates may help inducing the synthesis of enzymes involved in the degradation of these polymers. When treatment groups with leek and persimmon was compared, the total sugar content was found to be increased in leek containing ones, (Table 4.13.) ($p < 0.05$) since cellulose is the main principal component of leek, on the other hand persimmon has a high fructose content. When the protein content was compared, a significant increase was observed between all of the probiotic-prebiotic treated groups of wheat grass juice and control ($p < 0.05$). This may be due to the possible release of bacteriocins and reuterin in the related groups. Only a slight increase was observed between the ultrasonicated and the control (Table 4.13.).

5. CONCLUSION AND RECOMMENDATIONS

An increasing number of studies show that increased consumption of fruits and vegetables can reduce the risk of chronic degenerative diseases such as cardiovascular disease, cancers, and others. Nowadays, wheat grass and wheat grass juice are popular especially in Europe, United States and other developed countries and many researchers are showed that it is beneficial against many diseases such as cancer, ulcerative colitis etc. In this study, wheat grass juice was treated with combination form of prebiotics (leek and persimmon) and probiotics (*L. reuteri* and *L. acidophilus*) and ultrasonication because of increased functionality of wheat grass juice.

Ultrasonication consumes low energy when comparing to thermal food processing methods. And also, when ultrasound is used, no damage to health promoting substances such as vitamins, minerals, aminoacids is observed. In this study, we observed that ultrasonication process decreased the TMAB, yeast-mold (YM) and *E.coli* in wheat grass juice.

In general no any chemical or physical change was seen in all wheat grass juices treated with ultrasonication, probiotics and prebiotics.

In this study the TMAB and YM was found to be approximately decreased when a ten minute amplitude of 90 dB of ultrasonication was combined with freeze-dried leek or freeze-dried persimmon and *L. reuteri* or *L. acidophilus*. And also, lactic acid bacteria was increased when ultrasonication process was used. As a result, these findings suggested that ultrasonication and selected prebiotic-probiotic combination named “synbiotic” can demonstrate a suggestion status in food industry; to be used together, as well as the processing of wheat grass juice due to its efficacy on many diseases such as cancer, obesity and ulcerative colitis. The increase of lactic acid bacteria in wheat grass, not only by prebiotic supplementation but also the ultrasonication may play a role to ascend the popularity of the wheat grass juice.

6. REFERENCES

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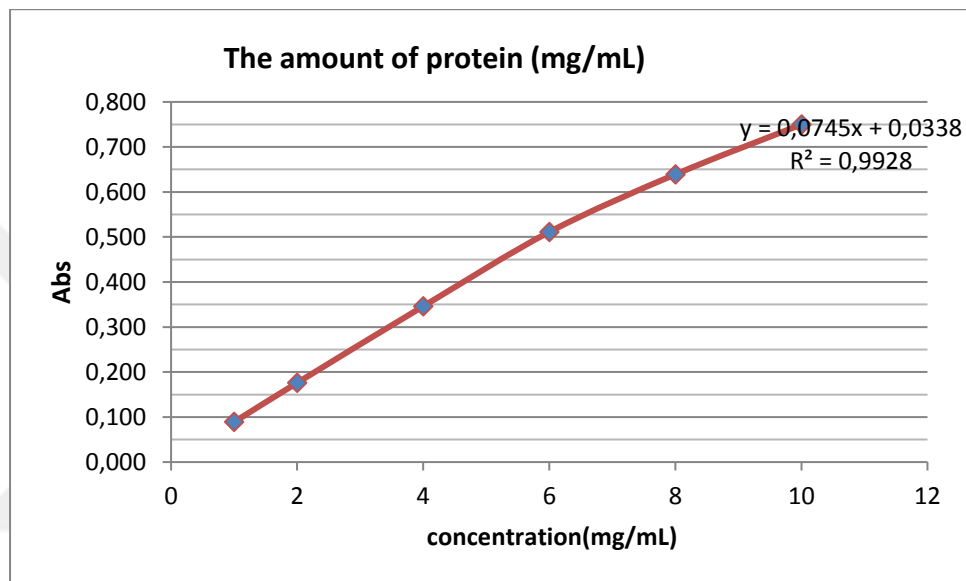


7. APPENDICES

A.1 The standards of protein (mg)

Std – 1	Std – 2	Std – 4	Std – 6	Std - 8	Std -10
0.089	0.176	0.346	0.511	0.639	0.750

A.2 Standard curve of protein (mg/mL)



Graph equation: $y=0.0745x +0.0338$

A.3 TMAB, YM and *E. coli* of the wheat grass juice (control group) Mean values were shown on the table.

