ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

CERTAIN CHEMICAL, PHYSICAL AND MICROBIOLOGICAL CRITERIA OF ULTRASONICATED AND PROBIOTIC ADDED WHEATGRASS JUICE

MASTER OF SCIENCE

ELİF YAŞAR

BOLU, SEPTEMBER 2016

ABANT IZZET BAYSAL UNIVERSITY

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APPROVAL OF THE THESIS

CERTAIN CHEMICAL, PHYSICAL AND MICROBIOLOGICAL CRITERIA OF ULTRASONICATED AND PROBIOTIC ADDED WHEATGRASS JUICE submitted by **ELİF YAŞAR** in partial fulfillment of the requirements for the degree of Master of Science in **Department of Biology, Abant Izzet Baysal University** by,

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To my family

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Elif YAŞAR

ABSTRACT

CERTAIN CHEMICAL, PHYSICAL AND MICROBIOLOGICAL CRITERIA OF ULTRASONICATED AND PROBIOTIC ADDED WHEATGRASS JUICE
MSC THESIS
ELİF YAŞAR
ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. SEYHUN YURDUGÜL

BOLU, SEPTEMBER 2016

Wheatgrass (*Triticum aestivum*) is an important source of energy and micronutrients in the developing world. However, wheatgrass juice can have a variety of microorganisms and can cause important problems in human beings. Pathogens such as *Salmonella* spp., *E. coli* which may be encountered in wheatgrass juice due to the soil contact of wheatgrass before harvesting induce food poisoning owing to the consumption. Nowadays, in order to combat these infections effectively, non-thermal technologies have gained great importance in food industry. Ultrasonication is an alternative way for these technologies without destruction or partial loss of health promoting substances present in wheatgrass juice such as antioxidants etc. In this study, sole ultrasonication for inactivating these pathogenic species, sole probiotics (*Lactobacillus acidophilus* and *Lactobacillus reuteri*) for promoting the wheatgrass juice for being an extraordinary functional food was used. The combination of probiotics and ultrasonication was also studied. The combination of 90 dB amplitude at 24 kHz frequency of ultrasound and 1 mL *L.acidophilus* resulted the most effective inhibition to total mesophilic aerobic bacteria and pathogens mentioned above, as per to the control, without disrupting the main chemical and physical properties of the wheatgrass juices.

KEYWORDS: Wheatgrass juice, *Salmonella* spp., Ultrasonication, Probiotics, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, Foodborne pathogens

ÖZET

ULTRASONİKASYON UYGULANMIŞ VE PROBİYOTİK EKLENMİŞ BUĞDAY ÇİMİ SUYUNUN BELİRLİ KİMYASAL, FİZİKSEL VE MİKROBİYOLOJİK ÖLÇÜTLERİ
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Buğday çimi (*Triticum aestivum*) gelişmekte olan ülkelerde önemli bir enerji ve mikro besin kaynağıdır. Ancak, buğday çimi suyu çeşitli mikroorganizmalara sahip olabilir ve insanlarda önemli problemlere sebep olabilir. Buğday çimini biçmeden önce toprakla temasına bağlı olarak buğday çimi suyunda karşılaşılabilecek olan Salmonella spp. gibi patojenler çim suyunun tüketilmesiyle gıda zehirlenmesine neden olurlar. Şimdilerde bu enfeksiyonlarla etkin mücadele edebilmek için ısıl olmayan teknolojiler gıda endüstrisinde büyük önem kazanmıştır. Ultrasonikasyon buğday çimi suyunda bulunan antioksidan gibi sağlığa yararlı maddelerin yıkımına veya kısmi kaybına sebep olmamasıyla birlikte bu teknolojilere alternatif bir yoldur. Bu çalışmada, patojenik türleri inaktive edebilmek için yalnızca ultrasonikasyon, buğday çimi suyunu özel fonksiyonel gıda olarak geliştirebilmek için yalnızca probiyotikler (*Lactobacillus acidophilus* ve *Lactobacillus reuteri*) kullanılmıştır. Ayrıca probiyotik ve ultrasonikasyon bileşimi de incelenmiştir. Ultrasonikasyonun 24 kHz frekanstaki 90 dB'lik genliği ve probiyotiğin (*Lactobacillus acidophilus*) 1 mL dozunun bileşiminin, kontrole göre fiziksel ve kimyasal özelliklerini bozmadan toplam mezofilik aerobik bakteriler ve yukarıda sözü geçen patojenler üzerinde en etkili inhibisyona yol açtığı bulunmuştur.

ANAHTAR KELİMELER: Buğday çimi suyu, *Salmonella* spp., Ultrasonikasyon, Probiyotikler, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, Gıda kaynaklı patojenler

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LIST OF ABBREVIATIONS AND SYMBOLS

^{0}C : Centigrade Degree

B. longum : *Bifidobacterium longum*

cm : Centimeter

$CuSO_4.5H_2O$: Cupric Sulphate

dB : Decibel

DNA : Deoxyribonucleic Acid

DNS : Dinitrosalicyclic Acid

E. coli : *Escherichia coli*

EMB agar : Eosin Methylene Blue Agar

FAC : 5-Fluorouracil, Doxorubicin and Cyclophospamide

FAO : Food and Agriculture Organization

FD : Factor Dilution

g : Gram

GCSF : Granulocyte Colony Stimulating Factors

GIT : Gastro Intestinal Tract

GRAS : Generally Recognized As Safe

h : hour

H_2O_2 : Hydrogen Peroxide

HHP : High Hydrostatic Pressure

HPP : High Pressure Processing

kGy : Kilogray

kHz : Kilohertz

KI : Potassium Iodide

kPa : Kilo Pascal

L. acidophilus : *Lactobacillus acidophilus*

L. Helveticus : *Lactobacillus helveticus*

L. johnsonii : *Lactobacillus johnsonii*

L. plantarum : *Lactobacillus plantarum*

L. reuteri : *Lactobacillus reuteri*

LAB : Lactic Acid Bacteria

Log : Logarithm

m : Mass

meq. wt. : Weight of the Standard

mg : Milligram

MHz : Megahertz

min. : Minute

mL : Milliliter

MPa : Mega Pascal

mPa.s : Mini Pascal Second

MRS agar : De Man, Rogosa and Sharpe Agar

MS : Manosonication

MTS : Manothermosonication

N : Normality

NaOH : Sodium Hydroxide

NH_3 : Ammonia

nm : Nanometer

O.D. : Optical Density

ORAC : Oxygen Radical Absorbance Capacity

PCA : Plate Count Agar

PEF : Pulsed Electric Field

PME : Pectin methylesterase

SOD : Superoxide Dismutase

spp. : Species

Std. : Standard

T. aestivum : *Triticum aestivum*

TA : Titratable Acidity

TMAB : Total Mesophilic Aerobic Bacteria

TNF : Tumour Necrosis Factor

UC : Ulcerative Colitis

UHP : Ultra High Pressure

UHT : Ultra High Temperature

USFDA : United States Food and Drug Administration

UV : Ultraviolet

V : Volume

W **:** Watts

WGJ **:** Wheatgrass Juice

WHO **:** World Health Organization

XLD agar : Xylose Lysine Deoxycholate Agar

YEA **:** Yeast Extract Agar

YM **:** Yeasts and Molds

µs **:** Microsecond

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1. INTRODUCTION

1.1 Food Preservation Methods

In the food industry, eliminating harmful microorganisms, and inactivating enzymes are important for food quality and also for public health. That is the reason why heat treatment is the most utilized method for stabilizing foods because of its capacity to destroy microorganisms and also to inactivate enzymes. However, since heat can alter the organoleptic properties of foods and diminish the contents or bioavailability of some nutrients, there is a growing interest in searching for methods that are able to reduce the intensity of the heat treatments needed for food preservation (Lopez et al., 1994).

In the last 20 years, consumer demand for high-quality foods that are microbiologically safe and stable has awakened a growing interest in nonthermal preservation techniques capable of inactivating microorganisms and enzymes (Mertens and Knorr, 1992; Barbosa-Canovas et al., 1998).

In order to reduce the detrimental effects of heat treatment, heat is combined with other physical and chemical agents to increase the lethal action; in addition, non-thermal alternatives are being tried. Some of the common non-thermal alternatives to conventional thermal processing of foods include; pulsed electric field inactivation (PEF), ionizing irradiation, high hydrostatic pressure (HHP) and ultrasonication (Crosby, 1982; Lopez et al., 1994; McClements, 1995).

To extend the use of nonthermal processing in the food industry, combinations of these technologies with traditional food preservation techniques are being studied. This is known as “hurdle technology”. It has already been applied successfully using traditional techniques of food preservation (Leistner and Gorris, 1995). Food preservation using combined methods involves successive or simultaneous applications of various individual treatments. Combined treatments are advantageous, principally because many individual treatments alone are not adequate

to ensure food safety or stability. In some circumstances, combined treatments allow a milder use of single treatments, with consequent improvement in food quality. The total preservation effect of combining several preservation techniques can be merely additive, but in terms of food quality and safety a synergistic effect is preferable.

1.2 The Juice Processing and Novel Methods Applicable To Juices

Fruits are highly perishable and have to be processed into juices to ensure year-round continuous supply. The highest juice quality is required to meet consumer needs and juice safety aspects are important considerations for prolonging shelf life. Fruit juices are one of the food products which are thermally sensitive and susceptible to chemical, physical and microbiological changes. In recent times, the demand from the consumer for fresh-like, minimally processed food products has been rapidly increasing. Currently, heat processing is the most commonly used hurdle for inactivating microorganisms and enzymes, thereby extending product shelf life. However, this process may have adverse effects on sensory and nutritional qualities of foods (Braddock, 1999). It may cause undesirable changes in various properties of fruit juices including physical, chemical, biological and organoleptic such as nutrients, colour and flavour (Bhattacherjee et al., 2011; Piasek et al., 2011; Giner et al., 2013; Mena et al., 2013). This process may also result in some nutritional compounds loss such as carotenoids (Fratianni et al., 2010) and flavonoids (Igual et al., 2011).

The pasteurization and UHT sterilization are common techniques to extend the shelf-life and raise the consumer safety while maintaining the fresh quality of juice (Puphaung et al., 2010; Jittanit et al., 2011).

