BIOLOGICAL AND CHEMICAL CHARACTERIZATION OF SHALGAM JUICE

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BIOLOGICAL AND CHEMICAL CHARACTERIZATION OF SHALGAM JUICE

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ABSTRACT

BIOLOGICAL AND CHEMICAL CHARACTERIZATION OF SHALGAM JUICE

Shalgam juice is a traditional fermented beverage, mostly produced and consumed in the southern east parts of Turkey. The main ingredient of shalgam juice is black carrot which is known for its high antioxidant capacity due to the presence of anthocyanins. Although shalgam juice's composition is widely studied, further characterization is needed to determine its potential health benefits. In this study, shalgam juice's chemical and biological composition was characterized by determining its antioxidant capacity, identifying its microflora with species-specific PCR based on 16S rDNA and checking its anti-proliferative effects on Caco-2 cell lines using MTS assay *in vitro*. The antioxidant capacities of 11 different commercially available shalgam juices were investigated using DPPH, ABTS and FRAP assays and were found as 2.43-4.36 µmol TE/mL in ABTS, 3.53-5.96 µmol TE/mL in DPPH, 2.01-3.61 µmol TE/mL in FRAP assays. Comparison of the species-specific PCR targeting 16S rDNA gene sequences results with data from NIH by BLAST search program revealed twenty one *Lactobacillus* species and subspecies. Some of the identified LAB was previously reported as probiotics suggesting that shalgam juice may have probiotic potential in addition to its nutritional properties. The reconstituted lyophilized powder of shalgam juice inhibited the growth of Caco-2 cells in a dose dependent manner (50-6400 µg/mL) with 55.5-91.4% cell viability. In conclusion, although this study provided detailed information about the antioxidant and anti-proliferative effects and microbial composition of shalgam juice *in vitro*, the mechanism behind health benefits of shalgam juice is still not clear and needs to be studied.

ÖZET

ŞALGAM SUYUNUN KİMYASAL VE BİYOLOJİK KARAKTERİZASYONU

Şalgam suyu, çoğunlukla Türkiye'nin güney doğu bölgelerinde üretilen ve tüketilen geleneksel fermente bir içecektir. Şalgam suyunun ana malzemesi, yüksek antioksidan kapasitede antosiyaninlere sahip olduğu bilinen siyah havuçtur. Şalgam suyunun kompozisyonu yaygın olarak bilinse de sağlığa olan potansiyel yararlarının belirlenebilmesi için daha geniş bir karakterizasyona ihtiyaç vardır. Bu çalışmada, şalgam suyunun kimyasal ve biyolojik kompozisyonu antioksidan kapasitesi, 16S rRNA'ya dayanan türe özel PZR ile mikroflorası ve *in vitro* şartlarda Caco-2 hücre hatları üzerindeki antiproliferatif etkisine MTS analizi ile bakılarak karakterize edilmiştir. On bir farklı ticari şalgam suyunun antioksidan kapasitesi DPPH, ABTS ve FRAP analizleri ile belirlenmiş ve ABTS analizinde 2.43-4.36 µmol TE/mL, DPPH analizinde 3.53- 5.96 µmol TE/mL ve FRAP analizinde 2.01-3.61 µmol TE/mL olarak bulunmuştur. 16S rDNA gen sekanslarını hedef alan türe özel PZR sonuçlarının NIH'daki BLAST arama programıyla karşılaştırılması sonucunda yirmi bir *Lactobacillus* türü ve alt türü tanımlanmıştır. Tanımlanan LAB'nin bazılarının daha önce probiyotik olarak kabul edilmiş olması, şalgam suyunun besleyici özelliklerinin yanı sıra probiyotik potansiyelinin de bulunabileceğini düşündürmektedir. Şalgam suyunun liyofilize edilip tekrar çözülmüş tozu Caco-2 hücrelerinin büyümesini doza bağlı bir şekilde (50-6400 µg/mL) %55.5-91.4 oranında hücre canlılığıyla inhibe etmiştir. Sonuç olarak, bu çalışma şalgam suyunun *in vitro* antioksidan ve antiproliferatif etkileri ile mikrobiyal kompozisyonuna dair geniş bir bilgi sağlamışsa da, sağlık üzerine olumlu etkilerinin arkasındaki mekanizma hala kesin değildir ve belirlenmesi gerekmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

ABTS	2,2'-azonobis(3-etilbenzothiazoline-6-sulfonat
ANOVA	One-way analysis of variance
BLAST	Basic local alignment search tool
Caco-2	Colorectal carcinoma
CFU	Colony forming unit
CUPRAC	Copper Reduction Antioxidant Capacity
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC50	Effective concentration 50
EDTA	Ethylenediaminetetraacetic acid
F-C	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
HAT	Hydrogen atom transfer
HPLC	High Performance Liquid Chromatography
LAB	Lactic acid bacteria
Lb.	*Lactobacillus*
Leu.	*Leuconostoc*
MRS	Man, Rogosa and Sharp
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
ORAC	Oxygen radical capacity
PBS	Phospate buffer saline
PDA	Photodiode array
PMS	Phenazine methosulfate
ROS	Radical oxygen species
SET	Single electron transfer
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine

TRAP	Total radical trapping parameter
TS	Turkish Standards

1. INTRODUCTION

Fermented fruit and vegetable juices are non-dairy fermented beverages produced generally by lactic acid fermentation and their microbiological and chemical compositions provide beneficial properties to them. The microbiological composition of lactic acid fermented products consists of mostly lactic acid bacteria, which are considered to be probiotics due to their beneficial health effects, such as antimicrobial, immunostimulatory and anticarcinogenic properties. Chemical composition, most importantly phenolic compounds of fruits and vegetables have also been found to be beneficial to health. Their antioxidant capacities result in functional effects related with oxidative stress such as anti-proliferative effects. Determination of the antioxidant capacities of fermented fruits and vegetable juices, as well as identification of beneficial lactic acid bacteria (LAB) is important in order to understand their health effects and functional properties.

Shalgam juice is a traditional fermented beverage, produced from black carrot and bulgur dough especially in southern eastern parts of Turkey. The chemical and microbiological composition of the product is affected by the production method as well as its ingredients. The main ingredient, black carrot has beneficial effects on health beyond its nutritional value due to its anthocyanins, which are bioactive compounds with antioxidant properties. Lactic acid bacteria that are responsible for fermentation also confer some health benefits. However the potential health effects of shalgam juice as a whole product is not widely known. Therefore, the biological and chemical characterization of shalgam juice composition need to be investigated to determine its functional properties such as antioxidant and anti-proliferative effects.

The aim of this study was to characterize shalgam juice's chemical and biological composition by determining its antioxidant capacity, identifying its microflora by way of a molecular approach using species-specific PCR, and checking its anti-proliferative effects on colorectal carcinoma (Caco-2) cell lines *in vitro*.

2. THEORETICAL BACKGROUND

2.1. FERMENTED BEVERAGES

Throughout centuries, fermentation has been used as one of the oldest methods for food preservation and production. Fermentation is a metabolic process of the break-down of carbohydrates and its derivatives to end-products such as organic acids, alcohol and carbon dioxide by the help of microorganisms (bacteria, yeasts and mycelia fungi) and their enzymes. Fermentation end-products provide fermented food and beverages their unique characteristics such as flavor, aroma and texture, as well as benefits like preventing spoilage or helping digestion [1].

More than 3,500 fermented foods and beverages are produced all over the world using several raw materials, manufacturing techniques and microorganisms, and most of these products are traditional products that are only produced in certain regions of the world usually by small scale businesses (Table 2.1) [2]. Fermented beverages can be classified as dairy or non-dairy fermented products [3]. Dairy fermented products are very common and commercially available in the market (Table 2.1) [2, 4-7]. Different type of milks are generally used as raw materials to produce fermented dairy beverages and produced with lactic acid fermentation [3]. However, consumer demand for non-dairy fermented foods are increasing in the western world due to an increase in vegetarianism and health problems like lactose-intolerance or high cholesterol [3]. Non-dairy fermented beverages are produced either from cereals and grains or from fruits and vegetables, with alcohol, acetic acid or lactic acid fermentation [2, 3]. Alcohol fermentation is carried out by yeasts, which convert carbohydrates to ethanol and CO_2. Alcoholic beverages such as beers and wines are produced by alcohol fermentation [8]. Acetic acid fermentation, which is carried out by bacteria of the *Acetobacter* genus results in production of acetic acid from carbohydrates and products such as vinegars and pickles [8]. Most of the non-alcoholic fermented beverages from fruit, vegetables and cereals are produced by lactic acid fermentation using Lactic Acid Bacteria (LAB). Lactic acid is the end-product of the fermentation and gives a refreshing, desirable taste and flavor to non-alcoholic beverages [2].

Table 2.1. Some fermented beverages produced in different regions of the world

Fermented beverage	Product	Raw material	Region	Reference
Dairy beverages	Airag	Mare or camel milk	Mongolia	4
	Gariss	Camel milk	Sudan	5
	Kefir	Milk	Eastern Europe	2
	Kishk	Sheep milk-wheat	Middle East, Iran	4
	Koumiss	Milk	Turkey, Mongolia, Kazakhstan and Kyrgyzstan	2
	Lassi	Cow milk	India, Nepal, Bhutan, Bangladesh, Pakistan	4
	Mohi	Cow milk	Nepal, India, Bhutan India	4
	Sethemi	Milk	South Africa	6
	Shubat	Camel milk	China	7
	Tarag	Cow, yak, goat milk	Mongolia	4
Non-dairy alcoholic beverages	Beer	Barley	Global	8
	Busaa	Maize	Nigeria, Gana	10
	Chikokivana	Maize and millet	Zimbabwe	10
	Chongju	Rice	Korea	10
	Kachasu	Maize	Zimbabwe	10
	Kaffir	Malt of sorghum, maize	South Africa	10
	Kaffir beer	Kaffir corn	South Africa	10
	Khaomak	Rice	Thailand	10

Table 2.2. Some fermented beverages produced in different regions of the world (continued)

Non-dairy alcoholic beverages	Merissa	Sorghum and millet	Sudan	10
	Mirin	Rice, alcohol	Japan	10
	Otika	Sorghum	Nigeria	10
	Pito	Maize, sorghum	Nigeria, Ghana	10
	Sake	Rice	Japan	10
	Seketeh	Maize	Nigeria	10
	Shaosinghjiu	Rice	China	10
	Sorghum beer	Sorghum, rice	South Africa	10
	Takju	Rice, wheat	Korea	10
	Talla	Sorghum	Ethiophia	10
	Tapai pulut	Rice	Malaysia	10
	Tapuy	Rice	Phillipines	10
	Tesgüino	Maize	Northern and North Western Mexico	10
	Wines	Grapes, fruits	Global	9
Non-dairy cereal beverages	Bagni	Millet	Caucasus	10
	Boza	Wheat, rye, millet, maise and other cereals mixed with sugar	Albania, Turkey, Bulgaria	11
	Braga	Millet	Romania	10
	Busa	Rice or millet	Syria, Egypt, Turkistan	10
	Bushera	Sorghum or millet flour	Uganda	8
	Chicha	Maize	South America	8

Table 2.3. Some fermented beverages produced in different regions of the world (continued)

Non-dairy cereal beverages	Darassum	Millet	Mongolia	10
	Hulumur	Millet	Sudan	10
	Mahewu	Corn meal	Africa, Arabian Gulf countries	8
	Mangisi	Millet	Zimbabwe	9
	Mbege	Malted millet acidic banana juice	Tanzania	10
	Munkoyo	Maize	Africa	8
	Pozol	Maize	South-eastern Mexico	8
	Soybean milk	Soybeans	China, Japan	10
	Tobwa	Maize	Zimbabwe	8
	Togwa	Maize flour and finger millet malt	Tanzania	3
Non-dairy fruit and vegetable beverages	Hardaliye	Grapes	Turkey	22
	Kombucha	Green tea, black tea	Asia	9
	Kanji	Black carrot	India	23
	Shalgam juice	Black carrot	Turkey	24
	Other fermented fruit and vegetable juices	Beet, cabbage, carrot, tomato, pineapple, orange, cranberry	Global	14-21

Non-dairy lactic acid fermented beverages are produced mostly in a traditional manner, especially in eastern parts of the world such as in African, Asian and Middle Eastern countries in small-scale industries or homes and villages and their properties are not widely known (Table 2.1) [8]. These products are gaining more attention because of their possible beneficial effects on health [3]. Cereals and grains or fruits and vegetables are generally used to produce non-dairy lactic acid fermented beverages and fermentation is carried out mostly by the natural microflora of the raw material [3].

Cereal based non-dairy lactic acid fermented beverages are produced generally from cereal grains like rice, wheat, maize or sorghum [8-11]. *Boza* is one of the commercially available cereal based fermented beverage made from wheat, rye, millet, maize and other cereals mixed with sugar. It is produced and consumed mostly in Turkey, Albania, Romania and Bulgaria [11]. *Bushera* is another traditional cereal based lactic acid fermented beverage, produced from sorghum and is commonly consumed by children and adults in Western highlands of Uganda [8]. *Mahewu*, a beverage consumed in Africa and some Arabian Gulf countries is produced from lactic acid fermentation of corn meal and maize and preferred mostly by adults [8]. Similar to *mahewu,* beverages such as *pozol, munkoyo* and *tobwa* are also produced from maize. Pozol, which is consumed in Mexico, is made from cooked maize and lime solution [8]. Munkoyo and tobwa are similar products to pozol, which are produced in Africa and Zimbabwe [8]. *Bagni*, (Caucasia) *braga* (Romania), *busa* (Syria, Egypt and Turkestan), *darassum* (Mongolia), *hulumur* (Sudan), *mangisi* (Zimbabwe) are some of the other cereal based lactic acid fermented beverages, which are very similar to each other. They are produced from millet in several parts of the world in a traditional manner and knowledge about their composition and properties is very limited [10]. *Chicha* is one of the widely consumed cereal based lactic acid fermented beverages of South America and produced from maize. It has a unique fermentation process, in which saliva serves as an amylase source [8]. Soybean is also used to produce non-dairy fermented beverages such as *soybean milk* in China and Japan [10].

Fermented fruit and vegetable products are also gaining interest due to their bioactive properties beyond nutritional properties [3, 12]. Beside their desirable taste and flavor, they are also sources of ingredients such as vitamins, minerals, dietary fibers, phenolic compounds with antioxidant properties and lactic acid bacteria with probiotic properties

and their consumption is increasing in many countries [13]. These lactic acid fermented beverages are produced mostly with bacteria from their natural microflora from various fruits such as pomegranates, cranberries, pineapples, oranges and vegetables such as beets, cabbages, carrots, tomatoes [14-21]. *Kombucha* is one of the traditional plant based beverages produced by lactic acid fermentation of green or black tea in Asian countries [9]. *Hardaliye* is another fermented fruit beverage produced from red grapes with mustard seeds and consumed in Thrace region of Turkey [2]. Alcohol production by yeasts is inhibited with the addition of benzoic acid and mustard seeds give the product its unique taste and flavor [22]. *Kanji* is a lactic acid fermented vegetable juice, produced using black carrot as raw material, with the addition of mustard in India [23]. A similar product to kanji, *shalgam juice*, is also produced with black carrot, using sourdough in fermentation process. It is a traditional beverage, mostly produced in southern east parts of Turkey [24].

Interest in fermented fruit and vegetable beverages is increasing due to their bioactive properties and possible beneficial effects on some chronic diseases like cancer and cardiovascular diseases which are mostly related with oxidative stress [13]. Beneficial health effects and unique properties such as flavor and taste of fermented fruit and vegetable juices are attributed to their microbial and chemical composition [3, 13]. Microbial composition of fermented fruit and vegetable juices mostly consist of LAB, which are considered to be healthy due to their probiotic properties and metabolic end-products such as organic acids produced during fermentation of juices [3]. On the other hand, the chemical composition of juices are also of interest since fruit and vegetables are the sources of phytochemicals such as phenolic compounds with antioxidant and other bioactive properties [13]. Therefore, knowing the chemical and microbial properties of fermented fruit and vegetable juices is important to understand the mechanism behind their functional properties.

2.1.1. Microbial Composition of Fermented Fruit and Vegetable Juices

Fermentation of fruits and vegetables are mostly governed by lactic acid bacteria (LAB), since they naturally host LAB in their flora (Table 2.2) [13-29]. LAB are a group of gram positive, facultative anaerobic bacteria, which use hexoses in fermentation to produce lactic acid as the main end-product [30]. Lactic acid gives fermented beverages their flavor

and aroma. Depending on their metabolism, LAB are divided into two groups; homofermentative LAB and heterofermentative LAB [30]. Homofermentative lactic acid bacteria, such as *Pediococcus, Streptococcus*, *Lactococcus* and some *Lactobacillus* species produce lactic acid as the only end-product in fermentation of carbohydrates. Heterofermentative LAB, like *Weisella*, *Leuconostoc* and most of the *Lactobacillus* species in fermented vegetable juice production produces acetic acid, CO_2 and ethanol in addition to lactic acid using a different pathway in fermentation [30]. LAB are also considered as probiotics. Probiotics are viable microorganisms that are beneficial to health beyond their nutritional value when consumed in sufficient quantities [31]. Lactic acid bacteria mostly from the genuses *Lactobacillus* and *Bifidobacterium* are widely investigated as probiotic microorganisms [32]. The potential benefits to human health include antimicrobial activities [33], antihypertensive properties and reduction of LDL-cholesterol levels [34] and anti-proliferative properties [35], however the mechanism behind these effects are mostly unknown [36].

Since LAB in fermented fruit and vegetable juices can be autochthonous, meaning that they can be found in the natural microflora of the raw materials, they cause spontaneous fermentation. On the other hand, LAB can also be added into either raw vegetables or mild-treated vegetables as starter cultures [21]. *Lactobacillus plantarum*, *Lb. acidophilus, Lb. delbrueckii, Lb. fermentum, Lb. brevis*, and *Lb. casei* are the main lactic acid bacteria found in fermented plant products (Table 2.2).