		0th day	20th day	40th day	60th day
TMAB	Mean	6.595	6.156	6.985	5.990
YEAST	Mean	6.711	5.910	6.716	6.409
<i>E.coli</i>	Mean	5.674	5.280	6.549	6.990

A.4 *L. reuteri* containing wheat grass juice and the TMAB, YM and LAB counts (logcfu/mL).

Groups	TMAB	YM	LAB
	Mean ± .Std	Mean ± .Std	Mean ± .Std
control	6.59 ^{ab} ± 0.432	6.71 ^b ± 0.591	N.D
0.5 mL <i>L reuteri</i>	5.91 ^a ± 0.476	5.81 ^a ± 0.724	6.27 ^b ± 0.581
1 mL <i>L. reuteri</i>	6.80 ^b ± 0.426	6.48 ^b ± 0.441	7.89 ^a ± 0.062

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05) N.D:Not determined.

A.5 *L. acidophilus* containing wheat grass juice and the TMAB, YM and LAB counts (logcfu/mL).

Groups	TMAB	YM	LAB
	Mean ± .Std	Mean ± .Std	Mean ± .Std
Control	6.59 ^c ± 0.432	6.71 ^b ± 0.591	N.D
0.5 mL <i>L. acidophilus</i>	4.50 ^a ± 0.379	5.56 ^a ± 0.388	6.14 ^a ± 0.095
1 mL <i>L. acidophilus</i>	5.03 ^b ± 0.053	6.13 ^{ab} ± 0.002	6.56 ^b ± 0.265

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05) N.D:Not determined.

A.6 *L. reuteri* and *L. acidophilus* inoculated with 0.1 and 0.5 g freeze-dried persimmon containing wheat grass juice: The TMAB, YM, *E.coli* and LAB counts (logcfu/mL).

Groups	TMAB	<i>E.coli</i>	YM	LAB
	Mean±.Std	Mean±.Std	Mean±.Std	Mean±.Std
Control	6.59 ^c ±0.432	5.67 ^c ±0.084	6.71 ^c ±0.591	N.D
T7*	4.69 ^a ±0.669	4.42 ^a ±0.597	5.74 ^{ab} ±0.493	4.81 ^a ±0.047
T9*	4.53 ^a ±0.088	4.23 ^a ±0.096	5.61 ^a ±0.475	5.60 ^b ±0.012
T2*	5.25 ^b ±0.034	5.14 ^b ±0.718	5.92 ^b ±0.805	6.32 ^d ±0.221
T4*	4.73 ^{ab} ±0.055	4.56 ^{ab} ±0.513	5.43 ^a ±0.090	5.98 ^c ±0.645

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05),N.D :Not determined.

*: According to Table 3.1.

A.7 *L. reuteri* and *L. acidophilus*, 0.1; and 0.5 g freeze-dried leek containing and the TMAB, YM; *E.coli* and LAB counts (logcfu/mL).

Groups	TMAB	<i>E.coli</i>	YM	LAB
	Mean±.Std	Mean±.Std	Mean±.Std	Mean±.Std
Control	6.59 ^d ±0.432	5.67 ^b ±0.084	6.71 ^d ±0.591	N.D
T6*	6.13 ^c ±0.304	6.01 ^c ±0.350	6.06 ^c ±0.493	6.26 ^{ab} ±0.103
T1*	5.27 ^a ±0.638	5.28 ^{ab} ±0.410	5.76 ^b ±0.226	5.99 ^b ±0.673
T8*	5.83 ^b ±0.423	5.15 ^a ±0.167	6.03 ^c ±0.457	6.37 ^a ±0.507
T3*	5.52 ^a ±0.397	5.49 ^b ±0.335	5.34 ^a ±0.300	6.39 ^a ±0.507

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05) N.D:Not determined. *: According to Table 3.1.

A.8 The TMAB counts of control and treatment groups for shelf life (log cfu/mL).