High intensity pulsed electric fields (PEF) is one of the most innovative technologies for processing thermosensitive products (Sitzmann, 1994; Qin et al., 1995; Pothakamury et al., 1996; Vega-Mercado, 1997). It is based on the delivery of pulses at high electric field intensity (5-55 kV/cm) for a few milliseconds (Jeantet et al., 1999). In several recent studies, apple juice has been used as a medium to evaluate the microbial inactivation following the application of PEF at various treatment times and field strengths (Evrendilek, Zhang and Richter, 1999; Evrendilek

et al., 2000; Lanoiselle, Nonus, Vorobiev and Arrayan, 2003; Liang, Cheng and Mittal, 2006). The microbial population in apple cider has been shown to decrease in response to increased PEF total treatment time and field strength. A 4.5 log cycle reduction of *Escherichia coli* was reported when processing reconstituted apple juice in a pilot scale PEF system at 34 kV/cm for a total treatment time of 166 μs (Evrendilek et al., 2000).

Gamma radiations and electron beams generate doses of 2-10 kGy. These technologies are commonly referred to as ionizing radiations (Farkas, 1998). Since radioactive isotopes are not used, electron-beam technology is currently developed as a safer alternative to gamma radiation. Ultraviolet (UV) energy is a non-ionizing radiation with germicidal properties at wavelengths in the range of 200-280 nm (Kuo et al., 1997; Bintsis et al., 2000).

The technology of high hydrostatic pressure (HHP), also referred to as high pressure processing (HPP) or ultra high pressure (UHP) has been known to be a potential preservation technique for more than a century (Hite, 1899). HPP subjects solid and liquid foods, with or without packaging, to pressures between 100 and 800 MPa. Process temperature during pressure treatment can be from below 0 ^{0}C to above 100 ^{0}C. Exposure times can range from a few seconds to over 20 min. Commercial production of pressurised foods has been reported for fruit jams, jellies, sauces, juices, rice wine, cake, avocado pulp, guacamole and cooked ham.

1.2.1 Ultrasonication

Ultrasound, in its most basic definition, refers to pressure waves with a frequency of 20 kHz or more (Brondum et al., 1998; Butz and Tauscher, 2002). Ultrasonication is mostly preferred as combination with the application of an external hydrostatic pressure of up to 600 kPa [manosonication (MS)] to increase substantially the lethality of the treatment. In addition, a combination of MS with temperature [manothermosonication (MTS)] is another treatment (Raso et al. 1998).

Generally, ultrasound equipment uses frequencies from 20 kHz to 10 MHz. High energy ultrasound, which is also known as power ultrasound has intensities

higher than 1 W/cm^{-2} and frequency range of 20 to 100 kHz. It has the ability to cause cavitation, which has uses in food processing to inactivate microorganisms. The power ultrasound is useful in invasive applications, which gives impact to physical, chemical and biological properties of foods in processing, e.g., to generate emulsions, disrupt cells, promote chemical reactions, inhibit enzymes, tenderize meat, and modify crystallization processes. It is also used for preservation and safety such as milk homogenisation (Bosiljkov et al., 2009), juice yield enhancement (Lieu and Le, 2010) and microbial inactivation (Gao et al., 2014). The low energy ultrasound has intensities of less than 1 W/cm^{-2} and frequencies of more than 100 kHz. It can be used in nondestructive analytical measurements and monitoring of composition and physicochemical properties of food during processing and storage for quality control purposes such as those in detection of honey adulteration (Alissandrakis et al., 2010), particle sizing (Lefebvre et al., 2013) and emulsion stability (Kaci et al., 2014).

Ultrasound which is used in pasteurization continues to be of great interest to the dairy industry. Some researchers has proved that ultrasonic method is effective for the destruction of *E. coli*, *Pseudomonas fluorescens* and *Listeria monocytogenes* with no detrimental effect on the total protein or casein content of pasteurized milk (Cameron et al., 2009).

The inactivation of enzymes such as pectinmethylesterase, polyphenoloxidases and peroxidases responsible for deterioration of fruit and vegetable juice and various enzymes pertinent to milk quality are effective in ultrasonication (O'Donnell et al., 2010; Tiwari et al., 2009; Vercet et al., 2002; Terefe et al., 2009). Tomato pectic enzyme (Lopez, Vercet, Sanchez and Burgos, 1998; Vercet, Sánchez, Burgos, Montañés and Lopez Buesa, 2002), soybean lipoxygenase (Lopez and Burgos, 1995a), horseradish peroxidase (Lopez and Burgos, 1995b) and orange PME (Vercet, Lopez and Burgos, 1999) are the other enzymes which is inactivated by using ultrasonic methods.

Many researchers have studied to understand the mechanism played by ultrasound on the disruption of microorganisms (Alliger, 1975; Baumann, Martin and Feng, 2005; Bermudez-Aguirre, Corradini, Mawson and Barbosa-Canovas, 2009; Earnshaw, Appleyard and Hurst, 1995; Garcia, Burgos, Sanz and Ordonez, 1989;

Guerrero, López-Malo and Alzamora, 2001; Guerrero, Tognon and Alzamora, 2005; Hughes and Nyborg, 1962; Lo'pez-Malo, Guerrero and Alzamora, 1999; Raso, Palop, Pagan and Condon, 1998; Wrigley and Llorca, 1992). It has been explained by acoustic cavitation and its physical, mechanical and chemical effects that inactivate bacteria and deagglomerate bacterial clusters or flocs (Joyce, Phull, Lorimer and Mason, 2003). It has also been shown that ultrasound frequency, wave amplitude and volume of bacterial suspension effect the mortality rate (Raso, Palop, Pagan and Condon, 1998). While a frequency of about 20 kHz is usually applied for microbial inactivation; the resistance to ultrasound treatment of spores, and Gram-positive and coccal cells are higher than vegetative, Gram-negative and rod-shaped bacteria (Feng, Yang and Hielscher, 2008). In addition, it also varies among different strains. For example, *Escherichia coli* and *Saccharomyces cerevisiae* were reduced by more than 99% after ultrasonication, whereas *Lactobacillus acidophilus* was reduced by 72% and 84% depending on the media used (Cameron, McMaster and Britz, 2008).

Some researchers investigated three treatments to evaluate the effect of ultrasound on the fermentation. They concluded that treatment with ultrasonic wave before inoculation resulted in an increase in water holding capacity and decrease in syneresis, treatment after inoculation resulted in a decreased fermentation time by 0.5 h and increased water holding capacity but arguably no beneficial effect on syneresis (Wu et al., 2001).

Ultrasound can improve the action of *Lactobacilli* in the rate of nearly 50% and also induced a sweetening effect in yogurt without increasing the caloric content (Toba, 1990).

The usage of ultrasound is recognised as an alternative technology to the conventional fruit juice processing technologies. Ultrasound is a potential technology for the requirements of USFDA. The US Food and Drug Administration requires fruit juices to meet a minimum of 5-log reduction in pertinent microorganisms to control the spread of food-borne illnesses (USFDA, 2001; Lee et al., 2013). To achieve the USFDA requirement, food-borne pathogens inactivation with ultrasound undergoes long processing time leading to high production cost and small throughput. Therefore, the combination of low frequency ultrasound with mild heat can help in reducing processing time and temperature by 16 and 55%, respectively,

minimizing the negative effects on fruit juice quality and makes the processing more economically feasible (Koshani et al., 2014).

1.3 Probiotics

Food and Agriculture Organization/World Health Organization (FAO/WHO) define probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002).

The heterogeneous group of lactic acid bacteria (*Lactobacillus*, *Lactococcus* etc.) and the genus *Bifidobacterium* are the genera which most probiotics belong (FAO/WHO, 2001; Holzapfel, Haberer, Geisen, Bjorkroth and Schillinger, 2001). It has been found that there are several mechanisms by which probiotics mediate their health benefits on the host, and can be categorized into three groups; (i) certain probiotics which have antimicrobial activity, can ban or inhibit pathogens; (ii) probiotic bacteria are believed to strengthen the intestinal epithelial barrier; and (iii) probiotic bacteria can modulate the host immune response (Ezendam and Loveren, 2006; Lebeer et al., 2008; Lebeer et al. 2010; Marco et al., 2006). The strains of *Streptococcus*, *Escherichia coli*, *Bacillus* and *Saccharomyces* are the other less commonly used probiotic micro-organisms.

Probiotic bacteria have many nutritional and therapeutic benefits, including lactose metabolism, control of gastrointestinal infection, cancer suppression, reduction of serum cholesterol, immune stimulation, and increased digestibility of foods (Gilliland, 1990; Collins et al., 1998; Saliminen et al., 1998). The therapeutic potential of probiotics depends on a number of factors, but most important are their survival during manufacture, storage and transit through the gastrointestinal tract, and the ability to proliferate in the large intestine (Tannock, 1998).

While there are many applications of probiotics, new innovative applications are also emerging. For example, some evidence suggests that the recovery of skin immune functions can be accelerated by orally consuming *L. johnsonii* La1 following UV exposure (Peguet-Navarro et al., 2008). It is also possible to see that *L. reuteri* DSM17938 can reduce crying time in colicky babies by improving gastric

emptying (Savino et al., 2010, 2007). Interestingly, it has even been found that a combination of *L. helveticus* R0052 and *B. longum* R0175 may reduce anxiety-related symptoms (Messaoudi et al., 2011) through a mechanism which may involve stimulation of the parasympathetic nervous system (Bravo et al., 2011). At last, studies are showing a positive effect of probiotics on body weight in healthy overweight adults (Kadooka et al., 2010) and cholesterol levels in hypercholesterolemic adults (Jones et al., 2011).

Recent data show that in addition to perform dietary function, probiotic strains also act as biocatalysts, by improving the properties of raw materials by fermentation or by performing specific biotransformations in the food production chain or during the passage through the GIT.

For instance, some probiotic bacteria produce esterases and have been used to transform methyl ferulate, ferulic acid and cinnamic acids (Lai et al., 2009; Szwajgier and Dmowska, 2010; Szwajgier and Jackubczyk, 2010). Some *Bifidobacteria* strains can be used to reduce the phytate content of whole wheat dough (Palacios et al., 2008), thus improving the bioavailability of iron from whole-grain diet.

1.3.1 *Lactobacillus acidophilus*

Lactobacillus acidophilus which is a nonpathogenic Gram-positive rod-shaped bacterium is a lactic acid bacteria (LAB) represented by many bacteriocin producing strains. It is a member of the normal intestinal microflora and it has health-promoting effects and antagonistic activity against food-borne disease agents (Gilliand and Speck, 1977).

Lactobacillus acidophilus which is a well-known probiotic strain, commonly used in the dairy industry. It is widely used for the production of fermented dairy products in the world. It has been suggested that ingestion of *L. acidophilus* exerts an immunomodulatory effect in the gastrointestinal system of both humans and animals (Tejada-Simon et al., 1999; Wheeler et al., 1997; Ishida et al., 2005; Gill et al., 2000).