Table 2.4. Lactic acid bacteria found in fermented fruit and vegetable juices

Fermented Product	**Ingredients**	**LAB strains in product**	**Reference**
Beet juice	Beet	*Lb. acidophilus* LA 39, *NCDO1748 and Ch-5* *Lb. casei* A4 *Lb.delbrueckii* D7 and ŁOCK 0854 *Lb. plantarum and* ŁOCK 0858 Brewer's yeast	16-18
Cabbage juice	Cabbage	*Lb. casei* A4 *Lb. debrueckii* D7 *Lb. plantarum* C3	14
Carrot juice	Carrot	*Lb. acidophilus* NCDO1748 Brewer's yeast *Lb. delbrueckii subsp. bulgaricus* DSM 20 081 and ATCC 11 842 *Lb. rhamnosus* DSM 20 711 *Lb. plantarum* RSKK 1062	13, 26, 27
Orange, pineapple and cranberry juice	Orange, pineapple and cranberry	*Lb. salivarius ssp. salivarius* UCC118L and UCC500 *Lb. paracasei ssp. paracasei* NFBC43338 *Lb. rhamnosus* GG *Lb. casei* DN-114 001 *Bifidobacterium animalis ssp. lactis Bb-12*	19
Tomato juice	Tomato	*Lb. acidophilus* LA 39 *Lb. casei* A4 *Lb.delbrueckii* D7 *Lb. plantarum* *W. cibaria/confusa* *Lb. brevis* *P. pentosaceus* *E. faecium/faecalis*	15, 20, 21
Pomegranate juice	Pomegranate	*Lactobacillus acidophilus DSMZ 20079* *L. plantarum DSMZ 20174* *L. delbrueckii DSMZ 20006* *L. paracasei DSMZ 15996)*	25
Red beet juice	Red beet	*Lb. plantarum 2142* *Lb. curvatus 2770* *Lb. casei pseudoplantarum 2745*	28

Table 2.5. Lactic acid bacteria found in fermented fruit and vegetable juices (continued)

Hardaliye	Grape and raw mustard seeds	*Lb. paracasei subsp. paracasei* *Lb. casei subsp. pseudoplantarum* *Lb. pontis* *Lb. brevis* *Lb. acetotolerans* *Lb. sanfransisco* *Lb. vaccinostercus*	22
Shalgam juice	Black carrot	*Lb. plantarum* *Lb. paracasei subsp. paracasei* *Lb. brevis* *Lb. fermentum* *Leu. mesenteroides subsp. mesenteroides* *Pediococcus pentosaceaceus* *Lb. delbrueckii subsp. delbrueckii*	29

Species such as *Lb. acidophilus*, *Lb. plantarum* are homofermentative LAB while most other the species are heterofermentative [37]. Although lactic acid bacteria can be found in the natural microflora of fruits and vegetables, some of the fermented products are produced by the addition of these species as starter cultures because of their applicability to fruit and vegetable juices which are ideal media for fermentation as well as their probiotic properties [19]. Beets, like many other vegetables have an autochthonous microflora consisting of lactic acid bacteria and yeast, however the small quantities of LAB are not sufficient enough to ferment the product [17]. Therefore, LAB such as *Lb. acidophilus*, *Lb. casei*, *Lb.delbrueckii* and *Lb. plantarum* strains are used as starter cultures to produce lactic acid fermented beet juice [16-18]. Other vegetable and fruit juices such as carrot, cabbage, pomegranate, cranberry and orange juices have also been fermented with these LAB species and they were found suitable for production of fermented beverages [14, 26]. Similarly, LAB found in shalgam juice fermentation such as *Lb. paracasei subsp. paracasei, Lb. casei subsp. pseudoplantarum, Lb. pontis, Lb. brevis, Lb. acetotolerans, Lb. sanfransisco, Lb. vaccinostercus, Lb. plantarum, Lb. fermentum, Leu. mesenteroides subsp. mesenteroides, Pediococcus pentosaceaceus* and *Lb. delbrueckii subsp. delbrueckii* mostly come from the dough rather than black carrot [22, 29]. Lactic acid fermented tomato juice has also been produced with LAB (*Lb. plantarum, Lb. brevis, Enterococcus*

faecium/faecalis, W. cibaria/confusa, Pediococcus pentosaceus) isolated from the natural microflora of tomatoes [21].

LAB species found in fermented fruit and vegetable juices have shown some health effects *in vivo* and *in vitro* (Table 2.3) and are considered probiotics. *Lb. casei* and *Lb. acidophilus* are commercially available probiotic bacteria, which were used in fermented beet, carrot, cabbage and fruit juices as starter cultures [14, 17, 19]. They inhibited the growth of colon, cervix, breast, brain, liver, bone and pancreas cancer cell lines (HT-29, HeLa, MCF-7, U-87, HepG-2, U2Os, PANC-1) 72-79% (*Lb. acidophilus* 606) and 80-85% (*Lb. casei* ATCC 393) *in vitro* [38]. These species also showed DPPH radical scavenging activity properties by inhibiting DPPH 36.05-52.06% at 10^8 CFU/mL concentration [38]. They have also shown antimicrobial effect on species *Helicobacter pylori, Escherichia coli, Salmonella typhimurium, Staphylococcus aureus* and *Clostridium perfringens*, which cause gastrointestinal infection [39]. *Lb. plantarum* is another species of LAB used as starter culture in fermented vegetable juices such as fermented cabbage and tomato juices or can be found in spontaneous vegetable beverage fermentations and is considered as a probiotic [15, 16, 24]. *Lb. plantarum* 299v strain was found to have immune system stimulating properties by down-regulating the expression of inflammatory cytokine, IL-8 in HT-29 colorectal carcinoma cell lines treated with TNF-α, a pro-inflammatory cytokine [40]. LDL-cholesterol and fibrinogen levels in blood were also reduced in subjects consuming a drink containing 5 x 10^7CFU/mL *Lb. plantarum 299v* for 6 weeks [41-43]. *Lb. brevis*, which can either be found in natural fruit and vegetable juice fermentation or used as starter culture in fermented vegetable juices, is not commercially available as a probiotic strain. However it is proposed as a probiotic due to its beneficial health effects such as immune system stimulation by increasing the expression of IFN-α in subjects consuming *Lb. brevis subsp. coagulans* and antimicrobial properties against *Bacillus cereus, Candida albicans* and *E. coli* [44, 45]. Strains of *Lb. delbrueckii* species, which are used as starter cultures in fermented vegetable juices, has shown antibacterial activity against *Salmonella* species, *S. aureus* and *E. coli* [46]. Another probiotic property of the members of this species is 127-3152 Trolox Equivalent/10^9 cells *in vitro* antioxidant capacity against free radicals in ORAC assay [47]. *Lb. rhamnosus* is another species found in vegetable fermentations and has shown antigenotoxic effects by inhibiting the generation (93.4%) of

4-Nitroquinoline-1-oxide, a genotoxic product, in vitro and antimicrobial properties against *E. coli*, *S. aureus*, *C. perfringens*, *C. albicans* and *Streptococcus mutans* [48, 49].

Table 2.6. *In vitro* and *in vivo* beneficial health effects of some LAB found in fermented fruit and vegetable juices

Lactic Acid Bacteria	**Probiotic properties**	**Reference**
Lb. acidophilus	Antimicrobial effect on *E. coli, S. typhimurium, S. aureus, C. perfringens* and *H. pylori*, antimutagenic and antioxidant properties, serum cholesterol lowering effects	38-40
Lb. casei	Antitumor, immunostimulatory and antimicrobial activities	39
Lb. plantarum	Cholesterol reducing, natural immune response improving properties, protective effects against intestinal infection	41, 43
Lb. brevis	Immune system stimulation, antagonistic effects towards potentially harmful microorganisms and adhesion properties to intestine	44, 45
Lb. delbrueckii	Antimicrobial, antioxidant properties	46, 47
Lb. rhamnosus	Antimicrobial and anticarcinogenict properties	48, 49

Because of these beneficial health effects, the isolation and identification of new lactic acid bacteria strains in natural complex microbial communities is important for their selection as probiotics in food products [50, 51].

2.1.1.1. Identification of Microflora

LAB and their fermentation end-products give fermented food and beverages their unique characteristics and probiotic properties. Therefore, identification and detection of LAB in natural microflora is important for their applicability in food products and safety of the product [50]. Identification of bacteria is traditionally dependent on cultivation and phenotypic methods such as their morphology, growth at different temperatures, fermentation of various carbohydrates. However, since these methods are dependent on environmental conditions, they can be time consuming and have poor reproducibility [52]. On the other hand, molecular identification methods can provide more accurate and reliable results on microbial diversity and composition [50]. The identification of microorganisms with molecular methods is based on the comparison of the sequence of

genes. These methods can be either fingerprinting methods or can be based on mostly 16S ribosomal RNA analysis of the genome, since it has a manageable size (1.5kb) and a large database of sequences [51, 52]. Fingerprinting methods, of which unique patterns of bacterial species can be formed, or Polymerase Chain Reaction (PCR) based methods are used for comparison [52]. Some of the most used methods for molecular identification of LAB are pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), use of probes and ribotyping, rep-PCR, amplification rDNA restriction analysis (ARDRA), denaturating gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), real-time PCR and sequence analysis of ribosomal DNA (rDNA) [52].

PFGE is a fingerprinting method, in which large DNA fragments are separated by increasing pulse times in electrophoresis and fingerprint profiles and patterns of bacterial species can be obtained. This method can be time consuming, however it is found to be effective to separate a wide variety of LAB such as *Lb. acidophilus* complex*, Lb. casei, Lb. delbrueckii, Lb. fermentum, Lb. helveticus, Lb.plantarum, Lb. rhamnosus* and *Lb.sakei* in subspecies and strain level [52, 53]. RAPD is a method also based on the fingerprinting analysis. DNA fragments are amplified with short sequenced primers randomly using PCR and patterns created by DNA fragments are used in identification. Although it is used to identify LAB in food products, low reproducibility is a major drawback for this identification method [50]. In a study investigating the diversity of a fermented eggplant product, RAPD-PCR, as well as PFGE was used and among 149 *Lactobacillus* species including *Lb. plantarum, Lb. brevis, Lb. pentosus* and *Lb. fermentum* strains, 97-98% of bacteria were identified with 86% or higher reproducibility with RAPD method [54]. In RFLP (or chromosomal DNA restriction analysis), banding patterns are observed from the DNA fragments cut with specific restriction enzymes and variations in banding patterns are used to discriminate between LAB species. For more accurate results, the use of computer aided analysis might be needed due to complex banding patterns [50]. In a previous study, seven *Lactococcus* and 12 *Lactobacillus* species could be identified with single restriction from wines [55]. One of the methods used for identifying LAB with fingerprinting is ribotyping. Nucleic acid probes are used to create hybridization patterns of ribosomal genes [52]. Fingerprinting patterns created with this method are more stable and easy to interpret compared to RFLP method. In a previous study, which investigated the

identification of *Lb. johnsonii, Lb. casei, Lb. rhamnosus, Lb. acidophilus, Lb. plantarum*, and *Lb. fermentum* it was found that ribotyping was discriminatory at the species level rather than at strain level [56]. ARDRA is another fingerprinting method, using DNA sequences, amplified and then digested by restriction enzymes [57]. ARDRA is used to identify LAB in musts and wines and the technique was found reliable enough to discriminate 342 isolates. Species such as *Lb. brevis*, *Lb. collinoides*, *Lb. coryniformis, Lb. hilgardii, Lb. mali, Lb. paracasei, Leu. mesenteroides, Oenococcus oeni, P. parvulusand* and *P. pentosaceus* could be identified with this method [58]. Another considerably new method used to create fingerprinting patterns for identification of LAB is repetitive PCR (rep-PCR). Primers targeting various repetitive DNA elements are used in amplification [59]. *Lactobacillus* species isolated from fermented dry sausages could be identified at subspecies and strain level using this method, and species such as *Lb. alimentarius, Lb. curvatus, Lb. plantarum, Lb. sakei subsp. carnosus* and *sakei* were first detected with this method in sausages [59]. Although fingerprinting methods provide detailed information on the microflora of fermented food products in subspecies level, they produce complex banding patterns which are difficult to interpret and pure colonies are needed for identification.

LAB in mixed bacterial population instead of isolates, can be identified using methods such as DGGE and TGGE. These methods are based on the discrimination between 16S rRNA genes of individual LAB in mixed populations by the differences in melting temperature (TGGE) or the chemical stability (DGGE). The rRNA sequences are first amplified with primers for wider regions and DGGE or TGGE is used to separate different sequenced amplicons of the same length which differs according to species [52]. LAB found in dairy products such as cheeses and fermented milks, could be identified using TGGE in group, species and at the subspecies level [60]. Quantification of LAB in food samples can be made using real-time PCR. Amplification of the target DNA can be measured in real time by monitoring the florescence and was found to be more sensitive than conventional PCR [52]. LAB species, *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* were identified using 16S rRNA primers and melting curve analysis of real-time quantitative PCR and this method was suitable for rapid and accurate identification of closely related species while providing information on the quantification [52, 61]. Another approach to identify LAB species is amplification of DNA fragments of bacteria and

sequence analysis [51]. Taxonomic information can be obtained from the DNA sequences when compared with database, thus providing accurate information on identification [51, 62].

Species-specific PCR is one of the molecular methods dependent on 16S rDNA/rRNA sequences. Primers designed specifically for a genus or species is used to amplify 16S rDNA of the microorganism and used mostly to distinguish between close species [63]. This method can also be used as a culture-independent analysis in mixed populations like food products, thereby having an advantage of analyzing the product as a whole [64]. According to the literature, species-specific PCR was found as one of the most suitable methods for discrimination between closely related species such as, *Lb. casei/Lb. paracasei, Lb. rhamnosus*, and *Lb. zeae* species of *Lb casei* group or *Lb. plantarum* group species in vegetable matrices [65, 66]. LAB species such as, *Lb. curvatus*, *Lb. plantarum*, *P. acidilactici, Lb. lactis, and Enterococcus faecium* have been identified with species-specific PCR in fermented sausages which has a complex microbial composition and it reduced identification time while increasing the number of identified species from the food product [67]. This method was also used for accurate identification of LAB of *Bifidobacteria* species and was found as highly sensitive [68]. However, this method cannot detect the LAB which are not the target of the primers, therefore selection of species-specific primers is important to identify LAB in complex microbial environments like food [68].

2.1.2. Chemical Composition of Fermented Fruit and Vegetable Juices

All fruits and vegetable juices have components which are nutritional such as carbohydrates, fats, dietary fibers, vitamins, minerals and proteins, however phytochemicals contributed greatly to their antioxidant properties and possible beneficial effects related to oxidative stress [13, 69]. Phytochemicals are secondary metabolites of plants and protective bioactive compounds [69]. Among bioactive compounds, phenolic compounds are widely known for their antioxidant effects and considered as beneficial [13].

2.1.2.1. Phenolic compounds

Phenolic compounds are plant secondary metabolites responsible for defense against pathogens or ultraviolet radiation in plants, and the interest in phenolics are increasing with the understanding of their antioxidant and bioactive properties on diseases related with oxidative stress, such as cancer, cardiovascular and neurodegenerative diseases [70, 71].

More than 8,000 molecules are identified as phenolic compounds in the plant kingdom, which are found in most of the fruits and vegetables such as berries, nuts, tomatoes, cereals, teas or their beverages such as juices and wines. They can be classified into several different groups based on their structural differences however all have a common phenol (C6) ring (Figure 2.1). The number and arrangement of the carbon atoms determine the class of the phenolics and these molecules are often conjugated with sugar and organic acid residues, forming more complex structures [72, 73].

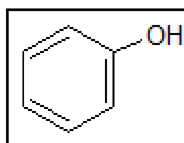


Figure 2.1. General structure of a simple phenol ring [72]

Phenolic compounds can be divided into classes and subclasses according to their structures and the phenol ring number they contain (Figure 2.2) [73]. Flavonoids represent the most common group of phenolics and they have a relatively low weight [72]. Their structure consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Figure 2.3) [72]. The major subclasses of flavonoids are flavonols, flavones, flavan-3-ols, isoflavones, flavanones and anthocyanidins [70-72].

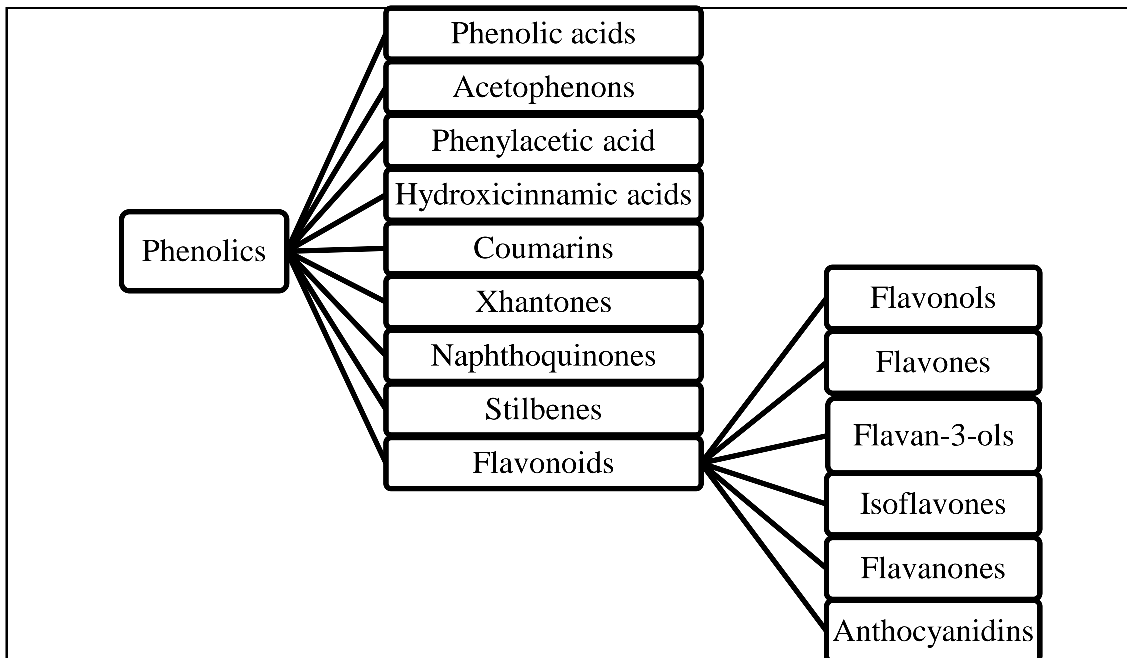


Figure 2.2. Phenolic classes according to their structure and phenol ring number [73]

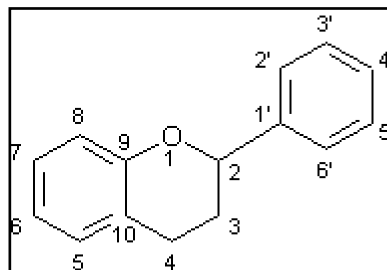
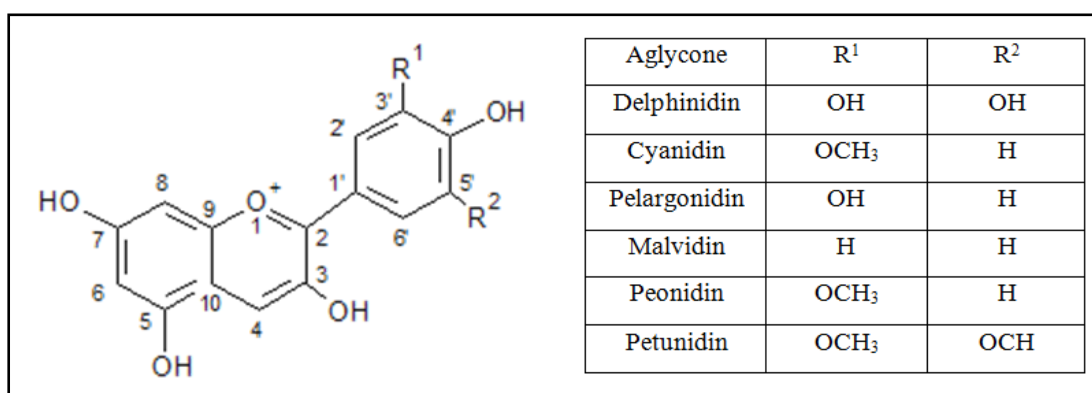


Figure 2.3. Flavonoid structure [73]

Most of the fruits and vegetables used in the production of lactic acid fermented beverages such as tomatoes, teas, pomegranates and black carrots are also rich in flavonoids. Tomatoes contain flavanones, which are mostly found in glycosylated forms in nature [70]. Flavonoids found in high concentration in green teas are flavanols. They can exist in two forms; monomer (catechins) and polymer (proanthocyanidin) forms [70]. Anthocyanidins can be found as glycosides and acylglycosides in red-blue colored fruits and vegetables, such as berries, grapes, black carrots and pomegranates. They are the most recognized group under flavonoids and are discussed further in the following sections [70, 74].