Groups	0 th day	20 th day	40 th day	60 th day
	TMAB	TMAB	TMAB	TMAB
Control	6.59 ^a ±0.432	6.15 ^b ±0.484	6.98 ^a ±0.563	5.99 ^{cd} ±0.164
10 min. 90 dB	5.09 ^d ±0.499	6.69 ^a ±0.087	6.00 ^c ±0.437	4.73 ^d ±0.369
T9*	4.53 ^e ±0.088	5.46 ^{de} ±0.063	6.72 ^b ±0.421	6.08 ^{ab} ±0.536
T18^	N.D	5.83 ^{bc} ±0.300	6.33 ^{ab} ±0.124	6.57 ^b ±0.234
T4*	4.73 ^e ±0.055	4.63 ^e ±0.904	N.D	N.D
T15^	N.D	N.D	5.24 ^d ±0.256	5.49 ^d ±0.334
T8*	5.83 ^f ±0.423	5.73 ^f ±0.140	6.48 ^{ef} ±0.172	6.67 ^e ±0.297
T17^	N.D	N.D	N.D	N.D
T3*	5.52 ^f ±0.397	5.20 ^f ±0.629	6.14 ^g ±0.327	6.47 ^g ±0.416
T14^	5.74 ^{fg} ±0.232	5.92 ^g ±0.206	5.26 ^g ±0.298	5.58 ^{fg} ±0.506
T11*	N.D	N.D	N.D	N.D
T20^	N.D	N.D	N.D	N.D
T12*	N.D	N.D	5.86 ^h ±0.220	6.42 ⁱ ±0.320
T21^	6.29±0.185	4.77 ^{hi} ±0.103	6.07 ⁱ ±0.083	4.33 ^{hi} ±0.577
T10*	6.43 ⁱ ±0.334	N.D	6.05 ^j ±0.495	4.59 ^{ij} ±0.363
T19^	5.69 ^k ±0.523	5.84 ^k ±0.104	4.50 ^{kl} ±0.577	N.D
T5*	5.72 ^l ±0.405	5.18 ^l ±0.832	6.02 ^m ±0.297	5.81 ^m ±0.152
T16^	6.60 ⁿ ±0.421	5.18 ^l ±0.597	6.49 ⁿ ±0.553	N.D
T13*	N.D	N.D	N.D	N.D
T22^	N.D	N.D	N.D	N.D

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05), N.D :Not determined.

*: According to Table 3.1. ^: According to Table 3.2.

A.9 The YM counts control and treatment groups for shelf life (log cfu/mL)

Groups	0 th day	20 th day	40 th day	60 th day
	YM	YM	YM	YM
Control	6.71 ^a ±0.591	5.91 ^b ±0.120	6.71 ^a ±1.000	6.40 ^a ±0.253
10 min. 90 dB	5.16 ^c ±0.364	6.64 ^a ±0.037	6.14 ^{bc} ±0.921	6.60 ^a ±0.339
T9*	5.61 ^d ±0.475	5.81 ^d ±0.304	6.82 ^a ±0.475	6.75 ^a ±0.326
T18^	N.D	5.91 ^e ±0.095	6.48 ^d ±0.392	6.49 ^b ±0.621
T4*	5.43 ^{cd} ±0.090	5.78 ^e ±0.113	N.D	N.D
T15^	4.34 ^e ±0.494	N.D	5.38 ^e ±0.741	5.40 ^c ±0.119
T8*	6.03 ^f ±0.457	5.97 ^e ±0.349	6.80 ^a ±0.511	6.72 ^d ±0.496
T17^	5.38 ^{ef} ±0.357	4.30 ^{de} ±0.012	N.D	N.D
T3*	5.34 ^{ef} ±0.300	5.09 ^d ±0.356	6.47 ^b ±0.515	6.36 ^b ±0.533
T14^	6.57 ^f ±0.553	5.77 ^e ±0.328	6.19 ^f ±0.544	5.86 ^c ±0.377
T11*	4.31 ^g ±0.405	5.45 ^f ±0.360	5.39 ^e ±0.139	5.73 ^e ±0.506
T20^	N.D	4.84 ^{ef} ±0.192	4.53 ^{ef} ±0.088	N.D
T12*	5.04 ^g ±0.460	5.68 ^f ±0.385	6.12 ^f ±0.518	6.71 ^d ±0.352
T21^	5.86 ^{ef} ±0.224	5.94 ^f ±0.087	6.80 ^g ±0.552	5.62 ^e ±0.417
T10*	6.53 ^g ±0.372	6.10 ^g ±0.378	6.13 ^f ±0.345	5.09 ^f ±0.024
T19^	5.81 ^{ef} ±0.369	5.82 ^f ±0.726	5.47 ^g ±0.375	N.D
T5*	6.52 ^g ±0.320	5.38 ^{ef} ±0.422	5.93 ^{fg} ±0.286	6.49 ^{de} ±0.476
T16^	7.03 ^h ±0.131	5.59 ^{ef} ±0.383	6.86 ^g ±0.471	N.D
T13*	4.45 ^h ±0.638	6.44 ^g ±0.895	4.81 ^h ±0.047	4.53 ^g ±0.681
T22^	N.D	N.D	5.02 ^h ±0.188	N.D