The majority of bacteriocins produced by the *L. acidophilus* group of LAB are heat stable, low molecular mass, non-lantibiotic peptides which belong to class II (Zamfir et al., 1999).

1.3.2 *Lactobacillus reuteri*

Lactobacillus reuteri a Gram-positive heterofermentative lactic acid bacterium, is found in the gastrointestinal tracts of humans and other animals (Reuter, 2001; Roos and Jonsson, 2002). It has the ability to decrease intestinal pH to a level which is unfavorable to most pathogenic bacteria, so it enhances the antimicrobial effect. *L. reuteri* demonstrate probiotic properties such as the lowering of blood cholesterol levels (Taranto et al., 2000), and show a direct anti-inflammatory activity (Ma et al., 2004; Madsen et al., 1999; Rosenfeldt et al., 2003).

It has been reported that during growth on glucose and in the presence of glycerol, some strains of *L. reuteri* produce and excrete reuterin (Talarico et al., 1988) and reutericyclin (Ganzle et al., 2000), which are water soluble, effective over a wide pH range, resistant to proteolytic and lipolytic enzymes, the broad-spectrum antimicrobial compound (el-Ziney and Debevere, 1998). It has been also shown that *L. reuteri* use glycerol as an external hydrogen acceptor source during fermentation (Peng et al., 2002).

Taranto et al. (2000) demonstrated that *L. reuteri* which possesses a GRAS (generally recognized as safe) status can synthesize vitamin B_{12}. Other studies have also showed that corrinoid synthesized by *L. reuteri* has the ability to prevent the disease caused by vitamin B_{12} deficiency in both pregnant mice and the weaned young (Molina et al., 2009; Molina et al., 2008; Watanabe et al., 2014).

1.4 Wheatgrass

Shoot of *Triticum aestivum* Linn. is called as wheatgrass. It belongs to Gramineae family. Triticum which yields various types of wheat is a genus of annual and biennial grasses. It is widely grown throughout temperate regions of North

America and Europe. *T. aestivum* Linn. common or bread wheat, is widely cultivated almost all over the world. As a good source of mineral nutrients, wheatgrass contains significant amount of iron, phosphorus, magnesium, manganese, copper and zinc. Wheatgrass is a rich source of tocopherols with high vitamin E potency and activates metabolism, restores alkalinity to the blood, moreover its abundance of alkaline minerals helps reduce over acidity in the blood. As a de-toxificant, wheatgrass also helps restore healthy cells (Fahey et al., 2005). In 1930s wheatgrass was brought by Charles F Schnabel for experimental trials (Murphy, 2002). As it is a non-toxic herb, it avoids Food and Drug Administration screening and allowance. Wheatgrass has been used as an herbal medicine in a number of serious diseases like thalassemia and myelodysplastic syndrome since ages (Marawaha, 2004; Mukhopadhyay, 2009). In addition, it has also been believed that it can strengthen the immune system and as it regress the spread of cancer cells, it can increase the life span of cancer patients (Moller et al., 1999).

Triticum aestivum (Wheatgrass) has some chemical constituents which make it valuable in additioning the health and vitality. The major chemical constituents present in are vitamins and minerals, enzymes and other special components (Kulkarni et al., 2006; Swati et al., 2010; Bar-sella et al., 1998). Vitamins A, B1, B2, B3, B5, B6, B8, and B12; C, E, and K, ascorbic acid, dehydrated ascorbic acid, carotene, sulfur, sodium, aluminium, copper, calcium, iodine, phosphorus, magnesium, alkaline earth metal, potassium, selenium, iron, zinc, boron and molybdenum are present in wheatgrass. It contains protease, amylase, lipase, cytochrome oxidase, transhydrogenase, superoxide dismutase (SOD) enzymes. Amino acids such as aspartic acid, threonine, asparagine, glutamine, proline, glycine, arginine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, tryptophan and serine, P4D1 (gluco-protein), muco-polysaccharides, and chlorophyll, bioflavonoids like apigenin, quercetin and luteonin, indole compounds, choline and laetrile (amygdalin) are the other special components that are present in wheatgrass.

1.5 Wheatgrass Juice

Wheatgrass juice (WGJ) is an extract which is squeezed from the mature sprouts of wheat seeds (*T. aestivum*). Dr. Ann Wigmore developed and popularized the use of WGJ for therapeutic purposes, as part of herb therapeutic nutritional approach (Wigmore, 1986). The nutritional content, including chlorophyll, vitamins (A, C, and E), bioflavonoids, iron, minerals (calcium and magnesium) and 17 amino acid, eight of which are essential have attributed to WGJ therapeutic qualities (Walters, 1992). Although WGJ have been recommended for four decades as a treatment for various diseases, yet very little clinical data exists to support its use. Nowadays comprehensive studies are carried on by scientists and clinicians in order to develop herbal medicines/dietary supplements as alternative or complimentary therapy with almost no side effects or adverse reactions for the treatment of chronic diseases. Towards this aim, few studies have been carried on to evaluate the effectiveness of wheatgrass (in form of powder or juice) in the treatment of chronic diseases like cancer (Dey et al., 2006), rheumatoid arthritis (Nenonen et al., 1998), ulcer (Ben-Arye et al., 2002) etc**.** Kulkarni et al. (2006) also reported that as WGJ contains such antioxidants as phenolic compounds and several flavonoids, it has high antioxidant activity. Phenolics and flavonoids have been shown to remove superoxide radicals in vivo so decrease the cell damage caused by oxidative stress (Cao et al., 1996). Wheatgrass extracts also has superoxide scavenging and ferric reducing abilities (Peryt et al., 1992; Kulkarni et al., 2006). Their ability to inhibit oxidative DNA damage was also confirmed (Falcioni et al., 2002).

1.5.1 Pharmacological Activities of Wheatgrass Juice

1.5.1.1 Anticancer Activity

The beneficial effect of wheatgrass juice (WGJ) was found during the control study on patients with breast carcinoma on chemotherapy. It was revealed that WGJ taken during FAC (5–fluorouracil, doxorubicin and cyclophospamide) chemotherapy may reduce myelo-toxicity, dose reduction and need for granulocyte colony

stimulating factors (GCSF) support without abating effectiveness of chemotherapy (Bar-Sela et al., 2007). Another study showed that wheatgrass juice contributed to improve the health status and lifespan of cancer patients (Dey et al., 2006). The studies showed that the extract of wheatgrass when applied to known chemical mutagens, decreased their cancer causing ability by up to 99 percent (Lai et al., 1978; Lai, 1979). It has also been found that the beneficial effect of wheatgrass might be due to antioxidant activity which prevents oxidative damage to deoxyribonucleic acid (DNA) and lipid peroxidation, stimulation of gap junction communication, effect on cell transformation and differentiation, inhibition of cell proliferation and oncogene expression, effects on immune function and inhibition of endogenous formation of carcinogens (Wheat et al., 2008; Mates et al., 2000).

1.5.1.2 Antiulcer Activity

The use of wheatgrass (*Triticum aestivum*) juice was found as a very effective and safe treatment of active distal ulcerative colitis (UC) by Ben-Arye et al. (2002) through study on WGJ. Studies also show that WGJ is a possible therapy for ulcerative colitis due to bioflavonoid in it. Bioflavonoids have both anti-inflammatory and antioxidant properties. One of these bioflavonoid, api-genin, has been shown to inhibit tumour necrosis factor (TNF) induced transactivation (Shah, 2007; Singha et al., 2004; Ben-Arye et al., 2002). The studies have also shown that chlorophyll balm and aqueous solution is useful in the treatment of skin ulcer (Gahan et al., 1943). Further chlorophyll derivatives which have anti-inflammatory activity have been used to treat various kinds of skin lesions, burns and ulcers where it acts as a wound healing agent, stimulating granulation tissue and epithelization (Chernomorsky et al., 1988).

1.5.1.3 Antioxidant Activity

The antioxidant activity of wheatgrass, consumed as a dietary supplement, was estimated at different levels. In this study a period of 6, 7, 8, 10 and 15 days, aqueous and ethanol extracts of wheatgrass were used. Researchers determined lipid

per oxidation and oxygen radical absorbance capacity (ORAC) and checked the potency of a few selected extracts. Tap water, tap water with nutrients, soil and tap water, and soil with nutrients were used as different conditions for growth. For comparison, a commercially available wheatgrass tablet was analysed. The results showed that the ORAC values of aqueous and ethanol extracts of day 10 with condition of soil with nutrient were found to be 39.9 and 48.2, respectively, being higher than those reported for many natural extracts or vegetables (Kulkarni et al., 2006; Siener et al., 2006).

1.6 The Relationship Between Probiotics and Cereals

It has been suggested that beverages such as fruit and vegetable juices are the developing category of food matrices to serve as carriers of probiotic bacteria. Furthermore, Martins et al. (2013) have been suggested that cereals are also potential viable substrates as they contain nutrients easily assimilated by probiotics. It has been demonstrated that cereals can improve *Lactobacilli* tolerance to the harsh conditions of the gastrointestinal tract, and they can support the growth of single and mixed culture fermentations of probiotic microorganisms (Charalampopoulos and Pandiella, 2010, Charalampopoulos et al., 2003 and Herrera-Ponce et al., 2014). Additionally, some studies have been done to prove potential of cereal substrates to support the growth of probiotic microorganisms. These have been associated with the risk reduction of chronic diseases such as obesity, cardiovascular disease, diabetes, and some cancers (Wang, He and Chen, 2014). Thus, the use of cereal substrates that promote the gastrointestinal health and can prevent chronic diseases has a great potential to develop novel functional beverages. If the cereal-based fermented beverages fulfill probiotic requirements and have acceptable physicochemical characteristics and organoleptic properties with probiotic characteristics, cereals can be another developing category of food matrices. Besides fruit juices and vegetables, cereals constitute an emerging segment of nonconventional dairy substrates for the design of probiotic beverages that will require sensory and physicochemical characterization for quality control and further product development. Most of the current work in the area of development of non-dairy probiotics has been performed in these products where sensory, colour and rheological evaluations have been

carried out (Chattopadhyay et al., 2013, Fonteles et al., 2013, Pereira et al., 2013 and Perricone et al., 2014).