2.1.2.2. Anthocyanins

Fruits and vegetables such as cranberries, grapes and red cabbages, which are used to produce fermented juices, are also rich in anthocyanins which confer some health benefits. Anthocyanins, a class of phenolic compounds that are glycosides and acylglycosides of anthocyanidins are responsible for blue, purple and red colors of fruits and vegetables [75, 76]. All naturally occuring anthocyanins of fruits and vegetables are O-glycosylated forms of anthocyanidins. When the sugar substitute number is higher than three they can be attached to basic molecules such as coumaric, caffeic, ferulic, p-hydroxy benzoic, synapic, malonic, acetic, succinic, oxalic, and malic acids with acyl linkages [77]. Most common anthocyanidins are delphinidin, cyanidin, pelargonidin, malvidin, peonidin and petunidin (Figure 2.4) [73, 78].



Aglycone	R^1	R^2
Delphinidin	OH	OH
Cyanidin	OCH_3	H
Pelargonidin	OH	H
Malvidin	H	H
Peonidin	OCH_3	H
Petunidin	OCH_3	OCH

Figure 2.4. Chemical structure of anthocyanidins [78]

Anthocyanins are more resistant to light, low pH and oxidation conditions in acylated and glycosylated forms. Their degradation is also prevented by esterification with various organic acids and formation of complexes with other flavonoids [71]. Anthocyanins can be found and consumed daily in red colored vegetables and fruits in different amounts (Table 2.4) [71, 79]. Anthocyanins found in red cabbages, blackberries and sweet potatoes, which are used in fermented fruit juice production, are mostly cyanidin-based anthocyanins. Cyanidin-based anthocyanins are substituted with sugars such as glucose, xylose, galactose and sophorose and most of these anthocyanins are found in acylated forms [80].

Table 2.7. Some major anthocyanin sources and their anthocyanin amounts

Anthocyanin Source	Anthocyanin Content	Reference
Aubergine	7500 mg/kg	71
Blackberry	1000-4000 mg/kg	71
Black currant	1300-4000 mg/kg	71
Blueberry	250-5000 mg/kg	71
Black grape	300-7500 mg/kg	71
Cherry	350-4500 mg/kg	71
Rhubarb	2000 mg/kg	71
Strawberry	150-750 mg/kg	71
Red wine	200-350 mg/L	71
Plum	20-250 mg/kg	71
Red cabbage	250 mg/kg	71
Black Carrot	10-980 mg/kg	79

Anthocyanins of fruit and vegetables such as cranberries, red cabbages and pomegranates used to produce fermented beverages have been found to have functional properties and are thought be beneficial to health when consumed as a part of the diet due to their bioactive properties. These possible effects on health as anticarcinogenic, antiatherogenic, antiviral, and antiinflammatory substances are based mostly on their antioxidant capacities [81]. *In vitro* studies have demonstrated that cranberry anthocyanins can reduce cancer cell proliferation at 25-200 μg/mL concentration in human oral (KB, CAL-27) breast (MCF-7), colon (HT-29, HCT116), and prostate (LNCaP) tumor cell lines and inhibit tumor formation via effects on cell cycle regulator proteins (p53, p21, p27, cyclin D1, cyclin A, etc.) [78, 81-84]. They had also inhibited TNF-α, IL-1, IL-6, IL-8 production in activated macrophages and acted as modulators of the immune response in vitro [85, 86]. Studies on bioactivity of anthocyanins had demonstrated that consumption of anthocyanins has positive effects on cardiovascular health by decreasing LDL-cholesterol levels in blood and enhancing capillary permeability and strength [87]. Similarly, red cabbage anthocyanins also reduced colorectal carcinoma initiated by 1,2-dimethylhydrazine in rats and these properties are mostly related with their antioxidant capacities [88].

The most important feature of anthocyanins like most of the phenolic compounds is their antioxidant properties [76]. Metabolic reactions in humans lead the body to produce free

radicals called Reactive Oxygen Species (ROS). ROS are atoms or molecules with an unpaired electron on their orbital which change the chemical structures of surrounding molecules while pairing their electrons. Excessive production of ROS causes damage and dysfunction of enzymes, cell membranes and genetic material by free radical attack to proteins, lipids, and DNA [89]. Diseases such as cancers, cardiovascular diseases or osteoporosis are often associated with oxidative damage [70]. Antioxidants reduce the oxidative stress either by interrupting the propagation of the free radical chain or by binding ions involved in free radical formation and this can be measured by several methods such as DPPH, ABTS and FRAP methods [31].

2.1.2.3. Determination of Antioxidant Properties of Anthocyanins

The antioxidant capacities, health promoting effects and preventive effects on diseases like cancer and cardiovascular diseases of anthocaynins are reported in many studies [80, 90]. The antioxidant potency of anthocyanins and other phenolics is widely studied with different methods *in vitro* using their ability to reduce different oxidants [91]. Several antioxidant methods are used to determine the antioxidant capacity according to antioxidants' response to free radicals *in vitro*, however a standardized antioxidant capacity determination method for food products is not available due to their complex structure and different antioxidant mechanisms [91]. *In vitro* antioxidant methods are either Hydrogen Atom Transfer (HAT) based or Single Electron Transfer (SET) based assays [91, 92].

HAT-based methods measure the antioxidant's ability to donate hydrogen atoms to free radicals. They are relatively faster than SET based methods and more independent from solvent and pH effects [91]. They are based on the competitive reaction kinetics and methods such as Oxygen Radical Capacity (ORAC), Total Radical Trapping Parameter (TRAP) and β-carotene bleaching assays use this mechanism to measure the antioxidant capacity. ORAC (Oxygen Radical Absorbance Capacity) method, measures the inhibition of peroxyl radicals such as 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) by antioxidants. A hydrogen atom is transferred to free radical from antioxidant and the antioxidant capacity is measured spectrophotometrically from fluoresence product produced by reaction of peroxyl radicals with probes [93]. Cyanidin-based anthocyanins from various sources such as blackberries, black carrots, red cabbages and sweet potatoes were investigated for their oxygen radical absorbing capacities (ORAC) and the

antioxidant capacity was found to be highly related to the anthocyanin concentration of the source [80].

SET-based methods measure antioxidant's ability to transfer one electron to free radical compounds. They are based on the percent decrease of free radical rather than kinetics, therefore are slower than HAT based methods. Methods such as Ferric Reducing Antioxidant Power (FRAP), Copper Reduction Assay (CUPRAC), ABTS/TEAC (2,2'-azonobis(3-etilbenzothiazoline-6-sulfonat) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays are used to determine the antioxidant capacity [91, 94-96]. The expression of antioxidant capacities in SET based methods is also not standardized and several methods are used to calculate and express the antioxidant capacity. One of the most common methods is using reference substances such as Trolox (6-hydroxy-2,5,7,8-tet-ramethychroman-2-carboxylic acid), a water soluble vitamin E derivative, gallic acid and catechin [70]. Another way to express antioxidant capacity is using EC_{50}, mostly in DPPH assays. EC_{50} is the initial concentration to decrease 50% of the free radical [92].

Among SET based methods, FRAP method measures the reduction of ferric salt Fe (III)$(TPTZ)_2Cl_3$ to Fe (II)$(TPTZ)_2Cl_3$ by antioxidants at acidic pH at 593 nm, which is the maximum absorbance wavelength [96] (Figure 2.5). Antioxidant capacities of fruit juices such as orange, apple, pineapple and grapefruit juices were determined with FRAP method and were found to be closely related to their total phenolic contents [97]. It is also reported that fruits containing anthocyanins such as berries, cherries and pomegranates has shown higher antioxidant capacity than other sources of plant phenolics such cereals and vegetables [97-100].

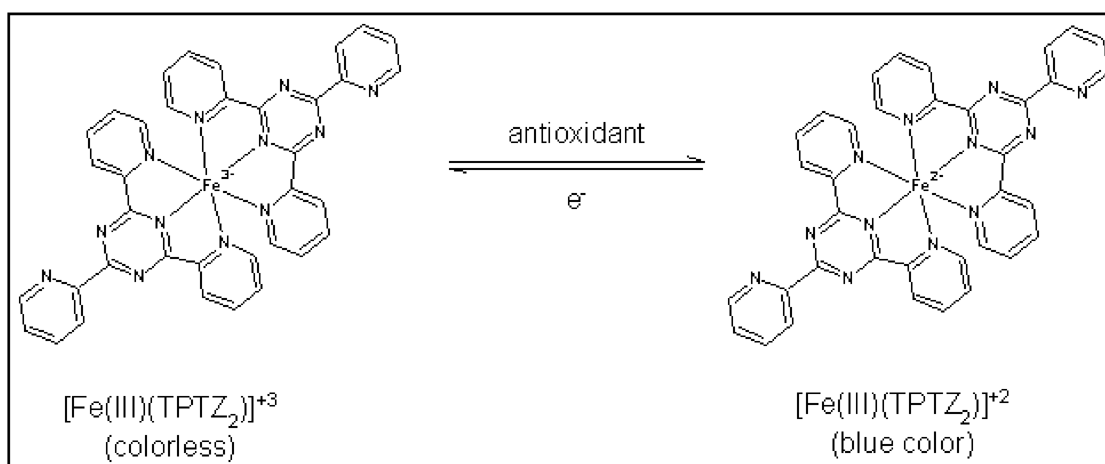


Figure 2.5. Reduction of ferric salt in the presence of an antioxidant [91]

ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity assay, which is also named as TEAC assay, is based on the measurement of reduction of previously formed blue colored radical ABTS•$^{+}$ (free radical form). ABTS•$^{+}$ is produced prior to reaction with antioxidant from ABTS with addition of potassium persulfate [94] (Figure 2.6). Among fruit beverages such as pomegranate, grape, orange and cranberry juices, anthocyanin rich beverages such as cranberry and pomegranate juices have shown higher antioxidant capacity than other juices in ABTS assay [99].

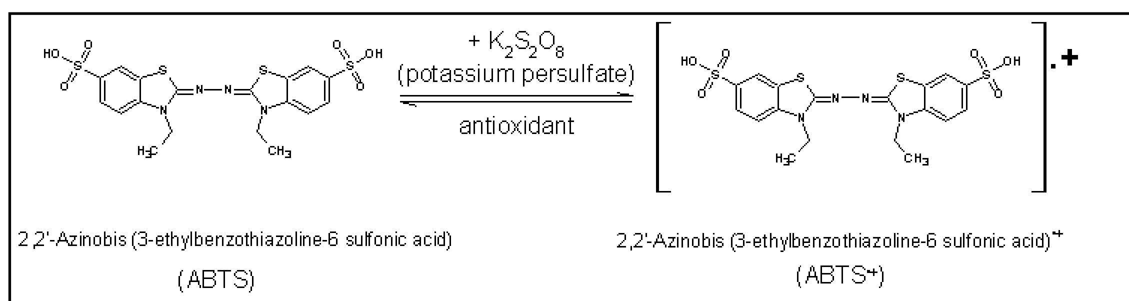


Figure 2.6. Formation of ABTS$^{\bullet+}$ radical in the presence of potassium persulfate [92]

DPPH (2,2-Diphenyl-1-picrylhydrazyl) method for antioxidant analysis measures antioxidants' ability to reduce DPPH•, thus the color change at 515 nm (Figure 2.7). The DPPH Radical Scavenging Activity assay was originally developed by Brand-Williams, Cuvelier, & Berset [95] and adapted to 96-well microplates by Fukumoto & Mazza [101].

Reduction of DPPH• radical in presence of antioxidants to DPPH-H is measured spectrophotometrically at 515 nm. Flavonoid rich extracts of fruits such as cranberries, has shown the highest activity among extracts of whole fruits and the EC_{50} value of these extracts varied from 17.3 to 34.6 µM in DPPH assay [83].

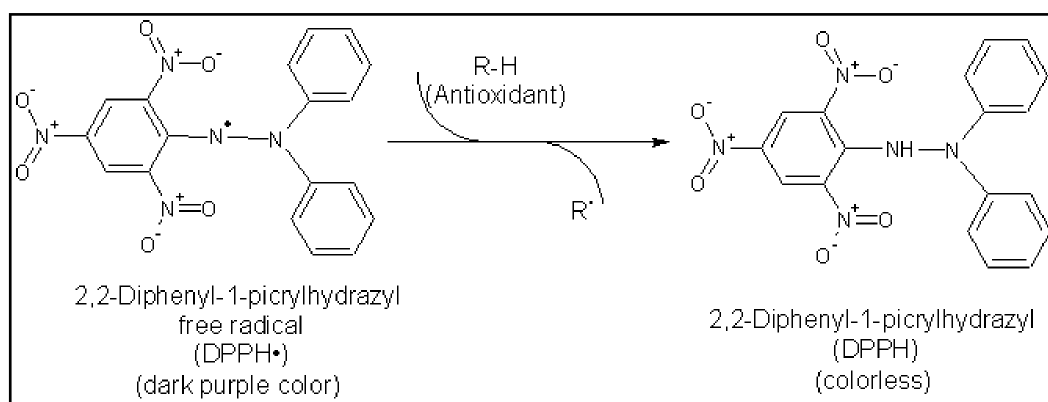


Figure 2.7. Reduction of DPPH• free radical to DPPH in the presence of an antioxidant [92]

Other than antioxidant capacity assays, total phenolic contents of anthocyanin rich products are determined, which are related to their antioxidant capacities. Folin-Ciocalteu reagent based assay is used widely for phenolic content determination and is similar to SET-based antioxidant methods [92]. The blue color formed when electrons were transferred from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes in the F-C reagent in alkaline environment is measured at 760 nm, however the exact chemical nature of F-C reagent is unkown [102].

2.2. SHALGAM JUICE

Shalgam juice, a dark red colored, cloudy and sour fermented beverage, has been a popular drink in western parts of Turkey, as well as in southern eastern region of Turkey, where it is mostly produced and consumed [24, 29, 103]. Shalgam juice is defined by Turkish Standards Institution (TSE) in TS 11149 standard as "The product produced by lactic acid fermentation of turnip (*Brassica rapa*), black carrot (*Daucus carota*), chilli powder and extract obtained from the lactic acid fermentation of bulgur flour, sourdough, drinking water and salt, which can be heat treated for preservation, if desired" [104]. Black carrot, bulgur flour, dough, salt and water are the main ingredients of shalgam juice [24, 29, 103]. Turnip is a minor ingredient and used only if available but the use of it gives positive sensory characteristics [24, 103]. The dark color of the beverage originates from black carrot anthocyanins and its sour taste originates from lactic acid which is produced by fermentation with lactic acid bacteria [24, 29].

2.2.1. Ingredients of Shalgam Juice

2.2.1.1. Black Carrots: Anthocyanins, Antioxidant Capacity and Health Effects

Carrot (*Daucus carota* L.), the main ingredient of shalgam juice, has been cultivated for thousands of years and used as a food source for human nutrition [24]. Carrot cultivars are botanically classified into two groups; Carotene group: the orange colored group grown worldwide and Anthocyanin group: dark purple-black colored group, grown in eastern countries like Turkey, Afghanistan, Egypt and India [79, 105]. Black carrot is cultivated in several parts of Turkey, mostly in the middle part of Anatolia, in Eregli (Konya) [106]. The amount of black carrot used in the production of shalgam juice has a determining effect on acidity and the color of the beverage. As seen from Table 2.5., the chemical composition of black carrot mainly consists of sugar, protein, and minerals such as calcium and sodium. It has a high amount of (142-425 g/kg) sugar [105] with sucrose as the predominant sugar, followed by fructose and glucose [79, 107]. However, the amount and the ratio of sugars change according to cultivation and storage conditions [107]. 10-20% (w/w) black carrot is necessary for shalgam juice production during fermentation [103].

The solid content, protein, and mineral content of black carrot also depends on the region and cultivation conditions [106] (Table 2.5.)

Table 2.8. Chemical composition of black carrot [79, 105-107]

	Amount (g/kg)
Sugar (Total)	142.0-397.0
Sucrose	41.1-381
Fructose	5.8-101
Glucose	6.9-131
Total solid content	142.3-159.6
Protein	7.0-13.8
Calcium	0.48-0.65
Sodium	0.3-0.45

Unlike orange colored cultivars, black carrots contain anthocyanins. Total anthocyanin amounts in black carrots were found between 17.4 and 45.4 g/kg dry matter and most of these anthocyanins were found in acylated forms with the proportion of 55% to 99% of total anthocyanins [79]. Five major anthocyanins were identified in black carrots. Two of them are non-acylated anthocyanins; cyanidin 3-xylosylglucosylgalactoside (cya 3-xylglcgal) and cyanidin 3-xylosylgalactoside (cya 3-xylgal). Three of them are derivatives of cya 3-xylglcgal, which are acylated with sinapic acid (cyanidin 3-sinapoylxylosylglucosylgalactoside), ferulic acid (cyanidin 3-feruloylxylosylglucosylgalactoside) and p-coumaric acid (cyanidin 3-p-coumaroylxylosylglucosylgalactoside) [79, 108, 109] (Figures 2.8&9). The acylated anthocyanins in black carrots have enhanced stability against heat and light and thus extended shelf-life compared to non-acylated anthocyanins [110]. The reason is explained by the protection of acylated anthocyanins from hydrophilic attack by their acyl moieties [111].