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05), N.D :Not determined.

*: According to Table 3.1. ^: According to Table 3.2.

A.10 The *E.coli* counts control and treatment groups for shelf life (log cfu/mL).

Groups	0 th day	20 th day	40 th day	60 th day
	<i>E.coli</i>	<i>E.coli</i>	<i>E.coli</i>	<i>E.coli</i>
Control	5.67 ^a ±0.084	5.28 ^b ±0.325	6.54 ^a ±0.388	6.99 ^b ±0.228
10 min. 90 dB	4.76 ^b ±0.270	5.70 ^{ab} ±0.835	N.D	N.D
T9*	4.23 ^b ±0.096	4.97 ^a ±0.032	N.D	6.49 ^a ±0.211
T18^	N.D	5.40 ^b ±0.276	6.24 ^b ±0.234	6.59 ^a ±0.357
T4*	4.56 ^{ab} ±0.513	N.D	N.D	N.D
T15^	5.48 ^{ab} ±0.764	N.D	5.35 ^c ±0.165	N.D
T8*	5.15 ^c ±0.167	5.88 ^d ±0.170	6.80 ^{ab} ±0.511	6.72 ^{ab} ±0.446
T17^	N.D	5.67 ^d ±0.508	N.D	N.D
T3*	5.49 ^{ab} ±0.335	N.D	N.D	N.D
T14^	5.71 ^c ±0.315	N.D	5.95 ^d ±0.205	5.60 ^c ±0.315
T11*	N.D	N.D	N.D	N.D
T20^	N.D	N.D	N.D	N.D
T12*	N.D	N.D	6.28 ^b ±0.272	6.39 ^d ±0.264
T21^	5.35 ^{cd} ±0.390	N.D	6.08 ^{bc} ±0.154	N.D
T10*	6.20 ^d ±0.223	4.73 ^{cd} ±0.369	5.66 ^d ±0.219	4.53 ^c ±0.088
T19^	4.45 ^e ±0.212	6.16 ^e ±0.320	4.53 ^c ±0.681	N.D
T5*	5.61 ^c ±0.310	6.23 ^e ±0.433	5.99 ^f ±0.185	6.11 ^{cd} ±0.362
T16^	6.26 ^f ±0.512	4.87 ^f ±0.562	6.56 ^g ±0.478	N.D
T13*	N.D	N.D	N.D	N.D
T22^	N.D	N.D	N.D	N.D

Data are means ± SD from two replicates

Values followed by different letters in the same column are significantly different (p<0.05), N.D:Not determined.

*: According to Table 3.1. ^: According to Table 3.2.

A.11 The LAB counts control and treatment groups for shelf life (log cfu/mL)