2. THE AIM AND SCOPE OF THE STUDY

The aim of the study is to examine the effects of ultrasonication, certain probiotics and their combination on certain chemical, physical and microbiological criteria of wheatgrass juice. To avoid destruction or partial loss of health promoting substances present in wheatgrass juice such as antioxidants etc., the effect of ultrasound will be investigated on certain chemical, physical and microbiological criteria of wheatgrass juice. The behaviour of probiotics will therefore promote the wheatgrass juice for being an extraordinary functional food by combining the free radical scavenging activity of probiotics with antioxidant properties of the wheatgrass juice. It is also expected to combat with the pathogens such as *Salmonella* spp., *E.coli* which may be encountered in wheatgrass juice due to the soil contact of wheatgrass before harvesting.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Wheatgrass

The wheatgrass seeds, purchased from a local vendor in İstanbul, TURKEY were awaited in a glass jar filled with tap water overnight at ambient temperature, and sown to pots containing three kilograms of soil enriched with fertilizer. Afterwards the grasses were kept for a period of ten days at room temperature and cut by scissors until reached up to 15 cm.

3.1.2 Wheatgrass Juice

The juice of wheatgrasses' was extracted by a juice extractor (Healthy Juicer, Lexen Inc., China).

3.1.3 Probiotics

The mixed-culture probiotics (*Lactobacillus acidophilus* Novaflor^{TM} and *Lactobacillus reuteri* BioGaia®) inoculated to the wheatgrass juice in the study were obtained from the Department of Food Engineering, Faculty of Engineering and Architecture at Abant İzzet Baysal University, in tablet form and kept in the refrigerator at +4° C before their activation in MRS broth (MERCK).

3.2 Method

3.2.1 The Treatment with Ultrasonication

10 mL of wheatgrass juices were ultrasonicated at a constant frequency of 24 kiloHertz (Hz), at the amplitudes of 30 and 90 dB for a period of 10 minutes (Hielscher Ultrasound Technology, UP 400S Ultrasonic processor, Germany). Temperature control during ultrasonication treatment was achieved by decreasing excess heat evolved, using ice bath placed to the periphery of the sample tubes. In each treatment the application temperature was detected. The detected temperatures were ranging between 15 to 24 ^{o}C through ulrasonication process.

3.2.2 The Treatment with The Probiotics

A tablet of *Lactobacillus acidophilus* and *Lactobacillus reuteri* was added into 100 mililiters of MRS broth (MERCK) and incubated at 37 ^{o}C for 48 hours in the incubator. Afterwards the growth period, 1.0 mL of prepared bacterial suspensions (approximately 10^{7} cfu/mL) were inoculated into 9.0 mL of each of the wheatgrass juices.

3.2.3 The Microbial Identification of Microflora and Microbial Counts

The microflora of wheatgrass juices were identified by the appropriate selective medium of bacteria, yeast and mold. The total mesophilic aerobic counts, *Salmonella* spp., *E. coli*, lactic acid bacteria and mold and yeast were detected by plate count (PCA) (Merck), XLD (Merck), EMB (Merck), MRS (Merck) and yeast extract agars (Merck), respectively.

The treated and untreated (control) juice samples were sequentially diluted with sterile distilled water to enumerate viable cells of the natural micropopulation. All bacterial enumerations were done in triplicates and the plates were incubated at 37^{o}C for 24-48 hours in the incubator. Yeast and filamentous fungi counts were

done by surface plating in triplicate plates on the yeast extract agar (YEA, Merck). The plates were incubated at 30 ^{o}C for two days. The microbial counts were calculated according to the following formula:

cfu/mL = (number of colonies x dilution factor) / aliquot plated (Mohideen, 2015).

Dilution Factor = 1 / dilution rate

The microbial counts were converted to logarithmic values.

3.2.4 Physical and Chemical Analysis

3.2.4.1 Total Soluble Solids (^{o}Brix)

The total soluble solid content of the wheatgrass juices were determined by using a portable refractometer (Miiler, RHB-10/ATC, Germany). The measurements were performed at 25 ± 0.5 °C and the results were expressed as ^{o}Brix. All measurements were performed in duplicates.

3.2.4.2 Titratable Acidity

In order to determine the titratable acidity (TA), samples of 5 mL from each of the wheatgrass juice and 15 mL distilled water were placed into a beaker. This solution was subsequently filtrated by filter paper. 5 mL of this filtrated solution was taken to another beaker. 1 g of phenolphthalein was dissolved with 100 mL of 95 % ethyl alcohol at an Erlenmayer flask and three drops of this phenolphthalein solution were added to filtrated solution in the beaker. Then this solution was titrated against standardised 0.1 N NaOH to the phenolphthalein end point (pH 8.2 ± 0.1) until the solution got pink. All measurements were performed in duplicates. The volume of NaOH was expressed as either citric acid or malic acid per 100 mL of juice and the total acidity was calculated according to the given formula:

TA%= (N × V / m) × 100 × FD × meq. wt.

According to this equation V is titrated volume of NaOH, m is mass of juice (g), meq. wt. is the weight of the standard, N is the normality of NaOH and FD symbolizes the factor of dilution.

3.2.4.3 pH

The pH of the wheatgrass juice samples was measured by using a digital pH meter (WTW Inolab pH 720, Germany). All measurements were performed in duplicates.

3.2.4.4 Viscosity

The viscosity of the samples was measured by a viscometer (AND Vibro Viscometer, SV-10, Japan) and all measurements were performed in duplicates.

3.2.4.5 Determination of Total Sugar Content

The total sugar content (in fructose) of the wheatgrass juice samples was determined by using dinitrosalicyclic acid (DNS) method (Miller, 1959). Firstly, DNS solution and 40% sodium potassium tartarate 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. Consequently 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). All measurements were performed in duplicates.

3.2.4.5.1 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 tartarate were dissolved in 1416 g of distilled water.

3.2.4.6 The Color Determination

The color of 2 mL of each of the juice samples were determined by a colorimeter according to CIE $L^* A^* B^*$ system (Konica Minolta, CR-400, Japan). The color values were expressed as L^* (whiteness or brightness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness). All measurements were performed in duplicates.

3.2.4.7 Determination of Protein Content

The total protein content of the wheatgrass juice samples was determined by Biuret method. First of all, Biuret reagent was prepared. In order to adjust spectrophometer to zero, solution containing 1 mL distilled water and 1,5 mL Biuret reagent was prepared. This solution was awaited for 10 minutes at 37 ºC. Both of the cuvettes of spectrophotometer were filled with this solution and the spectrophotometer was adjusted to zero at 540 nm. Afterwards, in order to plot standard calibration curve, stock solutions containing 1, 2, 4, 6, 8 % and 10 % Bovine serum albumin were prepared. 1 mL of each of these solutions were put into test tubes and 1,5 mL Biuret reagent was added to them. Then these samples were awaited for 10 minutes at 37 ºC. The absorbance was determined by using spectrophotometer at O.D.540 nm (Shimadzu, PharmaSpec UV-1700, UV-Visible Spectrophotometer, Japan).

3.2.4.7.1 The Preparation of Biuret Reagent

The Biuret reagent was prepared by adding 3 g of $CuSO_4.5H_2O$ and 9 g of sodium potassium tartrate to 500 mL of 0.2 N NaOH solution, followed by addition of 5 g of KI. The resulting solution was then brought to a total volume of 1 mL with distilled water.

3.3 The Statistical Evaluation

The data were shown as means and analyzed by the IBM SPSS Statistics 19 program. Anova Scheffe and Tukey test were used to detect the significance between treatment groups. Kruskal-Wallis test was used in the chemical analysis of wheatgrass juices to detect the significance between treatment groups.

The significant level was set to $p < 0.05$ at the beginning of the study.

4. RESULTS AND DISCUSSION

4.1 The Microbial Analysis of Wheatgrass Juices

The microflora of wheatgrass juices was found to be composed of mesophilic bacteria, gram negative bacilli, and certain yeast and mold species in plate count agar (PCA), in eosine methylene blue (EMB) agar and yeast extract agar (YEA) respectively. No *Salmonella* in XLD agar plates was observed throughout the study. All the other microbial counts were tabulated in Appendix A.5.

In the fresh juices sold without pasteurization, pathogenic microorganisms such as, *E. coli*, even at low doses were claimed to cause illness (D'Aoust et al., 2001, Meng et al., 2001, Swaminathan, 2001,Bell and Kyriakides, 2002a, Bell and Kyriakides, 2002b and Bell and Kyriakides, 2002c). Some LAB strains was reported to be used as probiotics which were able to prevent the growth of pathogenic bacteria, such as *Salmonella* spp. and *Escherichia coli* for human and animals (Chou and Weimer, 1999; Jin et al., 1996). In addition to these, application of sonication treatments has been recognized as a potential innovative technology that can meet FDA requirements for achieving 5-log reduction in the contaminant microorganisms (or pathogens) associated with fruit juices (Salleh-Mack and Robets, 2007). Ultrasound treatment has been reported to be effective in microbial inactivation in guava, orange and tomato juices (Valero et al., 2007; Adekunte et al., 2010; Cheng et al., 2007; Tiwari et al., 2010). Due to these findings, these microorganisms were targeted and methods were used throughout the study.

The results presented in Table 4.1 stated that when the counts of control and the ultrasound treated wheatgrass juice were compared, there was a significant reduction in the logarithmic counts of the total mesophilic bacteria on PCA agar for the treated samples. When the logarithmic yeast and mold counts were considered, it was less than the control in the treatment of 30 dB and 90 dB amplitude of 24 kHz frequency of ultrasonication. The combination of physical and chemical mechanisms which occur during cavitation could inactivate yeast. Oyane et al. (2009) reported

that free radicals and H_2O_2 which are formed during sonication, aids in microbial inactivation. This result indicates that ultrasonication is effective against not only mesophilic bacteria, but also yeast and molds ($p < 0.05$). When the logarithmic *E. coli* counts on EMB agar were considered, ultrasound treated samples contained less *E. coli* than control. These results were consistent with the studies conducted by Scherba et al. (1991) and Ince and Belen (2001) in terms of reduction of *E. coli.* Scherba et al. (1991) studied the effects of ultrasound on *E. coli* in an aqueous medium. In this study, significant reduction of the bacterial population was achieved (Scherba et al. 1991). Ince and Belen (2001) observed that sonication improved the inactivation of *E. coli.* Hua et al. (1995) reported the correlation of chemical reaction rates and ultrasonic intensity. *E. coli* inactivation most likely resulted from a combination of physical and chemical mechanisms which occur during acoustic cavitation, so it was expected that higher intensities would enhance inactivation rates.