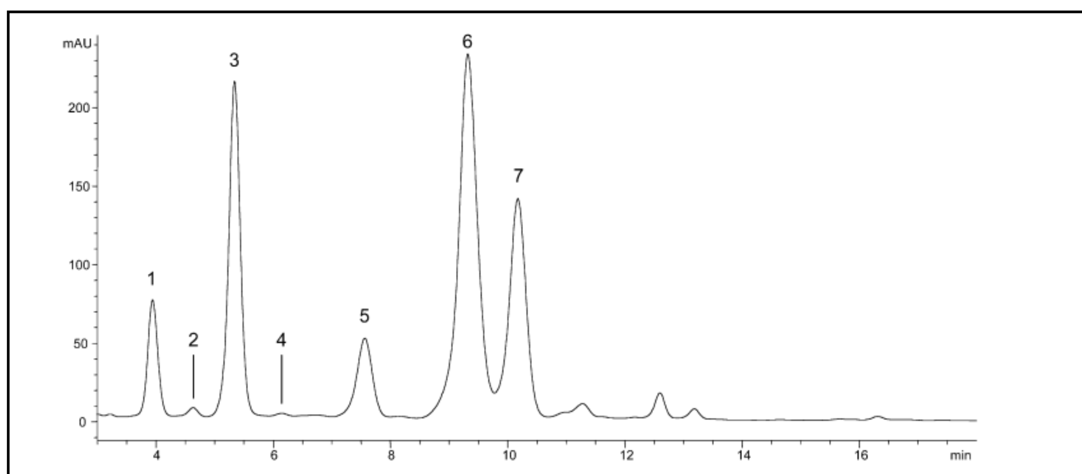
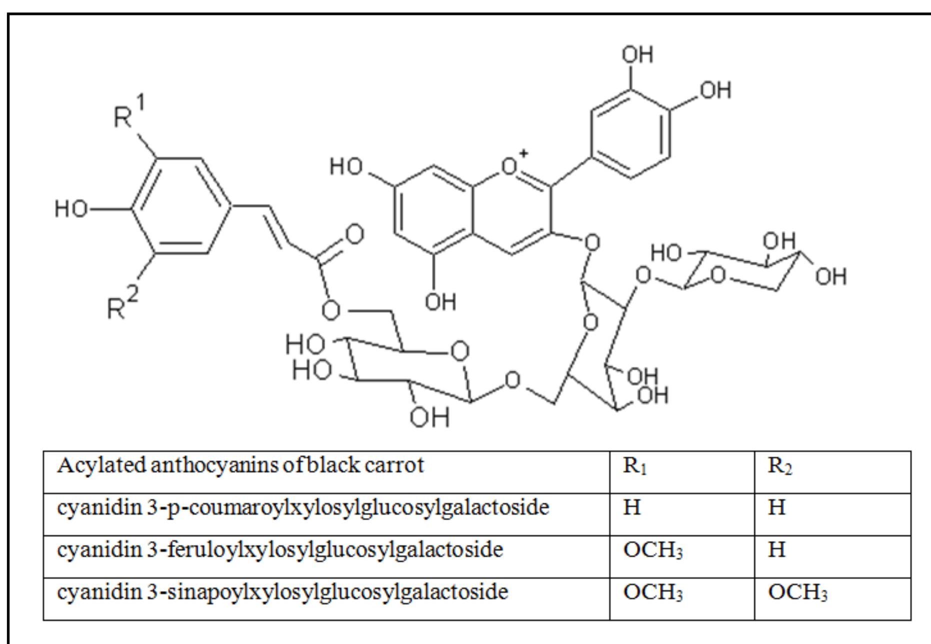


Figure 2.8. Anthocyanin profile of black carrot extracts are determined by High Performance Liquid Chromatography (HPLC) at 520 nm using a G1315A diode array detector. Peak assignment: (1) cyanidin 3-xylosylglucosylgalactoside (cya 3-xylglcgal), (2) caffeic acid derivative of cya 3-xyl-glcgal, (3) cya 3-xylgal, (4) p-hydroxybenzoic acid derivative of cya 3-xylglcgal, (5) sinapic acid derivative of cya 3-xylglcgal, (6) ferulic acid derivative of cya 3-xylglcgal, (7) p -coumaric acid derivative of cya 3-xylglcgal [79]

The stability of black carrot anthocyanins are also dependent on environmental factors such as temperature, solid content and pH [112]. Anthocyanins of black carrots were degraded with heat and degradation rate increased with increasing solid content of the carrot. Since stability of acylated anthocyanins increased in lower pH, black carrot anthocyanins which are mostly acylated with sinapic, ferulic and p-coumaric acid were found more stable in low pH [112].



Acylated anthocyanins of black carrot	R_1	R_2
cyanidin 3-p-coumaroylxylosylglucosylgalactoside	H	H
cyanidin 3-feruloylxylosylglucosylgalactoside	OCH_3	H
cyanidin 3-sinapoylxylosylglucosylgalactoside	OCH_3	OCH_3

Figure 2.9. Black carrot anthocyanins

The anthocyanins of black carrot have shown strong antioxidant capacities in numerous *in vitro* studies [81]. Black carrots were high antioxidant capacity containing group among Asian vegetables, when compared the antioxidant capacities of ethanol extracts with vegetables such as ginger (71.8%) and tomato (70.8%) [113]. The antioxidant capacity of ethanol and water extracts of black carrot were examined by β-carotene bleaching method and found as 73.0% and 61.8% antioxidant capacity, respectively [113].

The antioxidant capacity of black carrots is affected by processing and extraction methods [114-116]. Antioxidant capacity of black carrot extracts were determined using DPPH method after drying with different (microwave and hot air drying) methods [114]. While EC_{50} was found as 30.23 mg sample/mg DPPH in raw black carrot samples, it was 7.80 to 61.44 mg sample/mg DPPH in dried samples, which was explained by higher anthocyanin content in dried samples or blanching process used in raw black carrot samples. The antioxidant capacity of enzyme treated black carrot juice was also determined with FRAP and CUPRAC methods and the enzyme treated juice was found to have 30% greater antioxidant capacity than straight pressed one since recovery of anthocyanins are higher in enzyme-assisted extraction [115]. In another study, the effect of different lipophilic and hydrophilic extraction methods on the antioxidant capacity of different colored freeze dried

carrots were examined by ABTS and DPPH methods [116]. It was found that purple/black carrots have the highest antioxidant capacity in hydrophilic extracts among other varieties when compared with lipophilic extracts. The antioxidant capacity of hydrophilic extracts was 0.28 and 0.13 μmol TE/mg, in ABTS and DPPH assays, respectively [116].

The potential beneficial effect of black carrots on human cells regarding its antioxidant capacities has also been studied. In one of the studies, anthocyanin-rich extracts of black carrot concentrates have shown significant inhibition on the growth of HT-29 (colorectal adenocarcinoma) and HL-60 (promyelocytic leukemia) cells in a dose-dependent manner in vitro [117]. Acylated and non-acylated anthocyanins in extracts inhibited proliferation in both of the cancer cell lines and 80% suppression was observed at the highest concentration (2.0 mg/mL).

In another study, the bioavailability of black carrots was studied in healthy volunteers *in vivo* [118]. The bioavailability of black carrot anthocyanins in human body was determined by checking the anthocyanin concentration in plasma. The anthocyanins were detected in the first 8 hours and were related with the consumed concentration but the non-acylated anthocyanin concentration in blood was higher than acylated anthocyanins [118].

2.2.1.2. Bulgur Flour and Dough

Bulgur flour is another ingredient used in shalgam juice production. It is a special kind of flour obtained from boiled and ground wheat [24]. In bulgur flour the total sugar amount varies between 2.23 to 3.30 g/100g and starch amount varies between 4.45 to 5.84 g/100g (Table 2.6). Bulgur flour is the nutrient and carbon source of microorganisms that play a role in shalgam juice fermentation [24]. In lab-scale production, bulgur flour is fermented with sourdough (baker's yeast dough) before it is used by adding water [24].

On the other hand, in the commercial production of shalgam juice, the use of sourdough is not always necessary [119]. Generally, bulgur flour is fermented with the addition of water and incubated at 30-34ºC for 3 days without the presence of sourdough.

Table 2.9. Chemical composition of bulgur flour [103]

	Amount (g/100g)
Total sugar	2.23 - 3.30
Starch	4.45 - 5.84
Protein	12.4 - 17.6
Ash	2.32 - 3.59
Iron	0.58 – 0.8
Potassium	26.9 – 45.5
Calcium	13.95 – 15.98
Sodium	2.98 – 4.74

2.2.1.3. Other Ingredients

Other ingredients used in shalgam juice production are salt, drinking water and if available turnip. Some commercial shalgam juice producers uses turnip in production [24] which contains glucose (14.1 g/kg), fructose (11.0 g/kg), and sucrose (2.06 g/kg) [24, 120]. Salt used in shalgam juice production is rock salt. To control microbial growth and fermentation flora, 1-2% of salt is added to containers. Growth of pathogen microorganisms is inhibited by salt, while LAB growth is induced since *Lactobacillus* spp. are resistant to high concentration of salt in fermented products [30, 121]. Also, to control the spoilage and growth of yeasts and molds, sodium benzoate, a sodium salt of benzoic acid is used as chemical preservative in 0.02% (w/w) concentration and added at the end of the fermentation [122, 123]. Use of sodium benzoate in shalgam juice is regulated by Turkish Food Codex and maximum dosage is determined as 0.02% [123]. FDA is also determined the maximum dosage of sodium benzoate in food products as 0.1% [124].

2.2.2. Shalgam Juice Production

For shalgam juice production, a standard method is not available for commercial and home-scale production [24, 103]. The fermentation is spontaneous however addition of a starter culture for fermentation is also used in controlled lab-scale production but not preferred in commercial production since shalgam juice fermentation cultures are not commercially available [24, 106, 125]. Addition of 15% (w/w) shalgam juice from previous production is also a known technique [24, 103, 106].

The production of shalgam juice is based on the lactic acid fermentation and carried out by homo and heterofermentative lactic acid bacteria [29]. At the end of the fermentation, lactic acid is produced as a primary metabolite, pH of the carrot juice drops to 3.3-3.8 and this gives shalgam juice a sour taste. Ethyl alcohol, acetic acid, and carbon dioxide (CO_2) is also produced as secondary metabolites.

In shalgam juice production, two main production methods are used which are called "Traditional Production" and "Direct Production". Traditional production consists of two stages of fermentation and direct production consists of single stage fermentation by mixing all ingredients. Both of the methods are used in commercial, small-scale and lab-scale production, although most of the commercially available products are produced with traditional production [24, 106, 122].

The steps of the two-stage fermentation process, which is also called traditional production, are fermentation of dough (first fermentation) and fermentation of carrot (second fermentation) [24]. The first fermentation step is important for enrichment of LAB and yeasts. Bulgur flour, sourdough and water are mixed and left for fermentation for 3-5 days at room temperature or at 30-34 °C. During first fermentation the acid content of mixture increases and pH drops due to LAB activity. The bulgur dough extracts from first fermentation are combined in tanks or containers with chopped black carrots (10–20%, w/w), salt (1–2%, w/w), water and if available but not necessarily turnips (1–2%, w/w) for second fermentation [24, 103]. After 3-10 or 30-35 day fermentation at 10-35°C a red colored, sour beverage is obtained. Sodium benzoate, the chemical preservative is added after fermentation and shalgam juice is filtered before packaging (Figure 2.10).

In single stage production (direct method), all ingredients without a primary fermentation are mixed. Chopped black carrots, bulgur flour, salt and water are mixed and left for fermentation for 3-10 days at 10-35°C. After fermentation, sodium benzoate is added, beverage in tanks are filtered and bottled [24] (Figure 2.10).

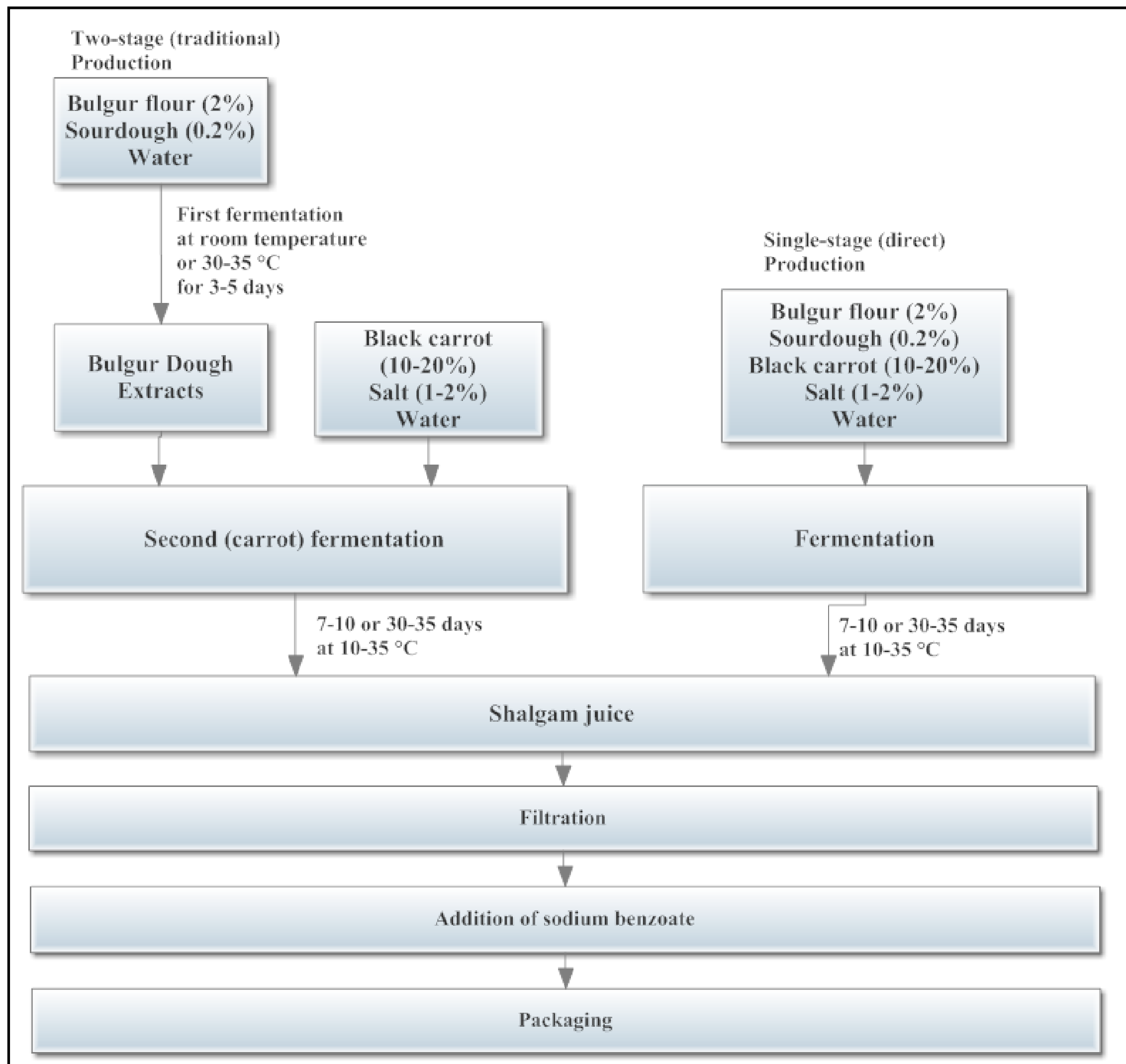


Figure 2.10. Shalgam juice production by two-stage (traditional) and single-stage (direct) production methods

2.2.3. Composition of Shalgam Juice

2.2.3.1. Chemical Composition of Shalgam Juice

The chemical composition of commercially available shalgam juices or juices produced in lab-scale using direct and traditional techniques, with the addition of starter cultures or other ingredients such as red beets, turnips are given in Table 2.7 with the standards for commercial shalgam juice established by Turkish Standards Institution [103, 104, 106, 122, 126-129].

Table 2.10. Chemical composition of different shalgam juices

	Ozler, 1995 [109][a]	**Tangüler, 2010 [93][b]**	**Güneş, 2008 [127][c]**	**Utuş, 2008 [128][d]**	**Canbas, 1984 [91]**	**Deryaoglu, 1990 [110]**	**Arici, 2004 [111]**	**Öztürk, 2009 [112]**	**Cakır, 2011 [113]**	**Tangüler, 2012 [126]**	**Turkish Standards [108]**
	Lab-scale produced shalgam juice						Commercial shalgam juice				
Total acidity as lactic acid (g/L)	5.2-8.9	6.36-9.27	4.95-7.45	7.15-7.75	3.9-10.7	6.64-9.91	1.06-7.18	3.92-10.85	6.3-12.6	6.24-7.25	≥6.0
Lactic acid (g/L)	-	5.50-8.17	-	5.6-6.3	-	5.18-8.44	0.57-3.63	2.61-8.75	-	2.66-4.74	4.5-5.5
Volatile acidity as acetic acid (g/L)	0.3-0.7	0.76-1.06	0.69-0.80	0.76-0.94	-	0.6-1.2	-	0.75-1.80	0.52-3	-	0.7-1.2
pH	3.34-3.77	3.43-3.56	3.39-3.49	3.45-3.53	3.35-3.85	3.33-3.67	3.16-3.60	3.26-3.86	3.31-4.13	3.28-3.48	3.3 – 3.8
Alcohol as ethyl alcohol (g/L)	0.5-4.0	4.21-5.90	3.29-4.12	3.0-3.72	-	1.32-7.30	≤ 5	0.19-4.76	-	0.79-5.03	-
Soluble solids (%) (w/w)	1.69-3.02	2.48-3.15	2.03-2.67	2.26-2.38	2.20-3.0	2.29-2.92	-	2.07-3.19	2.5-4	-	≥2.5
NaCl (%) (w/w)	0.82-1.77	0.95-1.15	1.16-1.20	1.2-1.35	1.17-2.05	1.38-1.98	-	1.12-1.78	1.17-2.57	-	≤.2.0
Protein (g/L)	0.38-1.82	1.85-2.65	-	-	-	0.88-1.83	-	-	-	-	-
Ash (%) (w/w)	0.85-2.23	1.17-1.46	1.27-1.52	1.41-1.62	-	1.46-2.07	-	1.12-2.07	1.32-1.97	-	≤2
Total carbohydrate (g/L)	0.1-0.6	0.5-0.8	0.26-0.29	0.09-0.2	-	0	-	-	-	-	-

a. Shalgam juice produced with the addition of ingredients such as red beet and turnip, b. Shalgam juice produced with starter cultures, c. Shalgam juice produced with various amounts of black carrot, d. Shalgam juice produced with various sizes of black carrot

Shalgam juice is a lactic acid fermented beverage, therefore lactic acid is produced by microorganisms as the major end-product of fermentation and total acidity of shalgam juice is expressed as lactic acid. In commercially available shalgam juices, total acidity was found between 1.06-12.6 g/L, which must be above 6.0 g/L in commercial products, according to Turkish Standards Institution [103, 104, 122, 128-130]. It was suggested that the difference between the total acidity values of shalgam juices obtained from various manufacturers was not dependent on the production time [103]. Total acidity of shalgam juice was found to be affected also by different formulations. The effect of the amount and the surface area (size) of the black carrot, the main ingredient of shalgam juice, on the chemical composition was investigated in controlled production studies. It was found that the higher amounts (10-20%, w/w) of black carrot increased the acidity [131]. However, the change in the acidity of shalgam juice produced with various sizes of black carrot (3-9 cm) was not significant [132]. The acidity was also not affected by the use of bulgur, instead of bulgur flour and pure yeast culture instead of sourdough [103]. On the other hand, addition of starter cultures such as *Lb. plantarum* species increased the acidity of shalgam juice while ingredients such as red beet and turnip decreased the acidity [126]. It was also suggested that products with these additional ingredients have very dissimilar organoleptic properties than traditional product and are not preferred [126, 133]

Lactic acid is the dominant acid in the product and was found between 0.57-8.75 g/L in commercial shalgam juices [122, 127, 128, 130]. It was also found to be increased by the addition of starter culture, *Lb. plantarum* and the surface area of black carrots used in fermentation in lab-scale production studies [106, 132].