Groups	0 th day	20 th day	40 th day	60 th day
	LAB	LAB	LAB	LAB
Control	N.D	N.D	N.D	N.D
10 min. 90 dB	N.D	N.D	N.D	N.D
T9*	5.62 ^a ±0.012	5.15 ^b ±0.275	5.84 ^a ±0.610	N.D
T18^	6.09 ^b ±0.165	N.D	5.88 ^a ±0.332	5.53 ^a ±0.465
T4*	5.98 ^b ±0.645	7.08 ^a ±0.458	N.D	N.D
T15^	7.06 ^d ±0.091	6.98 ^a ±0.318	7.28 ^b ±0.110	6.39 ^b ±0.310
T8*	6.37 ^{bc} ±0.507	6.31 ^c ±0.261	6.98 ^c ±0.042	6.59 ^c ±0.436
T17^	6.56 ^c ±0.505	N.D	N.D	N.D
T3*	6.39 ^{bc} ±0.507	6.80 ^{ab} ±0.369	N.D	7.17 ^d ±0.813
T14^	7.19 ^e ±0.475	7.25 ^d ±0.144	6.75 ^d ±0.301	7.05 ^{cd} ±0.379
T11*	7.21 ^f ±0.531	N.D	N.D	N.D
T20^	N.D	N.D	N.D	N.D
T12*	6.11 ^g ±0.507	N.D	N.D	6.10 ^e ±0.459
T21^	6.97 ^{fg} ±0.422	N.D	4.69 ^e ±0.065	N.D
T10*	6.19 ^g ±0.019	N.D	N.D	N.D
T19^	5.42 ^h ±0.106	5.72 ^e ±0.094	5.75 ^f ±0.236	5.26 ^f ±0.016
T5*	5.29 ^h ±0.216	6.21 ^f ±0.346	6.16 ^g ±0.234	6.08 ^g ±0.642
T16^	5.66 ^h ±0.552	5.56 ^e ±0.756	5.89 ^f ±0.216	N.D
T13*	6.03 ^{hi} ±0.482	N.D	N.D	N.D
T22^	6.87 ⁱ ±0.428	N.D	N.D	5.31 ^{fg} ±0.337

Data are means ± SD from two replicates

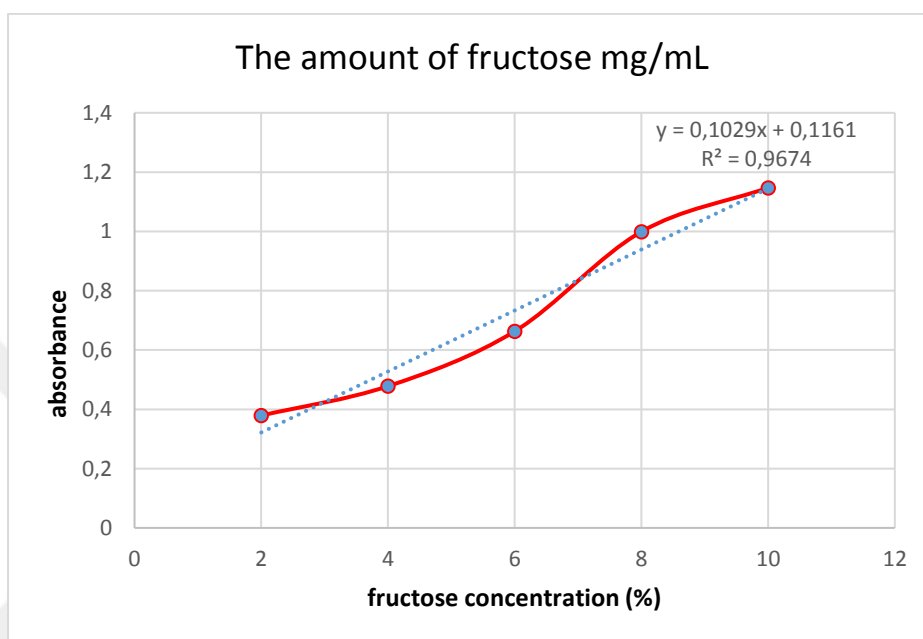
Values followed by different letters in the same column are significantly different (p<0.05), N.D:Not determined.

*: According to Table 3.1. ^: According to Table 3.2.

A.12 The standards of fructose (mg)

Std - 2	Std - 4	Std - 6	Std - 8	Std - 10
0.379	0.478	0.663	0.999	1.147

A.13 Standard curve of fructose (mg/mL)



Graph equation: $y=0.1029x + 0.1161$

8. CURRICULUM VITAE

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-Seyhun Yurdugül, Elif Tecen, Elif Yaşar, Merve Yavuz, Freeze-drying of persimmon fruit to extend the shelf life of wheat grass juice inoculated with probiotics, The 8th Asia-Pacific Drying Conference ADC 2015, Kuala Lumpur, Malaysia (in abstract book).

Hobbies (Optional) : playing table tennis, playing classical guitar, history, literature, cinema, gastronomy.