The results showed that there was an inverse relationship between frequency of ultrasonication and the counts of yeast and molds, mesophilic aerobic bacteria and *E. coli*. When the frequency of ultrasonication was increased the counts of yeast-mold, total mesophilic bacteria and *E. coli* were decreased. Bactericidal effects on microorganisms were reported during the initial stage with the bactericidal and bacteriostatic effects gradually increasing with the frequency of ultrasonication. Therefore this method was found to be effective for yeast-mold and total mesophilic bacteria ($p < 0.05$). For both bacterial species, the counts showed a decrease at 24 kHz which was ascribed to ultrasonic inactivation of bacterial cells (Joyce et al., 2003). It has been reported that the mechanism of microbial inactivation is mainly due to thinning of cell membranes, localized heating and production of free radicals (Butz and Tauscher, 2002; Fellows, 2000).

No *Salmonella* spp. was detected in the control and in the treatment of ultrasonication with the following doses of 30 dB and 90 dB amplitudes at 24 kHz frequency. This was mainly due to the sterilization of the soil for a period of one day, prior to the experiment.

Table 4.1. The counts of TMAB, YM and *E. coli* in the ultrasonicated wheatgrass juice according to doses of ultrasonication

	TMAB	**YM**	***E. coli***
Groups	**Mean±Std.**	**Mean±Sd.**	**Mean±Sd.**
Control	6.10^{b}± .148	6.21^{a}±.048	5.18^{a}±.506
30 dB amplitude at 24 kHz	6.00^{b}± .483	5.51^{a}±.483	3.69^{a}±2.479
90 dB amplitude at 24 kHz	4.60^{a}±.209	4.66^{a}± .209	2.27^{a}±2.621

Data are means ± Std. from three replicates. Values followed by different letters in the same column are significantly different (P< 0.05).

The results tabulated in Table 4.2 showed the counts of total mesophilic bacteria, yeast and mold and *E. coli* in control, 1 mL *Lactobacillus acidophilus* added wheatgrass juice and 1 mL *Lactobacillus reuteri* added wheatgrass juice. All the other microbial counts were tabulated in Appendix A.6. When the control and 1 mL *L. acidophilus* added wheatgrass juice was compared, the counts of TMAB, yeast and molds and *E. coli* was decreased by the addition of *L. acidophilus.* This may be due to the possible antimicrobial effect of *L. acidophilus* as a probiotic. On the other hand, when the control and 1 mL *L. reuteri* added wheatgrass juice was compared, the counts of these bacteria were increased by the addition of *L. reuteri.* Interestingly, there was an unknown inverse effect of *L. acidophilus* and *L. reuteri* on wheatgrass juice. We could suppose that a different factor played the major role. This idea was confirmed by many authors. Vandenberh (1993) and Rolfe (2000) suggested that probiotic bacteria had produced a variety of substances with antibacterial properties including organic acids, hydrogen peroxide, bacteriocins etc.

that affect not only the bacterial viability but may also affect bacterial metabolism or toxin production. All samples containing different *Lactobacillus* species showed activity against all or most of the other bacteria. However, such activity could be disappeared when the pH of the sample was approximated to 7. No lactic acid bacteria was observed in the control. Moreover, the counts of lactic acid bacteria was increased due to the addition of probiotics. Wheatgrass juice (as a source of simple sugars, minerals and vitamins of the B-complex) has provided the enhancement of both growth (i.e. higher viable counts and shorter generation times) and *L. acidophilus* activity (i.e. improved sugar utilization and lower pH). In fact, it was observed that *L. reuteri* was grown better than *L. acidophilus* in MRS agar. This increment could be due to the wheatgrass, serving suitable substrates for the growth of lactic acid bacteria (LAB) (Salovaara, 1996). Therefore, the antibacterial activity of these probiotics were seemed to be a consequence of the activity of different factors with different potency. In addition to this, cereals can be used as sources of non-digestible carbohydrates, besides promoting several beneficial physiological effects, selectively stimulating the growth of *Lactobacilli* (Andersson et al., 2001).

Due to the low pH and high sugar and organic acid content of fruit juices, they are highly susceptible to attack of yeast and mold, lactic acid bacteria (LAB) and thermo-acidophilic and acid-tolerant bacteria (Parish and Higgins, 1988; Hendrix and Red, 1995; Deak and Beuchat, 1996; Matsubara et al., 2002; Keller and Miller, 2006; Walker and Phillips, 2008). However, in this study, no wheatgrass juice samples having low pH were observed. It can be the reason why the bacterial inactivation decrease gradually.

Table 4.2. The total mesophilic bacteria, yeast and mold and *E. coli* counts of control, 1 mL *L. acidophilus* added wheatgrass juice and 1 mL *L. reuteri* added wheatgrass juice

	TMAB	**YM**	***E. coli***
Groups	**Mean±Std.**	**Mean±Std.**	**Mean±Std.**
Control	$6.10^{a} \pm .147$	$5.99^{a} \pm .049$	$5.18^{a} \pm .504$
1 mL *L. acidophilus* (10^7)	$4.54^{b} \pm .621$	$3.06^{a} \pm 3.540$	$4.58^{a} \pm .393$
1 mL *L. reuteri* (10^7)	$6.30^{a} \pm .106$	$6.21^{a} \pm .137$	$7.07^{b} \pm .504$

Data are means ± SD from two replicates. Values followed by different letters in the same column are significantly different ($p<0.05$).

When the control and the combination of 1 mL *L. acidophilus* and the independent treatment of ultrasonication was considered, it was observed that the total mesophilic aerobic bacteria, yeast and *E. coli* counts of the combination were gradually decreased. No total mesophilic aerobic bacteria and *E. coli* were determined when compared to the control ($p < 0.05$). The results of combination treatments of 1 mL *L .acidophilus* and an amplitude of 90 dB ultrasonication at 24 kHz frequency, was found to be very effective on providing the microbial quality of the wheatgrass juice respectively (Figure 4.1 and Table 4.3). The lethal effect of ultrasound has been proposed to be due to either an increase in the heat sensitivity of the microorganisms (Ciccolini et al., 1997) or to extreme pressure variations caused by cavitation (Sala et al., 1995). On the other hand, the counts of lactic acid bacteria were increased due to the increasing frequency of ultrasonication.

Table 4.3. The combination study of the ultrasonicated (90 dB at 24 kHz) and 1 mL *L. acidophilus* added, and the combination study of the 1 mL *L. acidophilus* added and ultrasonicated (30 dB at 24kHz) wheatgrass juice

Groups	**TMAB**	**YM**	***E. coli***	**LAB**
	Mean ± Std.	**Mean ± Std.**	**Mean ± Std.**	**Mean ± Std.**
control	6.10^{a} ± .148	6.09^{a} ± .161	5.18^{a} ± .506	N.D
1 mL *L. acidophilus* (10^7) added wheatgrass juice	4.54^{ab} ± .623	4.57^{a} ± .431	4.58^{a} ± .392	5.06^{b}± .449
1 mL *L. acidophilus* (10^7) added + 30 dB ultrasonicated wheatgrass juice	3.49^{b} ± 2.341	3.07^{a} ± 3.540	2.55^{ab} ± 2.943	5.47^{b} ± .120
1 mL *L. acidophilus* (10^7) added + 90 dB ultrasonicated wheatgrass juice	N.D	2.27^{a} ± 2.621	N.D	6.24^{a} ± .064

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.

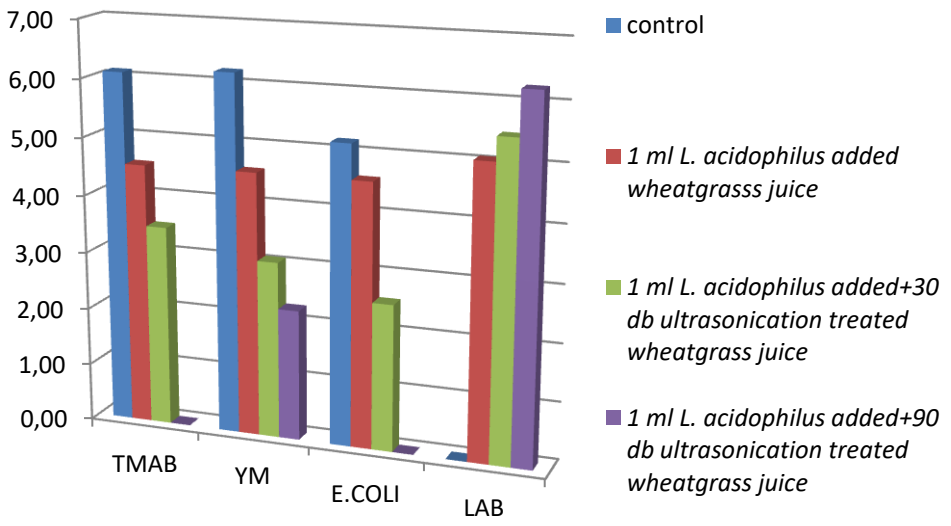

Figure 4.1. The total mesophilic aerobic bacteria, yeast-mold, *E. coli* and lactic acid bacteria (log cfu/mL) counts of *L. acidophilus* added, *L. acidophilus* and ultrasonication combination (30 dB - 1 mL), and combined *L. acidophilus* and ultrasonication (90 dB - 1 mL) treated wheatgrass juice

These results were consistent with the studies conducted by Zenker et al. (2003) in terms of reduction of microorganisms. Zenker et al. (2003) studied the effects of thermosonication, the simultaneous application of ultrasound combined with heat, (20 kHz, 48−60 °C) versus heat treatment alone (56−68 °C) on the inactivation of *Lactobacillus acidophilus* and *E. coli* in phosphate buffer (pH 7) in a batch-type reactor. The potential for selective inactivation of microorganisms by thermosonication offers promise for pasteurization of fermented foods (e.g. yogurt) whilst retaining the viability of probiotic cultures contained within the products (Knorr et al., 2004). A comparison of the improved effectiveness of thermosonication over heat treatment alone on inactivation of the microorganisms revealed that *L. acidophilus* was more resistant than *E. coli*. Additionally, Zenker et al. (2003) afford a continuous flow treatment system to show that *L. acidophilus* was more resistant than *E. coli* in several liquid foods, such as fruit juices and milk.