Volatile acids are produced by LAB during fermentation and acetic acid was found as the dominant volatile acid in shalgam juice production. Therefore volatile acidity of shalgam juice was expressed as acetic acid and found in the range of 0.6 and 3 g/L in commercial products [122, 127, 129]. However it is observed that unlike total acidity, the change in the volatile acid amount in shalgam juices produced with the addition of various sizes (3-9 cm) and amounts (10-20%, w/w) of black carrots, starter cultures and ingredients such as red beets and turnips, was insignificant and found between 0.3-1.60 g/L [106, 126, 131, 132].

pH of the commercial shalgam juices was found between 3.16 and 4.13 [103, 122, 127-130]. The pH of shalgam juice produced in controlled studies in lab scale (3.34-3.77) was also found to be affected by the amount of black carrot used in production and use of starter cultures and but not by the surface area of the carrot [105, 130, 131].

Shalgam juice fermentation is carried out by both homofermentative and heterofermentative LAB and ethyl alcohol is produced as an end-product of fermentation in low amounts (0.19-7.30 g/L) in commercial products [122, 127-130]. It was also found as 0.5-5.90 in controlled production studies and increased with the use of starter cultures and higher amounts of black carrots used in production [106, 126, 131, 132].

Soluble solid content of shalgam juice consists of organic acids, salt, protein and minerals and was found between 2.07 and 4.00% (w/w) in commercial products [103, 122, 127, 129]. Soluble solid content in controlled production studies (1.69-3.15%) has increased with the amount of black carrot but was not affected by the surface area of the carrot used in production [126, 131, 132]. Addition of starter cultures has also affected total solid content [106]. Sixty four per cent of solid content is salt which is added during fermentation to control the microflora in the concentration of 2% (w/w). It was found in the commercially available shalgam juices in the range of 1.12-2.57% and between 0.82 and 1.77% in lab-scale produced shalgam juices [103, 106, 122, 126-129, 131, 132].

The sugars in shalgam juice are in very low amounts because they are used in fermentation as carbon sources of lactic acid bacteria and are not specified in standard [24, 104]. Total carbohydrate amount of shalgam juices produced in controlled studies was found between 0.09 and 0.8 g/L and the amount or the size of black carrot used in fermentation, addition of ingredients such as turnips and red beets, and starter cultures had no effect on the sugar amount of shalgam juice [106, 126, 131, 132]. Proteins of shalgam juice were also not specified in standard and determined in low amounts (0.38-2.65 g/L) in commercially available and lab-scale produced shalgam juice [106, 126, 127]. Also, some minerals such as potassium (300-1000 mg/L), phosphorus (10.6-22.2 mg/L), calcium (89-173 mg/L) and iron (0.2-2.9 mg/L) were found in small amounts in lab scale produced shalgam juice [127].

In addition to other chemical components, as a black carrot beverage, shalgam juice contains anthocyanins that are present in black carrot [79, 125, 134]. The anthocyanin profiles of commercial shalgam juice samples were determined by high performance liquid chromatography (HPLC) and spectral measurements [125, 134]. The 5 anthocyanins in black carrot, cyanidin 3-xylosylglucosylgalactoside, cyanidin 3-xylosylgalactoside, cyanidin 3-sinapoylxylosylglucosylgalactoside, cyanidin 3-feruloylxylosylglucosylgalactoside and cyanidin 3-p-coumaroylxylosylglucosylgalactoside were present in shalgam juice [125, 134]. The effect of temperature during storage on shalgam juice anthocyanins, therefore on product quality was investigated and the degradation of anthocyanins was measured under different storage condition with various temperatures [125]. It was found that non-acylated anthocyanins in shalgam juice, cyanidin-3-xylosylgalactoside and cyanidin-xylosylglucosylgalactoside, were more sensitive to storage conditions than the acylated anthocyanins of shalgam juice as expected since acylated anyhocyanins are more stable to environmental conditions. Therefore, the anthocyanin profile and the quality of shalgam juice was affected by storage conditions [125]. In a more recent study of the same group, the only anthocyanin source of shalgam juice was determined as black carrot in different group of samples with various storage times by HPLC. In shalgam juice, anthocyanins found to be degraded with increasing storage time (0-17 months), as well as the storage temperature (0-40°C) [134].

2.2.3.2. Microbiological Composition of Shalgam Juice

Shalgam juice is a fermented vegetable juice and lactic acid bacteria are responsible from the fermentation by converting sugars to lactic acid and other byproducts [1]. Information on the complex microflora of shalgam juice is limited and recent [29, 128, 135].

In previous studies some of these microorganisms were identified using classical culturing techniques [29, 128, 135]. *Lactobacillus plantarum subsp. arabinosus*, *Lactobacillus brevis* and *Lactobacillus fermentum* species were identified from the colonies on agar plates inoculated with shalgam juice with biochemical tests [135]. In another study, LAB in 25 commercial shalgam juices were identified as *Lactobacillus rhamnosus, Lactobacillus paracasei ssp. paracasei, Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus fermentum* and their metabolisms such as lactic acid production properties, were determined [128].

In a more recent and detailed study, LAB of shalgam juice were isolated and identified from large and small scale productions with commercially available kits (API) based on the phenotypical methods [29, 130]. The occurrence and growth of bacteria during different fermentation steps was observed and *Lb. plantarum* and *Lb. paracasei subsp. paracasei* was found as the dominant LAB species in shalgam juice. The morphological, physiological and biochemical properties of these bacteria as well as other bacteria such as *Lb. brevis, Lb.fermentum, Lb. delbrueckii subsp. delbrueckii, Leuconostoc mesenteroides subsp. mesenteroides, P. pentosaceus*, which were found in the beginning of fermentation steps however couldn't survive during the fermentation process were used in identification of microflora [29]. Bacteria isolated from shalgam juice, *Lb. plantarum, Lb. paracasei subsp. paracasei* and *Lb. fermentum* were used as starter cultures to produce shalgam juice and the use of starter cultures found to affect the product quality, physical, chemical and sensory properties were also investigated [106].

2.2.3.3. Bioactive Properties of Shalgam Juice

Shalgam juice is a fermented vegetable juice rich in black carrot anthocyanins and lactic acid bacteria which are well known for their antioxidant properties and beneficial effects on health [32, 115, 117].

The antioxidant properties of ethanol extracts of shalgam juice compared to black carrot and black carrot juice concentrate extracts were studied in a study with DPPH, ABTS•+, metal chelating activity, linoleic acid emulsion system and reductive potential methods [136]. It was found that shalgam juice extracts had higher antioxidant capacity than black carrot extract but not black carrot juice concentrate extract in DPPH method with 33.57 μmol TE/g antioxidant capacity. However, the antioxidant capacity of shalgam juice in ABTS method showed lower values than DPPH method, thus suggesting the comparison between antioxidant capacity methods can't be directly made because they depend on different reactions and conditions [136].

Although chemical composition, microbiological composition and production techniques of shalgam juice were widely investigated, there is only one study on the antioxidant capacity and no study on the anti-proliferative effect of shalgam juice in the literature. To understand the possible beneficial effects and bioactive properties of shalgam juice, its

antioxidant capacity, its effects on human cells and anti-proliferative, apoptotic, immune stimulating properties, in relation to the chemical composition and probiotic potential, is still needed to be studied.

2.3. AIM OF THE STUDY

The aim of this study was to characterize shalgam juice's chemical and biological composition to understand further health effects and bioactive properties by determining its antioxidant capacity, identifying its microflora with molecular approach using species-specific PCR, and checking its anti-proliferative effects on colorectal carcinoma (Caco-2) cell lines in vitro using MTS assay.

3. MATERIALS AND METHODS

3.1. SHALGAM JUICE SAMPLES

Commercial shalgam juice, black carrot and dough samples were provided kindly by Company A, Adana, Turkey. According to information provided by the company, shalgam juice production was carried out using the traditional method. Shalgam juice production by Company A can be described as follows: Black carrots, which are stored at cold rooms after harvesting, are washed and chopped (3-6 cm) into pieces and packed in bags. Meanwhile, bulgur flour is fermented at 30-34°C for 3 days by addition of water. Then, 24 kg (0.7%, w/w) fermented bulgur dough was transferred into cotton bags and mixed with 600 kg (18%, w/w) black carrot in bags following addition of 45 kg (1.4%, w/w) salt by addition of water in 3.3 ton tanks for lactic acid fermentation. The production is made in small (200-300 L) or in 10 ton tanks. After 30-35 days at room temperature, shalgam juice in containers are filtered and transferred for packaging. For hot shalgam juice production, pickled hot pepper juice is added before packaging. (Figure 3.1)

While chemical composition, microflora identification and cytotoxicity studies were done using company A's shalgam juice (sample Aa1), antioxidant and total phenolic content assay analyses were carried out using products of different commercially available brands (A, B, C, D) and home-made (E) shalgam juice. All companies had both regular (A1, B1, C1, E1) and hot (A2, B2, C2, D2, E2) shalgam juices except Company D, which did not have regular shalgam juice. Besides, Company A had 2 different products coming from two different process lines (non-treated, Aa and heat-treated, Ab). According to information provided from companies, all shalgam juices were produced using traditional production method. Freshly squeezed black carrot juice and commercially available pomegranate juice were used as controls for the antioxidant analysis. Black carrots were supplied either from Company A or a local supermarket in Istanbul.

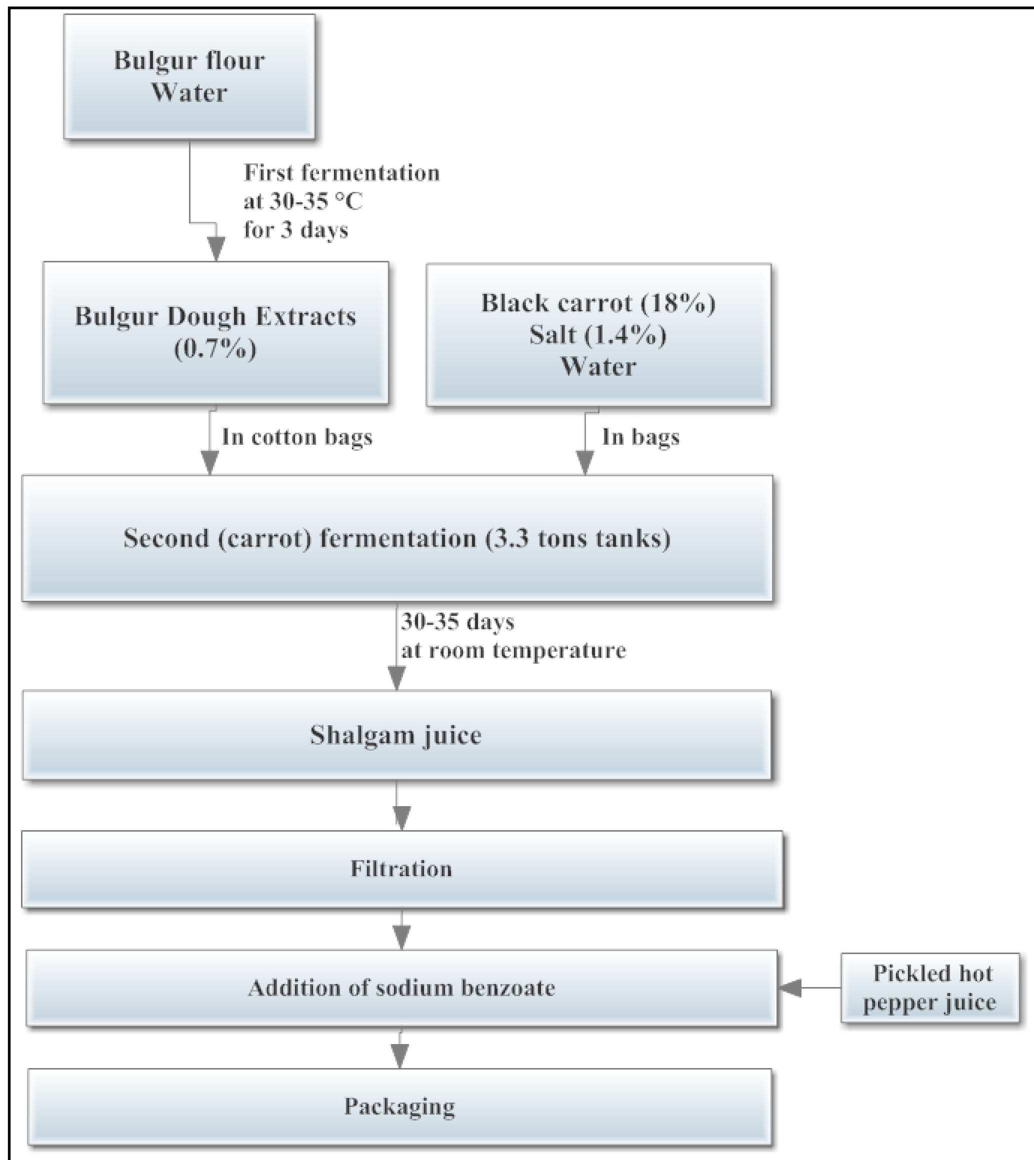


Figure 3.1. Production of shalgam juice of Company A, (Adana)

3.2. DETERMINATION OF THE CHEMICAL COMPOSITION OF SHALGAM JUICE

Chemical composition of the commercially available shalgam juice (Aa1), used in further microflora identification and cytotoxicity experiments was determined according to standards of Turkish Standards Institute (TS) and compared with the TS 11149, standard for shalgam juice [104].

3.2.1. pH

pH of the shalgam juice was measured with a Meterlab, pHM210 model pH meter (Radiometer Analytical, France) according to TS 1728 ISO 1842 [104].

3.2.2. Total Acidity

Total acidity of shalgam juice was determined with titration according to TS EN 12147 [104] and expressed as lactic acid, which is the primary acid in shalgam juice by using the factor appropriate for lactic acid, 0.090 [137].

3.2.3. Soluble Solid Content

Soluble solid content of shalgam juice was determined with the refractometric method using a Bellingham Stanley, sucrose indexed refractometer (Xylem, USA) according to the method of TS 4890 and AOAC [104, 137]. The results were expressed as per cent (w/w) dry substance. All determinations were made at 20.1°C.

3.2.4. Salt

The salt content of shalgam juice was determined with Mohr titration method according to TS 2664 [104] and was expressed as per cent (w/w).

3.2.5. Ash

The ash content of shalgam juice was determined according to standard TS EN 1135 of Turkish Standards Institute [104] inside an ashing furnace (Nabertherm, Germany) and was expressed as g/L.

3.2.6. Protein

The protein content of shalgam juice was determined with Kjeldahl method according to official AOAC method for fruit and fruit juices with some modifications [137]. An

automated system was used in Kjeldahl method with a Buchi SpeedDigester and Buchi Distillation Unit (Switzerland). In digestion potassium sulphate (K_2SO_4; Merck, Germany) was used instead of HgO or Hg and protein content of shalgam juice was expressed as % (w/w).

3.2.7. Total Carbohydrates

The carbohydrate content of shalgam juice was determined with phenol-sulphuric acid method of Dubois et al. [138] using glucose as standard and a standard curve of glucose was used to calculate the concentration of carbohydrates in shalgam juice. Total carbohydrate concentration of shalgam juice was expressed as g/L.

3.2.8. Determination and Quantification of Organic Acids and Sugars

The organic acid (lactic, acetic, citric, oxalic, succinic, L-ascorbic, butyric, propionic and malic acid) and sugar (glucose, fructose, sucrose and arabinose) content of shalgam juices of Company A (hot, Aa1 and regular Aa2) were determined by High Performance Liquid Chromatography (HPLC), using external standards.

For sugar analyses and quantification, HPLC system (Thermo Scientific, England) equipped with a Refractive Index Detector (Thermo Scientific, England) was used. A HyperREZ XP Carbohydrate Ca++ column (300 x 7.7mm x 8µm; Thermo Scientific, England) was used at 50°C and detector temperature was maintained at 35°C. Mobile phase was 0.005M H_2SO_4 with a 0.6 mL/min. flow rate [139]. External standards of D-(+)-Glucose (Supelco, USA), D-(-)-Fructose (Supelco, USA), D-(+)-Sucrose (Fluka, Germany) and D(-)-arabinose were prepared in 8 different concentrations (0, 30, 60, 90, 120, 150, 180, 210 mg/L) and concentrations of individual sugars in samples were calculated from calibration curves drawn from external standards.

For organic acid determination and quantification, HPLC system (Thermo Scientific, England) equipped with a Photodiode Array (PDA) detector (Thermo Scientific, England) set to 210 nm was used with a Hypersil GOLD C18 column (250 x 4.6mm x 5µm; Thermo Scientific, England). Mobile phase was 0.013 M phosphoric acid (pH=2.3; Sigma Aldrich,

Germany) with a 1 mL/min flow rate according to method of McFeeters et al. [140] with some modifications on the concentration, therefore on the pH of the buffer. Seven different concentrations of external standards of L-(+)-Lactic acid (0, 0.8, 1.7, 2.5, 3.3, 4.2, 5 g/L) and acetic acid, citric acid, oxalic acid, succinic acid, L-ascorbic acid, propionic acid, malic acid and butyric acid (0, 0.17, 0.33, 0.5, 0.67, 0.83, 1 g/L, Supelco, USA) were prepared and concentrations of individual organic acids in samples were calculated from calibration curves drawn from external standards. Shalgam juice juice samples were centrifuged (Hettich, Germany) at 10.000 x g for 5 min and filtered through a 20 µm NY filter (Minisart Sartorius, Germany) before injection. Samples were diluted with MilliQ water (1:4) in organic acid analysis. All analyses were repeated three times for three different bottle of each sample.