When the total mesophilic bacteria, yeast and mold, *E. coli* and lactic acid bacteria counts of the control and 1 mL *L. reuteri* added wheatgrass juice was considered, there was an increment of all bacteria. Due to these results, sole *L. reuteri* treatment was found to be ineffective on the counts of bacteria. When the combination treatment of 1 mL *L. reuteri* and ultrasonication was considered, the

counts of the total mesophilic bacteria, yeast and mold were decreased. However, *E. coli* and lactic acid bacteria counts were increased. Due to these results, the combination of 1 mL *L. reuteri* and ultrasonication was considered, when this combined treatment was found to be effective on the counts of total mesophilic bacteria and yeast and mold, it was found to be ineffective on the counts of *E. coli* and lactic acid bacteria (Figure 4.2 and Appendix A.7) ($p < 0.05$). However, when the effectiveness of the *L. acidophilus*, *L. reuteri* and the combination of these probiotics with ultrasonication was considered, *L. acidophilus* was found to be more effective than *L. reuteri* against all bacteria.

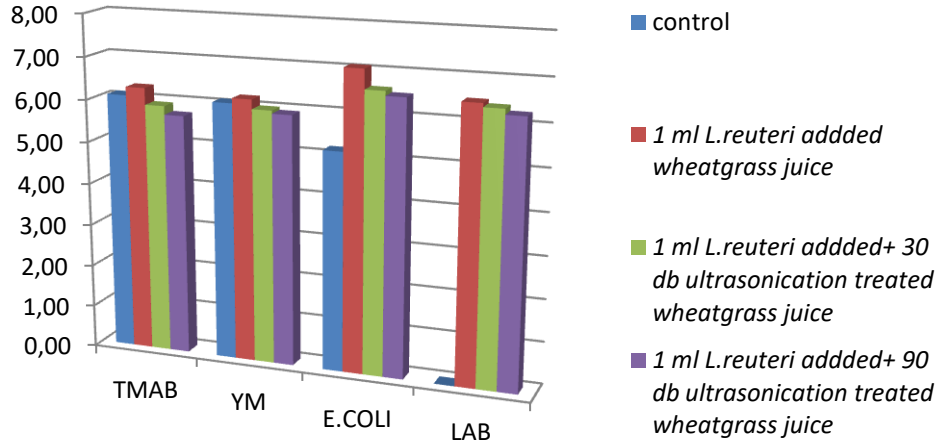

Figure 4.2. The total mesophilic bacteria, yeast and mold, *E. coli* and lactic acid bacteria (log cfu/mL) counts of the *L. reuteri* added, *L. reuteri* and ultrasonication combination (30 dB - 1 mL), and combined *L .reuteri* and ultrasonication (90 dB - 1 mL) treated wheatgrass juice

It has been documented that strains of *L. reuteri* have inhibitory effects against Gram-positive bacteria, e.g., *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*, and Gram-negative bacteria, e.g., *Escherichia coli*, *Yersinia enterocolitica* and *Pseudomonas fluorescens* (el-Ziney et al., 1999). However, in this study, the counts of total mesophilic bacteria, yeast and mold and *E. coli* was found to be increased when *L. reuteri* was added to the wheatgrass juice samples. This might be possibly explained by the case that during the period of disappearance of bacteria, *L. reuteri* was not found to be the dominating species only. Consequently, the total mesophilic bacteria, yeast and mold and *E. coli* counts of *L. reuteri* added wheatgrass juice samples were decreased by ultrasonication.

These results were consistent with the studies conducted by Tuorila and Cardello (2002). They suggested that fruit and vegetable juices could serve as a good medium for growing probiotics. It has also been reported that probiotic cultures, *L. reuteri*, *L. acidophilus* and *Bifidobacterium bifidum*, grew well in non-dairy oat-based products (Mårtensson, Öste and Holst (2002). In another study, wheat and barley extracts were found to exhibit a significant protective effect on the viability of *L. plantarum*, *L. acidophilus* and *L. reuteri* under acidic conditions (Charalampopoulos, Pandiella and Webb, 2003). It is important to have a significant number of viable lactic acid bacteria present in the finished product for maximum health benefits (Shah, 2001).

4.2 The Physical and Chemical Analysis of Wheatgrass Juices

Among all, the microbiologically effective treatments were selected for the physical and chemical analysis with respect to control. The pH of the wheatgrass juices were found to be 6.12, 6.17, 6.16, 5.28, 5.64, 5.00, 5.53, 5.44, and 5.34, for the control, 30 dB and 90 dB ultrasonication treated wheatgrass juice, *L. acidophilus* added wheatgrass juice, combination treatments of *L. acidophilus* with 30 dB and 90 dB, *L. reuteri* added wheatgrass juice, and the combination treatments of *L. reuteri* with 30 dB and 90 dB ultrasonication respectively (Table 4.4., Appendix 8). The pH of the probiotic added groups was found to be lower than the control and the sole ultrasonicated groups, especially reaching to more than one pH unit decrease since these microorganisms secrete lactic acid ($p < 0.05$). When the control and the sole ultrasonicated groups were compared, the difference between these groups were found to be almost the same. These results are in line with the experiments on sonicated orange and tomato juices (Adekunte et al., 2010; Tiwari et al., 2009).

Table 4.4. The physical and chemical analysis of nontreated, ultrasound treated and probiotic added wheatgrass juices

	Control	**An amplitude of 30 dB at 24 kHz**	**An amplitude of 90 dB at 24 kHz**	***L. acidophilus* added wheatgrass juice**	***L. reuteri* added wheatgrass juice**
Total Soluble Solids (Brix%)	$4.00^{ab} \pm .000$	$4.25^{b} \pm .354$	$3.25^{a} \pm .354$	$4.75^{b} \pm .354$	$4.75^{b} \pm .354$
Viscosity (mPa.s)	$1.46^{a} \pm .007$	$1.83^{c} \pm .007$	$2.08^{e} \pm .007$	$3.92^{j} \pm .007$	$3.25^{h} \pm .021$
pH	$6.12^{g} \pm .014$	$6.17^{h} \pm .007$	$6.16^{h} \pm .007$	$5.28^{b} \pm .007$	$5.53^{e} \pm .007$
L*	$24.08^{a} \pm .028$	$26.39^{b} \pm .007$	$27.44^{d} \pm .007$	$29.92^{g} \pm .014$	$26.48^{c} \pm .021$
a*	$-7.58^{d} \pm .106$	$-9.92^{b} \pm .120$	$-10.92^{b} \pm .049$	$-10.80^{b} \pm .028$	$-7.95^{cd} \pm 1.464$
b*	$8.18^{a} \pm .035$	$11.27^{de} \pm .021$	$11.92^{f} \pm .035$	$11.18^{d} \pm .007$	$9.04^{b} \pm .000$
Titratable Acidity	$0.71^{bcd} \pm .014$	$0.52^{a} \pm .099$	$0.59^{ab} \pm .007$	$0.68^{abcd} \pm .064$	$0.63^{abc} \pm .000$
Total Sugar Content (mg/mL)	$0.78^{ab} \pm .177$	$0.20^{a} \pm .021$	$0.18^{a} \pm .028$	$12.66^{d} \pm 1.018$	$11.38^{cd} \pm .262$
Total Protein Content (mg/mL)	$11.59^{a} \pm .410$	$15.30^{ab} \pm 1.075$	$13.94^{ab} \pm .898$	$28.82^{cd} \pm 2.920$	$28.31^{cd} \pm 4.540$

Data are means ± Std. from two replicates. Values followed by different letters in the same column are significantly different ($P < 0.05$).

Ultrasound treatment is reported to have a minimal effect on the quality of fruit juices, such as orange juice (Tiwari, Muthukumarappan, O'Donnell and Cullen, 2008), guava juice (Cheng, Soh, Liew and Teh, 2007), blackberry juice (Tiwari, O'Donnell and Cullen, 2009) and strawberry juice (Tiwari, O'Donnell, Patras and Cullen, 2008).

According to Table 4.4, an insignificant difference was found between the treatment groups of total soluble solid content of the wheatgrass juices with respect to control ($p > 0.05$). It has been suggested that if the wheatgrass is immersed in

distilled water or in a hypotonic solution, the amount of total soluble solids can be decreased with ultrasound application. In general, when the ultrasonic frequency is decreased, the loss of soluble solids increase. However, total soluble solids loss may be inhibited by ultrasound application due to reduction in microbial count (Cao et al., 2010). The release of possible antimicrobial substances from the wheatgrass, by the help of ultrasonication could also be considered.

When the titratable acidity was considered, a slight decrease was observed in all of the treatment groups with respect to control ($p > 0.05$). Total sugar content was observed to decrease insignificantly due to the ultrasound treatment ($p > 0.05$). These results were in agreement with the studies conducted by Tiwari, Muthukumarappan et al. (2008) for sonicated orange juice and Ugarte-Romero et al. (2006) for apple cider. However, when the total sugar content of probiotic added wheatgrass juice was considered, a slight increase was observed in all of the treatment groups with respect to the control due to probiotic treatment ($p < 0.05$). It has been suggested that *Lactobacillus acidophilus* reduced the level of stachyose, raffinose and sucrose while it increased the content of fructose, glucose and galactose (Wang, Yu, Yang and Chou, 2003).

The viscosity was observed to increase insignificantly due to the ultrasound and probiotic treatment ($p > 0.05$). This increase in viscosity might be possibly explained by the case that directly attributed to the sonication treatments that cause extraction of bound form of macromolecules and increase their concentration in the colloidal system and make the juice more viscous (Suárez-Jacobo et al., 2011). Previously, Bates et al. (2003) and Wu et al. (2008) has also observed an increase in viscosity in ultrasound treated vegetable purees and thermo-sonically treated tomato juice.

The lightness/darkness (L*) value in color assay in all treatment groups was found to be almost the same in the treatment groups compared to the control, whereas an insignificant difference was observed in a* (red/green) and b* (blue/yellow) value in all treatment groups with respect to the control ($p > 0.05$). It has been suggested that particulate fractions could be the reason of color changes or darkening in juices (Ugarte-Romero et al., 2006; Zarate-Rodriquez, Ortega-Rivas and Barbosa-Canovas, 2000). These results were also consistent with the studies

conducted by Valero et al. (2007) in terms of ultrasound effect on orange juice quality. Valero et al. (2007) studied the effect of ultrasonic treatments on color of orange juices. The authors reported that ultrasound treatment has almost no effect on the evolution of color scoring in the analyzed orange juices. Whereas a* (red/green) value was found to be insignificantly decreased due to probiotic treatment ($p > 0.05$), b* value (blue/yellow) value was found to be increased when compared with the control ($p < 0.05$). It has been suggested that changes in color could also be due to cavitation, which govern various physical, chemical or biological reactions, such as enzymes and microorganisms (Sala, Burgos, Condon, Lopez and Raso, 1995).