3.2.9. Anthocyanins

Anthocyanins in shalgam juice were determined using HPLC and identified by comparing with literature [79, 125, 134]. Shalgam juice samples were centrifuged (Hettich, Germany) at 10.000 x g and filtered through a 20µm filter (Minisart Sartorius, Germany). HPLC system (Thermo Scientific, England) equipped with a Photodiode Array (PDA) detector (Thermo Scientific, England) set to 520 nm was used with a Hypersil GOLD C18 column (250 x 4.6mm x 5µm; Thermo Scientific, England). Mobile Phase A was acetonitrile (Sigma Aldrich, Germany) and mobile phase B was 4% phosphoric acid (Sigma Aldrich, Germany) in MilliQ water [134]. The flow rate was 1.0 mL/min and gradient conditions were as follows: at 0 min. 0% A, %100 B; at 20 min. 20% A, %80 B; at 25 min. 40% A, 60% B; at 30 min. 0% A, 100%.

3.3. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF SHALGAM JUICE

3.3.1. Sample and Standard Preparation

Total phenolic content (TPC) and the antioxidant capacities of commercially available hot and regular shalgam juices of company A, B, C, D, and E were determined (Table 3.1). Black carrot juice (freshly peeled and squeezed) and a commercially available

pomegranate juice were used as controls for comparison of antioxidant capacities. All samples were filtered through a 0.45µm filter (Minisart Sartorius, Germany) after centrifugation (Hettich, Germany) at 10.000 x g for 5 min and dilutions from each sample were prepared with 80% methanol (Sigma-Aldrich, Germany) for antioxidant assays (1/20, 1/22, 1/25, 1/28, 1/33, 1/40, 1/50, 1/66, 1/100, 1/200) and with MilliQ water for total phenolic content assay (1/10 and 1/50). For blank, 80% methanol and MilliQ water was used for antioxidant capacity assays and total phenolic content assay, respectively. Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as a standard in antioxidant assays and gallic acid was used as standard in total phenolic content assay. Different dilutions (0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, 0.05 mg/mL) of Trolox (Sigma-Aldrich, Germany) and gallic acid (Sigma-Aldrich, Germany) were prepared in 80% methanol and MilliQ water. All samples or standards were prepared daily and all analyses were repeated three times for three different bottle of each sample.

Table 3.1. The samples used in antioxidant capacity and TPC determinations

Samples		Production Place
Aa1	Commercial regular shalgam juice (non-heat treated) (Company A)	Adana
Aa2	Commercial hot shalgam juice (non-heat treated) (Company A)	Adana
Ab1	Commercial regular shalgam juice (heat treated) (Company A)	Adana
Ab2	Commercial hot shalgam juice (heat treated) (Company A)	Adana
B1	Commercial regular shalgam juice (Company B)	Adana
B2	Commercial hot shalgam juice (Company B)	Adana
C1	Commercial regular shalgam juice (Company C)	Adana
C2	Commercial hot shalgam juice (Company C)	Adana
D2	Commercial hot shalgam juice (Company D)	İzmir
E1	Home-made regular shalgam juice	İstanbul*
E2	Home-made hot shalgam juice	İstanbul*
BC1	Freshly squeezed black carrot juice from black carrots	Adana
BC2	Freshly squeezed black carrot juice from black carrots	İstanbul
PJ	Commercial pomegranate juice	İstanbul
*Place of purchase		

3.3.2. Antioxidant Determination of Shalgam Juice

Shalgam, black carrot and pomegranate juices' antioxidant capacities were determined with ABTS, DPPH and FRAP assays, using Trolox as a standard. Analyses were carried out in triplicates for each dilution.

3.3.2.1. ABTS

ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity assay is based on the method of Miller et al. [141] modified by Re et al. [94]. Free radical, ABTS•+ was prepared daily with Milli-Q grade water by reacting 5 mL 7mM ABTS solution (Sigma-Aldrich, Germany) and 88 µL 140 mM potassium persulfate ($K_2S_2O_8$; Sigma-Aldrich, Germany) and the mixture was allowed to stand in the dark at room temperature for 12–16 h before use. The absorbance of ABTS•+ was adjusted to 0.70 (±0.05) at 734 nm with 80% methanol in a 96 well plate (Corning, USA) using a microplate reader (MultiScanGo, Thermo Scientific). For reaction, 50 µL Trolox standard or sample was added to 250 µL ABTS+ in a 96-well plate and readings were made for 15 min at 30°C at 734 nm. Since there was no change in the absorbance of Trolox in 15 min (Fig 3.2), absorbance values at the fourth minute were used according to literature for antioxidant capacity calculations [94].

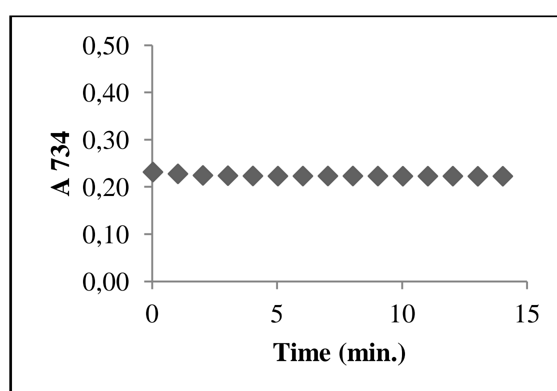


Figure 3.2. ABTS reaction rate at 734 nm for 150µM Trolox standard has not changed during the monitoring period

3.3.2.2. DPPH

The DPPH Radical Scavenging Activity assay was based on the modified method of Fukumoto & Mazza [101], which was adapted from the methods of Brand-Williams, Cuvelier, & Berset [95]. In the modified method, 80% methanol was used instead of 100% methanol and readings were made in 96 well plates. For DPPH assay 22 µL sample or Trolox standard and 200µL 150 µM DPPH solution (Sigma-Aldrich, Germany) was mixed in the wells of a 96 well plate and readings were made at 0 min, 30 min, 3 h and 5 h at 515 nm. Absorbance versus concentration curves with highest slope were used in the calculation of antioxidant capacity as described in section 3.3.4. (30 min for Trolox standard and 300 min for juice samples).

3.3.2.3. FRAP

The ferric reducing antioxidant power of shalgam, black carrot and pomegranate juices was measured according to the methods of Benzie and Strain with some modifications made for 96 well plate [96]. FRAP reagent was prepared daily by reacting 25 mL of 300 mM acetate buffer at pH 3.6 (3.1 g sodium acetate trihydrate ($C_2H_33NaO_2 \cdot 3H_2O$; Riedel-de-Haen) and 16 mL acetic acid ($C_2H_4O_2$; Sigma-Aldrich, Germany) per liter of solution), 2.5 mL of 20mM Iron-III-hexahydrate ($FeCl_3 . 6H_2O$; Carlo Erba) and 2.5 mL of 10mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine; Sigma-Aldrich, Germany). Readings were made at 593 nm by mixing 50 µL Trolox standard or sample with 250 µL FRAP reagent in a 96-well plate for 6 min. Similar to previous studies, there was no change in the absorbance for six minutes (Figure 3.3) [96] and fourth minute was used for antioxidant capacity calculations in FRAP method.

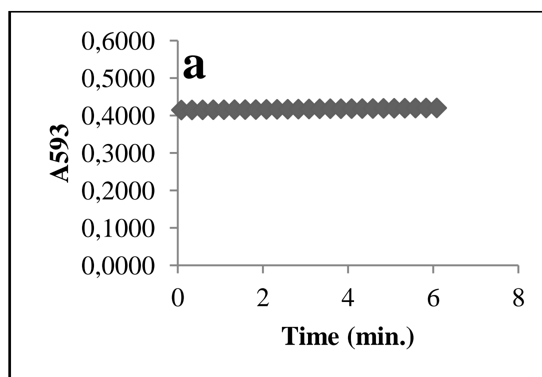


Figure 3.3. FRAP reaction rate at 593 nm for 100µM Trolox standard has not changed during the monitoring period

3.3.3. Total Phenolic Content

Total Phenolic Content (TPC) of juices was determined with the Folin-Ciocalteu method [102] with some modifications made for 96-well plate [142], using gallic acid as standard. Shalgam juice samples were diluted as 1/10 with water, black carrot and pomegranate juice samples were diluted as 1/50 and 100 µL sample or serially diluted standards (0-0.05 mg/mL) were added to 2 mL centrifuge tubes. Two hundred µL of 10% (vol/vol) Folin-Ciocalteu reagent (Sigma-Aldrich, Germany) was added to tubes and vortexed. Eight hundred µL of 700 mM sodium carbonate anhydrous (Na_2CO_3; Carlo Erba, Italy) was added and the assay tubes were incubated at room temperature for 2 h. After incubation 200 µL sample, standard or blank was transferred to a 96-well microplate and the absorbance was measured at 765 nm with a microplate reader (MultiScanGo, Thermo Scientific). Readings were made in triplicates for each sample.

3.3.4. Calculation of Antioxidant Capacity and TPC

For calculation of antioxidant capacities, the slope of the absorbance versus concentration curve of Trolox standard and samples were used by dividing the slope of the sample by the slope of the Trolox standard ($R^2 \geq 0.99$). Trolox Equivalent Antioxidant Capacities (TEAC) of juices were calculated as µmol Trolox Equivalent/mL juice. Total phenolic contents were calculated from the calibration curve of gallic acid and expressed as µg GA/mL.

3.4. ENUMERATION AND IDENTIFICATION OF LACTIC ACID BACTERIA IN SHALGAM JUICE

The microflora of shalgam juice was identified with species-specific PCR, however during production, microorganism profile of shalgam juice changes due to pH, salt and processing effects. Since species-specific PCR can identify both viable and non-viable microorganisms, cultivation is needed to determine the viable counts in the final product. Therefore, viable microorganisms of final product, shalgam juice were enumerated by cultivating on appropriate media. Then, DNA of the microorganisms in shalgam juice was isolated either directly from shalgam juice or from picked colonies on agar plates.

3.4.1. Enumeration of Microorganisms

The microbial load of shalgam juice mostly comes from bulgur dough used in fermentation [24]. That is why bacteria and mold/yeast counts of both shalgam juice and bulgur dough extract were determined by spread and pour plate methods. Ten mL shalgam juice or 10 g of dough was suspended in 90 mL peptone water (Conda, Spain) and 10-fold serial dilutions were made (10^0-10^{-7}). After dilutions, *Lactobacillus* species were cultivated on Man Rogosa Sharp (MRS) agar (Conda, Spain), *Lactococcus & Streptococcus spp.* were cultivated on M17 agar (Conda, Spain), and they were incubated at 37°C in a 5% CO_2 incubator (New Brunswick, UK) for 48 h. Yeast and molds were grown on Potato Dextrose Agar (Conda, Spain) at 25°C for 48 h. Total aerobic bacteria count was determined by spreading on Nutrient Agar (Conda, Spain) and incubated at 37°C for 24 h. Viable counts were performed and expressed in Log_{10} CFU mL^{-1}.

The identity of the strains was confirmed by Gram staining (Salubris Gram Staining Kit, USA) and by checking colony morphologies. Isolated colonies were cultured and stored in the appropriate growth medium containing 30% glycerol (Sigma, UK) at -80 °C freezer (Sanyo, Japan).

3.4.2. Bacterial DNA Isolation

DNA of the microorganisms in shalgam juice was isolated either directly from shalgam juice or from picked colonies on agar plates. To gather enough samples, 50 mL shalgam juice was centrifuged at 20.000 x g for 5 min (Sigma, Germany) repeatedly (to reach a total volume of 250 mL samples). After centrifugation, pellet was suspended in Phosphate Buffered Saline (PBS; GIBCO, USA) and collected in 2 mL microcentrifuge tube. For DNA isolation from colonies, colonies were picked from the agar plate and suspended in PBS. Homogenates were centrifuged (Hettich, Germany) at 20.000 x g for 1 min, the supernatant was removed and cell pellet was used in DNA isolation. DNA isolation for all sample homogenates was done, using Purelink Genomic DNA Mini Kit (Invitrogen, USA) according to manufacturer's instructions with some modifications. Additional lysozyme enzyme was used for disrupting the cell walls of gram-positive bacteria. Lysozyme (Bio Basic, USA) was suspended in lysozyme buffer (25mM Tris-HCl, Merck, Germany; 2.5mM EDTA, Merck, Germany; 1% Triton X-100, Merck, Germany) at a concentration of 20 mg/mL and 1 mL lysozyme suspension was added on cell pellets in microcentrifuge tubes. Tubes were incubated at 37°C for 30 min in a water bath and kit protocol was used for DNA isolation.

3.4.3. Quantification of DNA

The quantity of isolated DNA was determined using a spectrophotometer (Implen Nanophotometer, USA) at 260 nm. Elution buffer (Invitrogen, USA), used as suspension of final DNA, was used as a blank. DNA concentration was calculated from the absorbance at 260 nm as ng/µL. Since the ratio higher than 1.8 is indicative of a protein contamination (nucleic acids and proteins give maximum absorbance at 260 nm and 280, respectively), protein contamination of DNA samples were measured by calculating the A_{260}/A_{280} ratio. The DNA is also observed on 1% agarose gel (Sigma, UK), which was prepared with 1X TBE buffer [10.8 g/L Tris Base, (Merck, Germany); 5.5 g/L boric acid, (Sigma, UK); 0.02M EDTA, (Merck, Germany] and stained with ethidium bromide (Sigma-Aldrich, UK). DNA ran at 80V for 30 minutes (Cleaver, USA). Gels were visualized in UV Transilluminator Gel Imaging System (Bio-Rad, USA).

3.4.4. Species-Specific PCR

To identify microbial flora of shalgam juice with molecular methods, polymerase chain reaction (PCR) using species specific primers were performed. Bacterial DNA samples were amplified with primers (Invitrogen, USA), previously designed and reported based on the 16S-23S regions of lactic acid bacteria found in dough and vegetable products [1, 64, 66, 143-145] and are given in the Table 3.2. PCR mastermix was prepared in a final volume of 24 µL without the template DNA. The mastermix contained 1.5µL 10X Tag Reaction Buffer (Fermentas, USA), to a final concentration of 10mM; 1.5µL $MgCl_2$ (Fermentas, USA), to a final concentration of 1.5mM; 2µL dNTP (Fermentas, USA), to a final concentration of 200µM; 0,35µL Taq DNA Polymerase (Fermentas, USA), to a final concentration of 1.75U; 1µL of each primer, to a final concentration of 10pmol each; and 14.65 µL nuclease free distilled water (Gibco, USA) to reach a total 25µL volume. 17 DNA samples, 16 obtained from colonies on MRS Agar, 1 directly from shalgam juice were used as template DNA and added as 1 µL to each PCR tube. Different PCR amplification protocols (Table 3.3) were used according to primers' annealing temperatures [66, 143, 145] in PCR thermal Cycler (Bio-Rad My Cycler, USA).

Table 3.2. Species-specific primers used in DNA amplification

Species	Primer	Reverse/ Forward	Sequence (5’-3’)	Reference
Lb. acidophilus	La1 La2	F R	GATCGCATGATCAGCTTATA AGTCTCTCAACTCGGCTATG	[143]
Lb. johnsonii	Lj1 La2	F R	CACTAGACGCATGTCTAGAG AGTCTCTCAACTCGGCTATG	
Lb. delbrueckii	Ld1 Ld2	F R	ACATGAATCGCATGATTCAAG AACTCGGCTACGCATCATTG	
Lb. casei group	Lc3 Lc4	F R	GCGGACGGGTGAGTAACACG GCTTACGCCATCTTTCAGCCAA	
Lb. casei	Lc1 Lc2	F R	GTGCTTGCACTGAGATTCGACTTA TGCGGTTCTTGGATCTATGCG	
Lb. paracasei	Lp1 Lc2	F R	GTGCTTGCACCGAGATTCAACATG TGCGGTTCTTGGATCTATGCG	
Lb. rhamnosus	Lr1 Lc2	F R	GTGCTTGCATCTTGATTTAATTTT TGCGGTTCTTGGATCTATGCG	
Streptococcus thermophilus	St1 St2	F R	TTATTTGAAAGGGGCAATTGCT GTGAACTTTCCACTCTCACAC	
Lb. plantarum	Lfpr PlanII	F R	GCCGCCTAAGGTGGGACAGAT TTACCTAACGGTAAATGCGA	[66]
Lb. gasseri	GasI GasII	F R	GAGTGCGAGAGCACTAAAG CTATTTCAAGTTGAGTTTCTCT	
Lb. reuteri	Lfpr Reu	F R	GCCGCCTAAGGTGGGACAGAT AACACTCAAGGATTGTCTGA	
Lb. fermentum	Lfpr FermII	F R	GCCGCCTAAGGTGGGACAGAT CTGATCGTAGATCAGTCAAG	
Lb. sharpeae	ShaI ShaII	F R	GATAATCATGTAAGAAACCGC ATATTGTTGGTCGCGATTCG	
Saccharomyces cerevisiae	NS1 ITS2	F R	GTAGTCATATGCTTGTCTC GCTGCGTTCTTCATCGATGC	[144]
Lb.brevis		F R	CTTGCACTGATTTTAACA GCTGCGTTCTTCATCGATGC	[145]
Lb. bulgaricus	Bulgfor Bulgrev	F R	TCAAAGATTCCTTCGGGATG TACGCATCATTGCCTTGGTA	[64]

Table 3.3. PCR conditions for each species

Primer sets	**PCR conditions**
Lb. acidophilus *Lb. johnsonii* *Lb. delbrueckii* *Lb. casei group* *Lb. casei* *St. thermophilus*	94°C – 3min 35 cycle (94°C -30s, 60°C-30s, 72°C-90s)
Lb. paracasei	94°C – 3min 35 cycle (94°C -30s, 65°C-30s, 72°C-90s)
Lb. rhamnosus	94°C – 3min 35 cycle (94°C -30s, 50°C-30s, 72°C-90s)
Lb. plantarum *Lb. gasseri* *Lb. reuteri* *Lb. fermentum*	92°C – 2min 35 cycle (95°C -30s, 55°C-30s, 72°C-30s) 72°C-1min
Lb. sharpeae	92°C – 2min 35 cycle (95°C -30s, 58°C-30s, 72°C-30s) 72°C-1min
Lb. brevis	94°C – 2min 35 cycle (94°C -1min, 40°C-1min, 72°C-1min) 72°C-10 min
Lb. bulgaricus	92°C – 3min 35 cycle (95°C -30s, 60°C-20s, 72°C-20s) 72°C-5min
S. cerevisiae	92°C – 3min 35 cycle (95°C -30s, 60°C-30s, 72°C-3min) 72°C-7min

3.4.5. Visualization of PCR Products

PCR products were visualized on 2% agarose gel, prepared with 1X TBE buffer. Gels stained with ethidium bromide and run at 90V for 35 min, were visualized in UV Transilluminator Gel Imaging System (Bio-Rad, USA). Amplified products were quantified with 100-1000bp DNA ladder (Fermentas, USA).

3.4.6. Sequencing Analysis

The amplified fragments were diluted to a concentration, of which the bands can be seen clearly (30 µL) and sequenced with the primers (5 pmol) used in amplification by Refgen Company (Ankara, Turkey). Sequence results were analyzed by Sequence Scanner Software v1.0 (Applied Biosystems) and each base sequence of the sample was compared with the rDNA sequences present in the nucleotide sequence database of National Centre for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST) search program. ClustalW software was used for phylogenetic tree presentation based upon the alignments.

3.5. CYTOTOXICITY ASSAY

In vitro cytotoxic effect of shalgam juice, of which chemical and microbial properties were determined (Aa1, Adana), and black carrot juice (freshly peeled and squeezed; Adana) was determined using colorectal carcinoma (Caco-2) cells.

3.5.1. Cell Culturing

3.5.1.1. Cell Passaging

Caco-2 cells (Caco-2/An1) were obtained from the cell culture collection of Sap Institute (HÜKÜK, Şap Enstitüsü, Ankara) and were grown in Dulbecco's Modified Eagle Medium (DMEM; with 2 mM L-glutamine; GIBCO, USA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS; GIBCO, USA); 100 units/mL penicillin, 100 µg/mL streptomycin (Gibco, USA) at 37°C in 5% CO_2 incubator (New Brunswick, UK) in T-25 flasks.

Cell passaging was performed when cells reached 80% confluence. Media was removed from flask and cells were washed once with 1 mL PBS solution (Gibco, USA) and detached by adding 1.5 mL trypsin (0.5 g/L)-EDTA (0.2 g/L) solution (Gibco, USA) to flask and incubating flask for 4 min at 37°C with 5% CO_2 humidified incubator. Detached cells were collected in a 15 mL centrifuge tube after addition of 3 mL growth media to neutralize trypsin-EDTA. The tube was centrifuged (Hettich, Germany) at 300 x g for 5

min and supernatant was discarded. Cell pellet was resuspended in 5 mL growth media and cells were seeded in a new T-25 flask.

3.5.1.2. Cell Counting

Cell counting was performed using a hemocytometer (Sigma, Germany). Cell pellets to be counted were suspended in PBS solution (instead of growth media, since serum proteins in media may cause false results by staining) and trypan blue dye exclusion method was used according to manufacturer's instructions (Wisent Bioproducts, Canada). One mL of cell suspension was mixed with one mL of trypan blue dye. Ten µL of this mixture was added to the hemocytometer. The cells were counted using inverted phase contrast microscope (Nikon, USA). The circled area on the hemocytometer represents 1/10000 of 1mL (Figure 3.4).

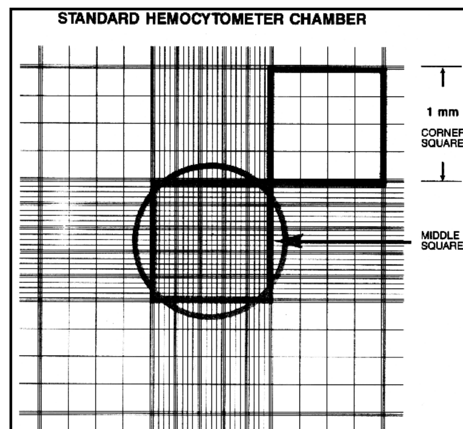


Figure 3.4. Hemocytometer chamber [146]

Cells in this square were counted 3 times and average of these numbers was used to calculate the number of cells per mL using the Equation (3.1).

$$\text{“Cells/mL} = \text{the average number x } 10^4 \text{ x dilution factor”} \tag{3.1}$$

3.5.1.3. Cell Freezing

After cell counting, cell suspension was centrifuged at 300 x g for 5 min and supernatant was removed. Cell pellet was suspended in freezing media containing 90% (v/v) FBS and

10% dimethyl sulfoxide (DMSO; Santa Cruz, USA) at a concentration of $1x10^6$ cells/mL. One mL of this suspension was transferred to cryovials and stored in liquid nitrogen.

3.5.1.4. Cell Thawing

Frozen cell vials taken from liquid nitrogen storage, were thawed rapidly at 37°C. Cell solution in cryovial was transferred to a 15 mL centrifuge tube and five mL of growth media was added slowly with gentle shaking. Cell suspension was transferred into a T-25 flask and incubated at 37°C with 5% CO_2 incubator. Following 20th hour, media was removed from flask and cells were washed with PBS solution. Fresh media was added and cells were incubated at 37°C with 5% CO_2 until they reach 80% confluence.

3.5.2. Cell Proliferation Assay

Cell proliferation rate, thus the cytotoxicity in Caco-2 cells incubated with shalgam juice was determined with CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay – MTS (Promega, USA). The assay is composed of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate; PMS). MTS is reduced into a formazan product in metabolically active cells by dehydrogenase enzymes and the absorbance of the formazan product can be measured at 490 nm, spectrophotometrically. The number of living cells, which is cell viability, in culture medium can be measured by measuring quantity of formazan product via absorbance.

Shalgam and black carrot juices were freeze dried in a lyophilizer (Christ, Germany) and suspended in PBS at a concentration of 6400µg/mL. Nine dilutions were prepared from each suspension with growth media (0, 50, 100, 400, 800, 1600, 3200, 6400 µg/mL) and 50 µL of dilutions were added to the wells of a 96-well plate. The plate was equilibrated at 37°C with 5% CO_2 humidified atmosphere while harvesting the cells for assay. Cells in T-25 flasks were harvested, counted and suspended to a final concentration of 1×10^5 cells/mL in growth media. Fifty µL of cell suspension was dispensed into all wells containing juice suspensions and plate was incubated for 48 h at 37°C with 5% CO_2 humidified atmosphere. After incubation, 20µL/well MTS/PMS solution was added to wells and plate was incubated for another 3 h. The absorbance at 490 nm was measured

using ELISA plate reader (Bio-Tek, USA) to determine the amount of soluble formazan produced by cellular reduction of the MTS. Inhibition of cell proliferation was determined through cell viability using Equation (3.2):

$$\text{Cell viability (\%)} = [(\text{sample absorbance with cells} - \text{sample absorbance without cells})/(\text{control absorbance with cells} - \text{control absorbance without cells})] \times 100 \quad (3.2)$$

3.6. STATISTICAL ANALYSIS

Minitab version 16 statistical software was used in statistical analyses. For chemical analyses and microbial enumeration data, One-way analysis of variance (ANOVA) and student t-test was applied. For antioxidant data, ANOVA and Tukey's test were applied to determine significant differences among different brands of shalgam juice ($P<0.05$).

4. RESULTS AND DISCUSSION

4.1. CHEMICAL COMPOSITION OF SHALGAM JUICE

As stated in the materials section, the chemical composition of Aa1 was determined, since it was used in further cytotoxicity and microflora identification studies. The chemical composition of Aa1 is given in Table 4.1.

Table 4.1. Chemical composition of Aa1 shalgam juice and Turkish Standard Institution's standard for shalgam juice

Properties	**Shalgam Juice**	**TS 11149**
Total Carbohydrate, (g/L)	0.29±0.02	-
Soluble Solid, % (w/w)	3.40±0.00	≥ 2.50
Salt, % (w/w)	2.00±0,06	≤ 2.00
Ash, % (w/w)	1.86±0.05	≤ 2.00
pH	3.43±0.00	3.30 – 3.80
Total Acidity as Lactic Acid, (g/L)	6.38±0.11	≥ 6.00
Protein, (g/L)	2.80±1.14	-

As expected, the total carbohydrate in shalgam juice samples was low and found as 0.29 g/L. The carbohydrate content of shalgam juice comes from the ingredients used in production such as black carrot and bulgur dough which are utilized by LAB during fermentation for production of aroma and flavor compounds. In a previous study, Güneş [131] investigated the most suitable quantity of black carrot for the production of shalgam juice by examining the total carbohydrate amount of bulgur flour and black carrot used in fermentation and found that while total carbohydrate amount of bulgur flour was 18.2 g/kg, total carbohydrate amount of black carrot was 70.9 g/kg at the beginning of fermentation. Throughout the fermentation, total carbohydrate concentration decreased to 0.25-0.29 g/L and affected by the amount of black carrot used in production (10-20%) [131]. In another study [132], the effect of black carrot size on the quality of shalgam juice was studied and total carbohydrate in bulgur flour and black carrot was also determined as similar to study of Güneş [131]. Similarly, the total carbohydrate concentration decreased in the end-

product (0,09-0.20 g/L), while there were no significant differences in total carbohydrate contents in shalgam juices produced with different sizes (3-9 cm) of black carrot [132]. In a more recent study, the effect of the addition of starter cultures in shalgam juice production was investigated [106]. It was found that total carbohydrate amount of shalgam juices produced in lab scale with the addition of starter cultures such as *Lb. plantarum* species was between 0.50-0.82 g/L and there were no significant differences compared to shalgam juice produced without starter culture [106].

The solid content of shalgam juice was 3.4% (w/w). Soluble solid content of shalgam juice consists of organic acids, salt, protein and minerals and 62% of the solid content is salt [103]. In previous studies, the solid content of commercially available shalgam juices were found between 2.2 and 4.0% [103, 122, 127-130]. According to TSI, solid content of shalgam juice should be above 2.5%, which was comparable to our result [104]. In controlled production studies of shalgam juice in laboratory condition in which the effect of black carrot amount on product quality was investigated [131], the solid contents of both black carrots and bulgur flour used in production were determined and found as 89.1-90.9% (w/w) for bulgur flour and 11.0-11.3% for black carrot (w/w). It was stated that soluble solid content of shalgam juice was found to be affected by the amount of the black carrot used in production [131]. The solid content increased (2.03-2.67%) with the higher concentration of black carrot (10-20%) used in production [131]. In another study, it was found that the use of different starter cultures was also effective on soluble solid concentration of shalgam juice (1.69-3.15%) [106].

The salt content of shalgam juice was found as 2.0% and was within the limits of the standards (2%, w/w). Fermented vegetable products, such as kimchi and sauerkraut also contains 1.8-3% (w/w) salt [37]. Salt in shalgam juice is added during production to control the microflora of fermentation and prevent spoilage since most of the LAB found in shalgam juice fermentation are salt tolerant bacteria, the growth of gram negative bacteria causing spoilage is inhibited with salt [24]. Similarly in previous studies, salt concentration was found in the range of 1.12-2.57% in commercial products and 0.82-1.77% in shalgam juices produced in lab-scale [103, 104, 106, 122, 126-132].

The ash amount of shalgam juice was found as 1.86% (w/w) which was within the limits of standards (2%) [104]. The ash content of shalgam juice depends on anionic and cationic ions of ingredients [127] and minerals of water used in the production, therefore it is affected by the quality of the water and amount of the ingredients. The ash amount of commercial shalgam juices were found in the range of 1.12-2.07%, similar to our study [103, 104, 122, 127-130]. In a previous study, the effect of black carrot amount was investigated and the ash amount of shalgam juice was found to be increased with increasing amounts of black carrot used in production (1.27-1.52%) [131].

The pH of the shalgam juice samples was found as 3.43 and was within the limits of standards (3.30- 3.80). [104]. The pH is related to the acid content of shalgam juice and similarly was found between 3.16 and 3.86 in commercially available shalgam juices [103, 104, 122, 127-130]. Also, in several controlled production studies at lab scale [106, 131, 132], the change in the pH of the dough (first) and black carrot (second) throughout fermentation was screened. During dough (first) fermentation, pH decreased from 5.23-5.93 to 4.20-5.01 and the decline in the pH continued during the black carrot (second) fermentation until the end of the fermentation. In final product, pH was found in the range of 3.39-3.56 [106, 131, 132]. In a previous study [131], pH of the shalgam juice was found to be affected by the amount of black carrot used in production and stated that the pH (3.39-3.49) of the shalgam juice decreased with higher amounts of black carrots (10-20%) used in fermentation. In another study, pH was found to be affected by the size of the black carrot used in the production (3.43-3.53) [132]. In the study of Tangüler [106], the effect of the use of starter cultures such as *Lb. plantarum* and *Lb. paracasei subsp. paracasei* species on the chemical composition was investigated and it was found that the pH (3.43-3.56) of the shalgam juices produced with the addition of starter cultures increased compared to shalgam juices produced without a starter culture. In vegetable fermentations, most of the spoilage and pathogenic microorganisms are eliminated by the decrease in the pH with the production of organic acids [37]. Also, microbial growth patterns and LAB in fermentation effects the change in pH [37].

Shalgam juice is produced with lactic acid fermentation, thus the predominant acid in shalgam juice is expressed as lactic acid. In our study, total acidity of shalgam juice was found as 6.38 g/L, which was consistent with other studies, of which total acidity of

commercially available shalgam juices was reported between 1.06 and 12.6 g/L [103, 104, 122, 127-130]. Our findings also met the standard requirement determined by TSI ($\geq$ 6.00 g/L) [104]. In previous lab scale production studies [106, 131, 132], the change in the acidity during and at the end of the fermentation was screened. In dough (first) fermentation, the acidity of bulgur dough increased from 2.90-5.30 g/kg to 8.55-11.9 g/kg. Total acidity of black carrots (0.48-2.16 g/L) increased during (second) fermentation with the addition of bulgur dough from the first fermentation to 4.88-8.30 g/L [106, 131, 132]. In the study of Güneş [131], the effect of black carrot amount on shalgam juice quality was investigated and total acidity was found to be affected by the amount of black carrot used in fermentation. Using higher concentrations of black carrots (10-20%) in shalgam juice production increased the acidity (4.95-7.45 g/L) in the final product [131]. In another study, effect of the starter cultures on the acidity was studied and it was found that addition of starter cultures such as *Lb. plantarum* species increased the acidity of shalgam juice (6.36-9.27 g/L) [106]. On the other hand, using additional ingredients such as red beet and turnip decreased the acidity (5.20-8.90 g/L) in the end product [126]. Similar to shalgam juice, in lactic acid fermented vegetable juices such as carrot and cabbage juices, the total acidity was found in the range of 4.38-7.40 g/L in the final product and the acidity increased with the fermentation time [14, 26]

The protein content of shalgam juice is determined with Kjeldahl method according to standard method of Association of Official Analytical Chemists (AOAC) since TS standard TS 11149 for shalgam juice does not specify the protein amount for shalgam juice [137]. The protein amount was found as 2.80 g/L, which is higher than previous findings of protein concentrations in commercially and lab-scale produced shalgam juices (0.88 g/L-1.83g/L) [106, 127]. The proteins in shalgam juice are in low amounts and are soluble proteins of ingredients, bulgur flour and black carrot, therefore it may be affected by the cultivar of black carrot used in production [106, 127].

4.1.1. Organic Acid and Sugar Determination by HPLC

Organic acids produced during fermentation decrease the pH and inhibit the growth of gram negative bacteria acting as antimicrobial substances in fermented vegetable products [147]. They also influence the organoleptic properties of the beverage (flavor, aroma,

color) [148]. Specifically, individual organic acids of shalgam juice from Company A (regular, Aa1 and hot, Aa2) were determined with HPLC using external standard curves ($R^2 \geq 0.999$) (Table 4.2). Lactic acid was found as the predominant acid and it was significantly different among regular and hot products with 8.90±0.59 g/L concentration in regular (Aa1) and 4.19± 0.09 g/L concentration in hot shalgam juice (Aa2) (Figure 4.1). Lactic acid found in shalgam juice is mostly L(+)-Lactic Acid. Less frequently D(-)-Lactic acid or a combination of both are present [1]. Since LAB in fermentation of shalgam juice are both homo and heterofermentative LAB, other organic acids such as acetic acid (1.29±0.13 g/L in Aa1 and 2.65±0.04 g/L in Aa2), citric acid (1.25±0.06 g/L in Aa1 and 1.10±0.01 in Aa2) and succinic acid (0.22±0.06 g/L in Aa1 and 0.22±0.02 g/L in Aa2) were also found in lower amounts, as fermentation end-products [29]. The significant difference between the lactic acid and acetic acid concentrations of hot and regular shalgam juices can be explained by the addition of pickled hot pepper juice at the end of the fermentation in hot products [119]. Addition of pickle juice is considered as an adulteration in the production, however to produce hot shalgam juice, it is specifically added to the end product [119, 134]. Pickled hot peppers are made with vinegar which contains high concentration of acetic acid [149], therefore the addition to shalgam juice may have increased the acetic acid concentrations in hot products. It was also reported that the acetic acid concentration in shalgam juice increased while lactic acid concentration decreased during storage and the organic acid content and quality of the shalgam juice changed from batch to batch, seasonally and yearly [134]. This may suggest that hot shalgam juices used in this study were from different batches and stored longer on the shelf. Similar to our findings, in previous studies lactic acid was found as the dominant organic acid of shalgam juice, followed by acetic acid and citric acid [106, 122, 130, 132, 134]. The concentration of organic acids was found as 2.61-8.75 g/L lactic acid, 0.34-1.48 g/L acetic acid and 0-3.41 g/L citric acid in commercial shalgam juices [122, 130, 134]. Our results have shown similar organic acid amounts among regular commercial products except lactic acid which had slightly higher concentration than other commercial products. Use of various sizes of black carrot, addition of starter cultures and production of shalgam juice without a dough fermentation affected the organic acid concentration of shalgam juices [106, 132]. In lab scale production, the highest lactic acid concentration (8.17 g/L) was found in shalgam juice produced with the addition of *Lb. plantarum* species while lowest lactic acid concentration (5.50 g/L) was measured in shalgam juice produced

without a prior dough fermentation [106]. The acetic acid concentration was also found to be affected by the production method and starter culture used in the fermentation [106]. The acetic acid concentration in shalgam juice produced with *Lb. fermentum* was higher (0.83 g/L) than the acetic acid concentration in shalgam juice produced without addition of starter culture (0.57 g/L) [106]. The effect of black carrot size (3-9 cm) on organic acid content of shalgam juice was also studied [132]. The lactic acid concentration was found in the range of 5.6-6.3 g/L with highest concentration in shalgam juices produced with 6 cm black carrots and acetic acid concentration was found in the range of 0.48-0.56 g/L with highest concentration in shalgam juices produced with 3 cm black carrots [132].