When the total protein content of ultrasound treated wheatgrass juice was considered, a slight insignificant increase was observed in all of the treatment groups with respect to control ($p > 0.05$) (Table 4.4). Iida et al. (2008) suggested that the rupture of yeast cells due to cavitation bubbles and the release of intercellular protein from yeast cells by ultrasonic action could be the reason of protein content increment. So, there are no significant differences present in the main physical and chemical analysis of nontreated and ultrasound treated wheatgrass juice ($p > 0.05$). When the total protein content assays of probiotic added wheatgrass juice were considered, a slight increase was observed in all of the treatment groups with respect to the control due to probiotic treatment ($p < 0.05$). These results were consistent with studies conducted by Donkor, Henriksson, Vasiljevic and Shah (2006) in terms of effect of *Lactobacillus acidophilus*. It has been obtained that probiotic bacteria released greater amounts of amino acids with the amount of free NH_3 groups. So, there are no significant changes present in the main physical and chemical analysis of non-treated and probiotic added wheatgrass juice except total sugar and protein assays. In addition to this, studies had shown that the metabolism of carbohydrates by *Lactobacillus* spp. varies according to the species and depends on the substrate and fermentation time (Mousavi et al., 2011; Nancib et al., 2009; Hedberg et al., 2008).

Table 4.5. The physical and chemical analysis of wheatgrass juice, *L. acidophilus* added wheatgrass juice and combined treatment of ultrasonication and *L. acidophilus* wheatgrass juice

	***L. acidophilus* added wheatgrass juice**	***L. acidophilus* added and 30 dB ultrasound treated wheatgrass juice**	***L. acidophilus* added and 90 dB ultrasound treated wheatgrass juice**
Total Soluble Solid (Brix%)	4.75^{b}±.354	4.00^{ab}±.000	4.00^{ab}±.000
Viscosity (mPa.s)	3.92^{j}±.007	1.74^{b}±.007	2.79^{g}±.000
pH	5.28^{b}±.007	5.64^{f}±.000	5.00^{a}±.000
L^*	29.92^{g}±.014	30.90^{h}±.000	29.65^{f}±.000
a^*	-10.80^{b}±.028	-13.64^{a}±.028	-10.30^{b}±.014
b^*	11.18^{d}±0.007	16.30^{g}±0.042	11.32^{e}±.007
Titratable Acidity	0.68^{abcd}±.064	1.08^{e}±.000	0.84^{d}±.035
Total Sugar Content (mg/mL)	12.66^{d}±1.018	20.49^{e}±1.407	12.53^{d}±3.387
Total Protein Content (mg/mL)	28.82^{cd}±2.920	30.79^{cd}±2.475	22.17^{bc}±1.068

Data are means ± Std. from two replicates. Values followed by different letters in the same column are significantly different ($P< 0.05$).

According to Table 4.5, the total soluble content of the wheatgrass juice was found to be almost the same in the treatment groups with respect to the control in Table 4.4 ($p > 0.05$). When the pH was considered, an insignificant decrease was observed in all of the treatment groups with respect to the control ($p > 0.05$) (Appendix 8). The titratable acidity was observed to increase insignificantly due to ultrasound treatment in probiotic added wheatgrass juice ($p < 0.05$). These results are in agreement with Muthukumarappan et al. (2008) for sonicated orange juice and Ugarte-Romero et al. (2006) for apple cider since no significant differences ($p > 0.05$) were found due to ultrasound treatment.

The lightness/darkness (L^*) value in color assay in all treatment groups was found to be insignificantly increased, on the other hand a^* (red/green) value was

found to be insignificantly decreased in the treatment groups compared to the control. The b^* (blue/yellow) value was observed to increase insignificantly compared to the control ($p > 0.05$) (Table 4.4., Table 4.5., Appendix 8). The viscosity of *L. acidophilus* added wheatgrass juice was observed to increase insignificantly when compared to control (Table 4.5., Appendix 8), but the other groups showed insignificant decrease compared with *L. acidophilus* added wheatgrass juice ($p > 0.05$). When the total sugar and the total protein assays were considered, an increase was observed in the probiotic treated groups compared to the control (Table 4.4., Table 4.5., Appendix 8). However, these results reached the highest score in combined treatment of *L. acidophilus* added and 30 dB ultrasound treatment. Ewe et al. (2012) and Fernandes et al. (2009) suggested that physical treatments, such as sonication, have been reported as a strategy to enhance membrane permeation, allowing higher mass transfer rates and consequently higher extractability of sugars from the cells. This could be the reason of an increase in sugar content.

The viscosity of wheatgrass juices were found to be 1.46 mPa.s, 1.83 mPa.s, 2.08 mPa.s, 3.92 mPa.s, 1.74 mPa.s, 2.79 mPa.s, 3.25 mPa.s, 2.02 mPa.s, and 2.61 mPa.s, for the control, 30 dB and 90 dB ultrasonication treated wheatgrass juice, *L. acidophilus* added wheatgrass juice, combination treatments of *L. acidophilus* with 30 dB and 90 dB, *L. reuteri* added wheatgrass juice, and the combination treatments of *L. reuteri* with 30 dB and 90 dB ultrasonication respectively (Table 4.4., Table 4.5., Appendix 8).

Table 4.6. The physical and chemical analysis of *L. reuteri* added wheatgrass juice and combined treatment of ultrasonication and *L. reuteri* wheatgrass juice

	***L. reuteri* added wheatgrass juice**	***L. reuteri* added and 30 dB ultrasound treated wheatgrass juice**	***L. reuteri* added and 90 dB ultrasound treated wheatgrass juice**
Total Soluble Solid (Brix%)	4.75^{b}±.354	4.50^{b}±.000	4.50^{b}±.000
Viscosity (mPa.s)	3.25^{h}±.021	2.02^{d}±.028	2.61^{f}±.007
pH	5.53^{e}±.007	5.44^{d}±.000	5.34^{c}±.014
L*	26.48^{c}±.021	33.06^{j}±.007	28.08^{e}±.014
a*	-7.95^{cd}±1.464	-15.10^{a}±.028	-9.53^{bc}±.014
b*	9.04^{b}±.000	18.90^{h}±.064	10.18^{c}±.000
Titratable Acidity	0.63^{abc}±.000	0.75^{bcd}±.035	0.77^{cd}±.000
Total Sugar Content (mg/mL)	11.38^{cd}±.262	20.21^{e}±1.669	6.43^{bc}±1.846
Total Protein Content (mg/mL)	28.31^{cd}±4.540	33.55^{d}±.000	15.35^{ab}±2.178

Data are means ± Std. from two replicates. Values followed by different letters in the same column are significantly different (P< 0.05).

According to Table 4.6, no significant differences were found in the treatment groups of total soluble solid content of the wheatgrass juices with respect to the control, shown in Table 4.4 ($p > 0.05$). The titratable acidity was found to be almost the same in the treatment groups compared to the control ($p > 0.05$). The viscosity was found to be increased due to *L. reuteri* treatment compared to the control, probably due to the slime formation of these lactic strain ($p < 0.05$), however the viscosity of combination treatment of *L. reuteri* and ultrasound on wheatgrass juice showed insignificant decrease due to ultrasound treatment compared with sole *L. reuteri* added wheatgrass juice ($p > 0.05$) (Table 4.4., Table 4.6, Appendix 8). When the pH was considered, an insignificant decrease was observed in all of the treatment groups with respect to the control ($p > 0.05$). The lightness/darkness (L^*) value in color assay in all treatment groups was found to be insignificantly increased compared with the control, whereas a^* (red/green) value was found to be

insignificantly decreased compared with the control ($p > 0.05$) (Table 4.4, Table 4.6., Appendix 8). b^* (blue/yellow) value was found to be increased in all treatment groups with respect to the control ($p < 0.05$) (Table 4.4, Table 4.6., Appendix 8). When the total sugar content and total protein content assays were considered, an increase was observed in all of the treatment groups with respect to the control ($p < 0.05$) (Table 4.4, Table 4.6., Appendix 8), however the highest value was obtained in the combined treatments of *L. reuteri* added and 30 dB ultrasonicated wheatgrass juice.

5. CONCLUSIONS

It is reported that wheatgrass juice is a powerful concentrated beverage. In this study ultrasonication and probiotics (*Lactobacillus acidophilus* and *Lactobacillus reuteri*) were studied for promoting the wheatgrass juice for being an extraordinary functional food. Although sole ultrasonication reduced up the microbiological counts of the total mesophilic aerobic bacteria and *E. coli*, the combined treatment of 90 dB amplitude at 24 kHz frequency of ultrasonication and 1 mL *Lactobacillus acidophilus* completely destroyed these bacteria which are found in wheatgrass juice. In general no any chemical or physical change was observed in wheatgrass juices treated with ultrasonication and probiotics. Wheatgrass juice therefore, may play a role as a cereal beverage, bearing potential viable substrates due to its nutritional content easily assimilated by probiotics. Consequently, these findings indicated that the combination treatment of ultrasonication and probiotic, especially, *L. acidophilus* can show recommendable status in industrial processing of wheatgrass juice due to their effectiveness and low cost, therefore it was verified by the statistical analysis.

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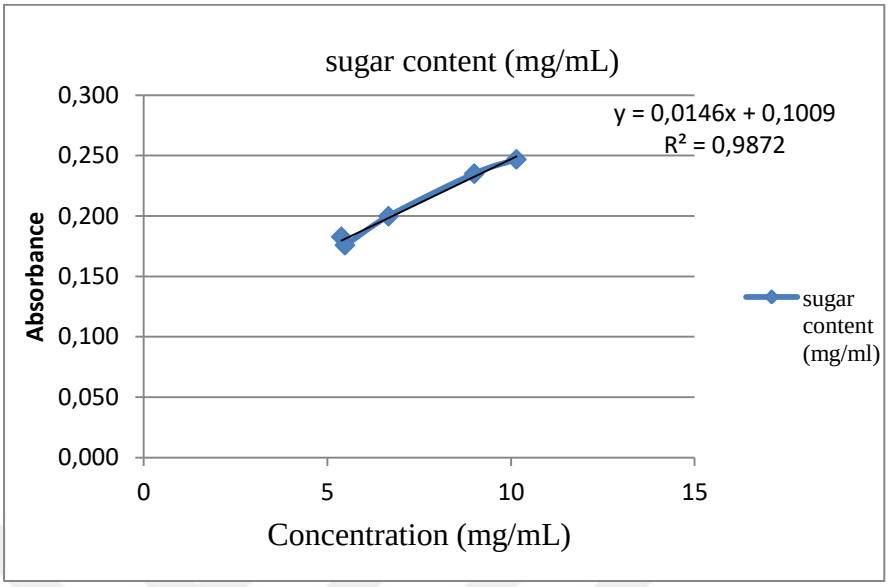
APPENDICES

6. APPENDICES

A.1 The Standards of Sugar (mg)

Std – 1	**Std - 2**	**Std – 3**	**Std – 4**	**Std - 5**
0,379	0,478	0,663	0,999	1,147

A.2 Standard Curve of Sugar (mg/mL)



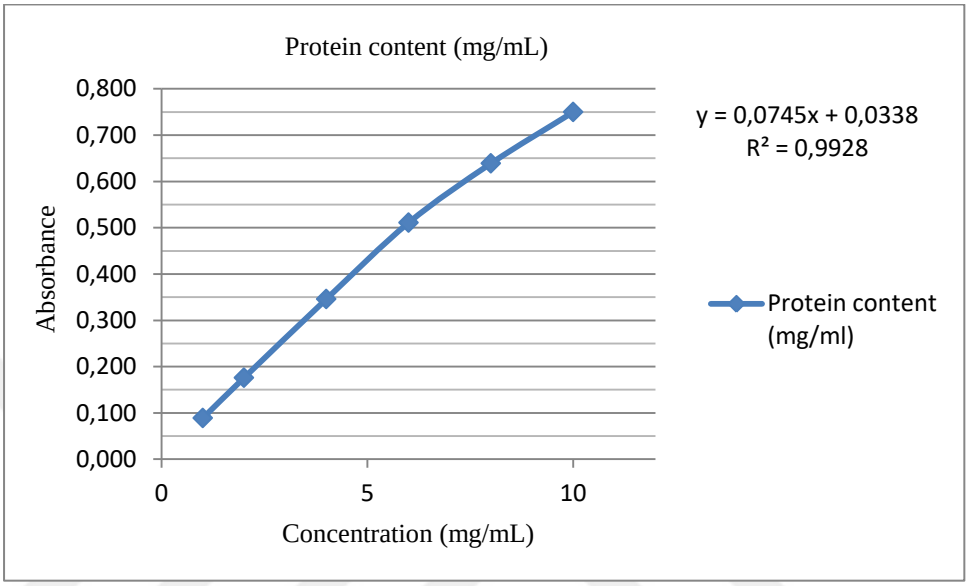
The amount of total sugar content can calculate that each sample's absorbance value put in the equation on graph.

Graph equation: y = 0,0146x + 0,1009

A.3 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.4 Standard Curve of Protein (mg/mL)



The amount of protein content can calculate that each sample's absorbance value put in the equation on graph.

Graph equation : y = 0,0745x + 0,0338

A.5 The Total Mesophilic Aerobic Bacteria (TMAB), Yeast-Mold and *E. Coli* Counts of the Wheatgrass Juice (Control)

		Wheatgrass juice (Control)
TMAB	**Mean**	1530000
	log cfu/mL	6,18
YM	**Mean**	1635000
	log cfu/mL	6,21
E. coli	**Mean**	232500
	log cfu/mL	5,37

A.6 The Microbial Counts of 1 mL *L. acidophilus* and 1 mL *L. reuteri* Added Wheatgrass Juice

1 mL *L. reuteri* (10^7)	1 mL *L. acidophilus* (10^7)	Control	Groups	
6.30^{a} ± .106	4.54^{b} ± .621	6.10^{a} ± .147	**Mean ± Std.**	**TMAB**
6.21^{a} ± .137	3.06^{a} ± 3.540	5.99^{a} ± .049	**Mean ± Std.**	**YM**
7.07^{b} ± .504	4.58^{a} ± .393	5.18^{a} ± .504	**Mean ± Std.**	***E. coli***
6.40^{c} ± .530	5.06^{b} ± .447	N.D	**Mean ± Std.**	**LAB**

Data are means ± Std. from two replicates. Values followed by different letters in the same column are significantly different ($P< 0.05$).

A.7 The Combination Study of the Ultrasonicated (90 dB at 24 kHz), and 1 mL *L. reuteri* Added, and the Combination Study of the 1 mL *L. reuteri* Added and Ultrasonicated (30 dB at 24kHz) Wheatgrass Juice

Groups	**TMAB**	**YM**	***E. coli***	**LAB**
	Mean ± Std.	**Mean ± Std.**	**Mean ± Std.**	**Mean ± Std.**
control	6.10^{ab} ± .148	6.09^{ab} ± .161	5.18^{b} ± .506	N.D
1 mL *L. reuteri* (10^7) added wheatgrass juice	6.30^{a} ± .106	6.21^{a} ± .048	7.07^{a} ± .066	6.27^{a} ± .196
1 mL *L. reuteri* (10^7) added + 30 dB ultrasonicated wheatgrass juice	5.91^{bc} ± .079	5.99^{ab} ± .137	6.61^{a} ± .306	6.49^{a} ± .723
1 mL *L. reuteri* (10^7) added + 90 dB ultrasonicated wheatgrass juice	5.71^{c} ± .0,084	5.92^{b} ± .076	6.50^{a} ± .791	6.40^{a} ± .530

Data are means ± SD from two replicates. Values followed by different letters in the same column are significantly different ($p<0.05$).

A.8 The Physical and Chemical Analysis of Wheatgrass Juice, *L. acidophilus* added Wheatgrass juice, Combined Treatment of Ultrasonication (30 dB at 24 kHz, 90 dB at 24 kHz) and *L. acidophilus* Wheatgrass Juice, *L. reuteri* Added Wheatgrass Juice, and Combined Treatment of Ultrasonication (30 dB at 24 kHz, 90 dB at 24 kHz) and *L. reuteri* added Wheatgrass Juice

	Control	**30 dB at 24 kHz ultrasound treated wheatgrass juice**	**90 dB at 24 kHz ultrasound treated wheatgrass juice**	***L. acidophilus* added wheatgrass juice**	***L. acidophilus* added and 30 dB ultrasound treated wheatgrass juice**
Total Soluble Solids (Brix%)	$4.00^{ab} \pm .000$	$4.25^{b} \pm .354$	$3.25^{a} \pm .354$	$4.75^{b} \pm .354$	$4.00^{ab} \pm .000$
Viscosity (mPa.s)	$1.46^{a} \pm .007$	$1.83^{c} \pm .007$	$2.08^{e} \pm .007$	$3.92^{j} \pm .007$	$1.74^{b} \pm .007$
pH	$6.12^{g} \pm .014$	$6.17^{h} \pm .007$	$6.16^{h} \pm .007$	$5.28^{b} \pm .007$	$5.64^{f} \pm .000$
L*	$24.08^{a} \pm .028$	$26.39^{b} \pm .007$	$27.44^{d} \pm .007$	$29.92^{g} \pm .014$	$30.90^{h} \pm .000$
a*	$-7.58^{d} \pm .106$	$-9.92^{b} \pm .120$	$-10.92^{b} \pm .049$	$-10.80^{b} \pm .028$	$-13.64^{a} \pm .028$
b*	$8.18^{a} \pm .035$	$11.27^{de} \pm .021$	$11.92^{f} \pm .035$	$11.18^{d} \pm .007$	$16.30^{g} \pm .042$
Titratable Acidity	$0.71^{bcd} \pm .014$	$0.52^{a} \pm .099$	$0.59^{ab} \pm .007$	$0.68^{abcd} \pm .064$	$1.08^{e} \pm .000$
Total Sugar Content (mg/mL)	$0.78^{ab} \pm .177$	$0.20^{a} \pm .021$	$0.18^{a} \pm .028$	$12.66^{d} \pm 1.018$	$20.49^{e} \pm 1.407$
Total Protein Content (mg/mL)	$11.59^{a} \pm .410$	$15.30^{ab} \pm 1.075$	$13.94^{ab} \pm .898$	$28.82^{cd} \pm 2.920$	$30.79^{cd} \pm 2.475$

A.8 The Physical and Chemical Analysis of Wheatgrass Juice, *L. acidophilus* added Wheatgrass juice, Combined Treatment of Ultrasonication (30 dB at 24 kHz, 90 dB at 24 kHz) and *L. acidophilus* Wheatgrass Juice, *L. reuteri* Added Wheatgrass Juice, and Combined Treatment of Ultrasonication (30 dB at 24 kHz, 90 dB at 24 kHz) and *L. reuteri* added Wheatgrass Juice (continued)

L. reuteri added and 90 dB ultrasound treated wheatgrass juice	*L. reuteri* added and 30 dB ultrasound treated wheatgrass juice	*L. reuteri* added wheatgrass juice	*L. acidophilus* added and 90 dB ultrasound treated wheatgrass juice
$4.50^{b} \pm .000$	$4.50^{b} \pm .000$	$4.75^{b} \pm .354$	$4.00^{ab} \pm .000$
$2.61^{f} \pm .007$	$2.02^{d} \pm .028$	$3.25^{h} \pm .021$	$2.79^{g} \pm .000$
$5.34^{c} \pm .014$	$5.44^{d} \pm .000$	$5.53^{e} \pm .007$	$5.00^{a} \pm .000$
$28.08^{e} \pm .014$	$33.06^{j} \pm .007$	$26.48^{c} \pm .021$	$29.65^{f} \pm .000$
$-9.53^{bc} \pm .014$	$-15.10^{a} \pm .028$	$-7.95^{cd} \pm 1.464$	$-10.30^{b} \pm .014$
$10.18^{c} \pm .000$	$18.90^{h} \pm .064$	$9.04^{b} \pm .000$	$11.32^{e} \pm .007$
$0.77^{cd} \pm .000$	$0.75^{bcd} \pm .035$	$0.63^{abc} \pm .000$	$0.84^{d} \pm .035$
$6.43^{bc} \pm 1.846$	$20.21^{e} \pm 1.669$	$11.38^{cd} \pm .262$	$12.53^{d} \pm 3.387$
$15.35^{ab} \pm 2.178$	$33.55^{d} \pm .000$	$28.31^{cd} \pm 4.540$	$22.17^{bc} \pm 1.068$

Data are means ± SD from two replicates. Values followed by different letters in the same column are significantly different ($p<0.05$).

7. CURRICULUM VITAE

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Yurdugül S, Tecen E, **Yaşar E**, Yavuz M. Freeze-drying of persimmon fruit to extend the shelf life of wheatgrass juice inoculated with probiotics, The 8th Asia-Pasific Drying Conference ADC 2015, Kuala Lumpur, Malaysia (in abstract book, not presented).