Table 4.2. Organic acid concentrations of regular (Aa1) and hot (Aa2) shalgam juices

Organic acid	**Concentration in regular shalgam juice (Aa1) (g/L)**	**Concentration in hot shalgam juice (Aa2) (g/L)**
Lactic acid	8.90±0.59	4.19± 0.09
Acetic acid	1.29±0.13	2.65±0.04
Citric acid	1.25±0.06	1.10±0.01
Succinic acid	0.22±0.06	0.22±0.02

In addition to other organic acids, an area which has similar retention time to L-Ascorbic acid was also detected. This suggests, there may be L-ascorbic acid in shalgam juice, however due to the interference in chromatography, it couldn't be quantified and should be investigated in further studies (Figure 4.1). In literature, L-Ascorbic acid was determined in shalgam juice in the concentration of 0.71-3.37 mg/100g using titration method [126] and stated that the source of ascorbic acid was assumed to be the black carrot used in production [126]. However, there is no published data on the detection of L-ascorbic acid content with HPLC in the literature. The ascorbic acid content of shalgam juice is important since sodium benzoate is used as a preservative in the end product [119, 123]. The studies has shown that ascorbic acid may cause production of benzene, a carcinogenic product, by decarboxylation of benzoic acid [150]. Oxalic acid was another organic acid detected in our study however similar to ascorbic acid due to the interference in chromatography, it couldn't be quantified (Figure 4.1). Oxalic acid in shalgam juice was determined and quantified in a previous study in the concentration of 14.37-62.20 mg/L

[106]. However, in the recent publication of the same group, presence of oxalic acid was not confirmed and not detected among organic acids of shalgam juice [130].

There was no determination of malic acid, propionic acid and butyric acid in shalgam juice in our study. For determination of other organic acids seen in chromatography (Figure 4.1) as well as quantification of L-ascorbic and oxalic acid, further method development is needed.

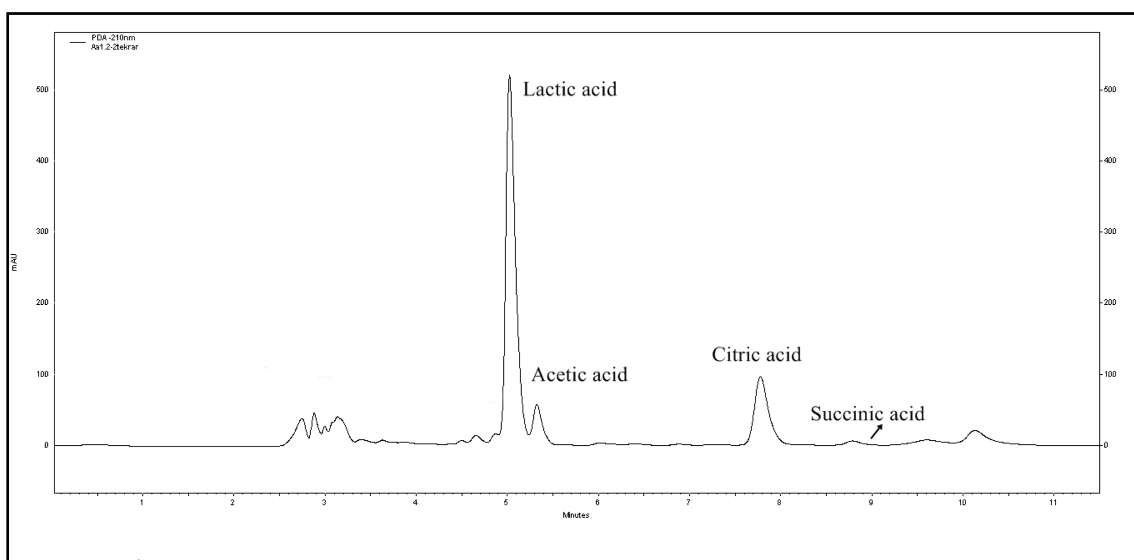


Figure 4.1. Organic acid profile of shalgam juice

Sugars of shalgam juice were also determined with HPLC using external standard curves ($R^2 \geq 0.999$) (Table 4.3). Sugars in black carrots are used in fermentation by LAB, therefore as expected their amount in final product was found in low amounts [130]. Fructose was found as the dominant sugar in shalgam juice (0.104 g/L), followed by sucrose (0.075 g/L) and glucose (0.041 g/L). No arabinose was determined. Sugar profile of shalgam juice was reported in several studies and sucrose (0.01-1.14 g/L), glucose (0.09-1.902 g/L) and fructose (0.006-4.0 g/L) was found in low levels as sugars of commercially available shalgam juices [122, 130]. The effect of production method and the addition of starter cultures on sugar concentration of shalgam juice was also investigated [106]. Glucose, fructose and sucrose concentrations were found as 0.048-0.191 g/L, 0.027-0.148g/L and 0.040-0.206 g/L, respectively with highest concentration in shalgam juice produced without the previous dough fermentation [106]. The sugars of shalgam juice are from the

ingredients and same three sugars were found with sucrose as the predominant sugar (4.11 g/100g), followed by glucose (0.69 g/100g) and fructose (0.58 g/100g) [107] in black carrots. In contrast to our study and black carrot studies, Tanguler [106] checked for presence of arabinose and found in low amounts (0.134 g/L-0.193 g/L) in shalgam juice with HPLC using external standards.

Table 4.3. Individual sugar concentration of regular shalgam juice (Aa1)

Sugars	**Concentration in regular shalgam juice (Aa1)**
Fructose	0.104 g/L
Sucrose	0.075 g/L
Glucose	0.041 g/L

4.1.2. Anthocyanins

Anthocyanin profile of shalgam juice was determined by HPLC. Due to the lack of standards, the peaks were identified using the information in literature [79, 125, 134]. As seen from Figure 4.2, same five major anthocyanins, cyanidin-3-xylosylglucosylgalactoside (cya 3-xylglcgal) (peak 1), cyanidin-3-xylosylgalactoside (cya 3-xylgal) (peak 2), cyanidin-3-xylosylglucosylgalactoside acylated with sinapic acid (peak 3), cyanidin-3-xylosylglucosylgalactoside acylated with ferulic acid (peak 4) and cyanidin-3-xylosylglucosylgalactoside acylated with coumaric acid (peak 5) were identified in shalgam juice [68, 107, 115].

Anthocyanins in shalgam juice are cyanidin-based anthocyanins, which can be found in blackberries, elderberries, black carrots, sweet potatos and red cabbages [80]. Cyanidin-based anthocyanins are substitued with sugars such as glucose, xylose, galactose and sophorose and most of these anthocyanins are found in acylated forms [80].

The anthocyanin cyanidin-3-xylosyl-glucosyl-galactoside acylated with ferulic acid was found as the dominant anthocyanin with the ratio of 47.81% (area). The acylated anthocyanins were found as 69.7% of the total anthocyanins in shalgam juice. This is also supported with the literature which demonstrates that black carrots are rich source of

acylated anthocyanins [79]. The dominant anthocyanin of black carrots was determined as cyanidin-3-xylosyl-glucosyl-galactoside acylated with ferulic acid with the ratio of 43-84% of total anthocyanins, similar to our findings [79]. Acylated anthocyanins in black carrots were also found in the range of 55-99% of total anthocyanins [79]. The anthocyanin profile of shalgam juice was shown to have similar individual anthocyanins in literature [79, 125, 134]. In a previous study investigating the relation between the anthocyanin content and product quality in shalgam juice, black carrots were determined as the only sources of anthocyanins in shalgam juice [125]. Cyanidin-3-xylosyl-glucosyl-galactoside acylated with ferulic acid was found as the dominant anthocyanin in shalgam juice with the concentration of 48.4% of total anthocyanins and the ratio of acylated anthocyanins to nonacylated anthocyanins was determined as 2.7 [125]. It was also stated that non-acylated anthocyanins in shalgam juice were degraded faster than acylated anthocyanins, which were found as dominant anthocyanins in shalgam juice and are more stable to heat, pH and environmental conditions because of their ability to prevent nucleophilic attack of water [79, 125, 134]. Storage temperature as well as storage time was determined as a factor affecting the anthocyanin stability in shalgam juice [125, 134]. The rate of degradation of anthocyanins in shalgam juice was highest when samples stored at 40 °C, compared to samples stored at 4°C and 25°C [125]. Degradation of anthocyanins also increased with time (0-17 months) and nonacylated anthocyanins degraded more rapidly than acylated ones during storage [134].

For more information on the properties of anthocyanins, quantification of individual anthocyanins is needed. Although there is no quantitative data on the individual anthocyanins of shalgam juice, in literature total anthocyanin amount was found in the range of 67.5-168.23 mg/L as cyanidin-3-glucoside using spectrophotometric methods in commercial and lab-scale produced shalgam juices [106, 122, 131, 132]. In a previous study [131], it was found that total anthocyanin concentration (129-149 mg/L) was affected by the black carrot amount in production. Since black carrot is the source of anthocyanins in shalgam juice, increasing amounts of black carrots resulted with higher concentration of anthocyanins in final product [131]. In the study of Utuş [132], the effect of various sizes of black carrot usage on the anthocyanin amount was investigated and highest concentration (145.60 mg/L) was found in the shalgam juices produced with 3 cm black carrot, while lowest concentration (120.18 mg/L) was found in the shalgam juices

produced with 9 cm black carrot. In another study, the effect of starter cultures on the total anthocyanin content was determined and the highest anthocyanin amount (168.23 mg/L) was found in shalgam juices produced without a starter culture, while lowest anthocyanin concentration (104.04 mg/L) was found in shalgam juices produced without a bulgur dough (first) fermentation [106].

As a fermented juice, microflora of shalgam juice may also affect the phenolic contents of the product [151]. Although there is no directly related study about the effect of lactic acid bacteria on anthocyanins, structure of other phenolic compounds such as phenolic acids can be changed by enzymes such as decarboxylase enzymes produced by *Lb. plantarum* species [152]. Anthocyanins may also affect LAB viability and growth in fermentation by either inhibiting or promoting the growth of microorganisms [151, 153]. However, in this study, the effect of microorganisms on anthocyanins in shalgam juice was not examined and still needs to be studied.

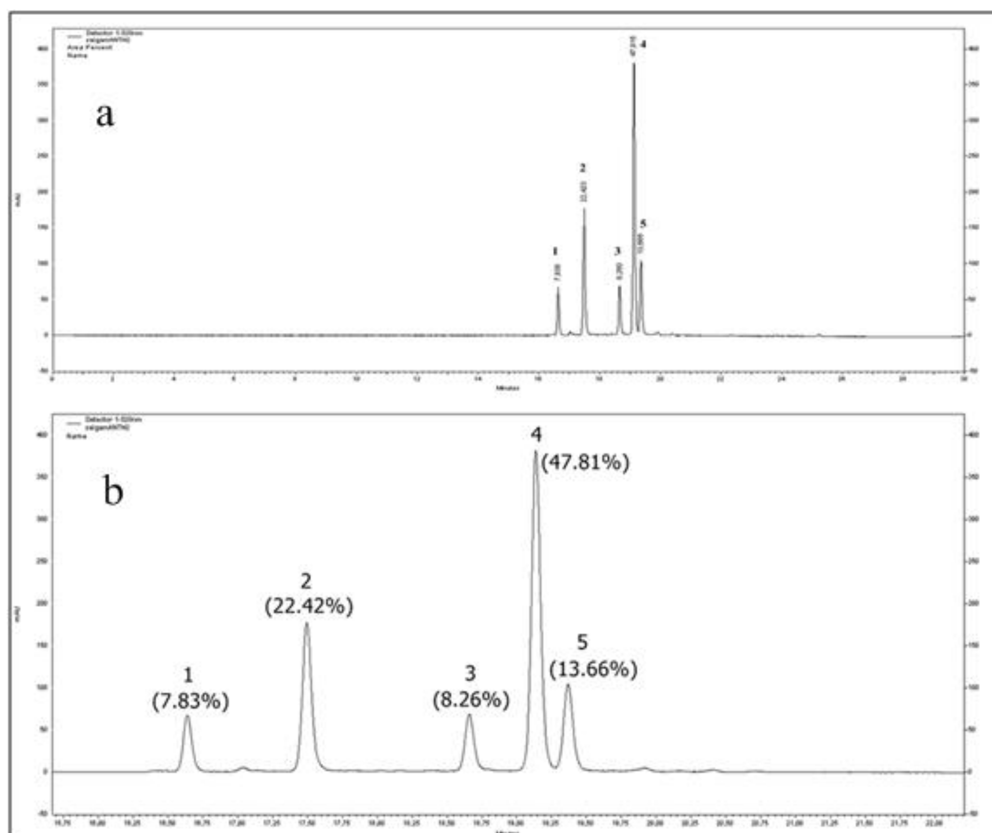


Figure 4.2. a. Anthocyanins in shalgam juice were determined as (1) cyanidin-3-xylosyl-glucosyl-galactoside, (2) cyanidin-3-xylosyl-galactoside, (3) cyanidin-3-xylosyl-glucosyl-galactoside acylated with sinapic acid, (4) cyanidin-3-xylosyl-glucosyl-galactoside acylated with ferulic acid and (5) cyanidin-3-xylosyl-glucosyl-galactoside acylated with coumaric acid. b. The ratios of individual anthocyanins

4.2. TOTAL PHENOLIC CONTENT AND ANTIOXIDANT PROPERTIES OF SHALGAM JUICE

Total phenolic contents (TPC) and antioxidant capacities as ABTS and DPPH radical scavenging activity, and ferric reducing ability (FRAP) of commercially available shalgam juices is given in Table 4.4. As stated before, antioxidant capacity and total phenolic content of black carrot juice and a commercially available pomegranate juice is used for comparison of the shalgam juice results (Table 4.4).

Table 4.4. Antioxidant capacity and total phenolic content of shalgam, black carrot and pomegrate juices

Samples	**ABTS (µmol TE/mL)**	**DPPH (µmol TE/mL)**	**FRAP (µmol TE/mL)**	**TPC (µg GA/mL)**
Aa1	3.42±0.09^{abc}	4.44±0.65^{ab}	2.26±0.32^{cd}	517.21±72^{bcd}
Aa2	2.98±0.15^{bc}	4.12±0.15^{ab}	2.01±0.17^{d}	448.71±10^{cd}
Ab1	3.32±0.09^{abc}	4.54±0.09^{ab}	2.66±0.25^{bcd}	463.79±18^{cd}
Ab2	3.51±0.09^{abc}	4.30±0.29^{ab}	2.23±0.11^{cd}	460.71±20^{cd}
B1	3.58±039^{abc}	3.82±0.02^{ab}	2.49±0.12^{bcd}	516.20±13^{bcd}
B2	3.63±1.21^{abc}	4.18±0.16^{ab}	2.49±0.69^{bcd}	445.39±181^{cd}
C1	3.96±0.16^{ab}	5.96±2.39^{a}	2.85±0.03^{abc}	570.41±30^{bc}
C2	3.39±0.09^{abc}	4.95±0.09^{ab}	2.90±0.19^{abc}	561.23±14^{bcd}
D2	2.43±0.09^{c}	3.53±0.27^{b}	1.91±0.04^{d}	387.24±36^{d}
E1	4.37±0.14^{a}	4.21±0.44^{ab}	3.61±0.23^{a}	754.44±9.59^{a}
E2	3.94±0.46^{ab}	3.89±0.14^{ab}	3.19±0.05^{ab}	684.24±11.42^{ab}
*Black carrot juice (Adana)	10.68±0.15	15.26±1.68	7.48±0.54	1785.40± 63,8
*Black carrot juice (Istanbul)	6.95±0.42	10.16±0.54	3.72±0.00	-
Pomegranate juice	17.63±0.98	24.10±2.45	13.52±1.12	2498.05±77

*Juices were freshly peeled and squeezed from black carrots

$^{a-d}$Means ± SD followed by the same letter, within a column, are not significantly different ($p<0.05$)

Aa1-C1: commercial regular shalgam juices, Aa2-D2: commercial hot shalgam juices, E1: regular home-made shalgam juice, E2: hot home-made shalgam juice

In ABTS radical scavenging activity assay, the change in the absorbance by antioxidants was measured at 734 nm at 4th minute. As seen from Table 4.4, ABTS values of different brand shalgam juices ranged between 2.43 µmol TE/mL and 4.37 µmol TE/mL. The highest antioxidant capacity was found in E1, home-made regular shalgam juice with 4.37 µmol TE/mL ABTS value. It had significantly higher antioxidant capacity when compared to shalgam juices with lowest antioxidant capacity, such as D2 and Aa2 ($p<0.05$). The reason might be coming from home-made shalgam juice is produced in small scale and may have not been stored as long as commercial products suggesting that shorter storage time might be effective on antioxidant capacity since anthocyanins of shalgam juice was affected by the storage conditions [125]. Also, when compared to shalgam juices with high antioxidant capacity, D2, commercially available hot shalgam juice produced in Izmir had

lower antioxidant capacity in ABTS assay. However, the black carrots used in production were supplied from the same area (Konya) as others suggesting that the storage conditions, cultivar of black carrots or production method used by this company might be different than others.

On the other hand, as expected, the antioxidant capacities of black carrot juices were significantly higher than shalgam juices ($p<0.05$). The black carrot juice provided from Company A showed significantly higher ABTS value (10.68 µmol TE/mL) than black carrot bought from market (6.95 µmol TE/mL). The reason might be the differences in the cultivar and storage conditions of the black carrots. Because of the high antioxidant capacity [99], commercially available pomegranate juice is also used as a control. In our studies, the ABTS result of pomegranate juice was found as 17.63 µmol TE/mL which was in the same range with literature [154]. According to our results, while shalgam juice had similar antioxidant capacity to apple (3.6 µmol TE/mL) and orange juices (4.2 µmol TE/mL) in ABTS assay, the antioxidant capacity of black carrot juice was comparable to that of cranberry juice (10.4 µmol TE/mL) [99].

DPPH method for antioxidant analysis measures antioxidants' ability to reduce DPPH•, thus the change of color at 515 nm. The antioxidant capacity of shalgam juice, determined with DPPH assay was found in the range of 3.53 µmol TE/mL and 5.96 µmol TE/mL as seen in Table 4.4. The highest antioxidant value was obtained from Company C's regular shalgam juice, with 5.96 µmol TE/mL DPPH value, compared to that of shalgam juices with lower antioxidant capacity, such as D2 ($p<0.05$). The antioxidant capacity of shalgam juice is related to the anthocyanin content of black carrot which can be affected by pH and storage conditions [80, 125]. Therefore, the differences between different brands of shalgam juices can be explained by their production method and storage conditions. The antioxidant capacity of black carrot juices was 10.16 and 15.26 µmol TE/mL, which was significantly higher than shalgam juices and similar to ABTS assay, black carrot juice provided from Company A showed higher DPPH value than black carrot juice obtained from the supermarket (Table 4.4). DPPH radical scavenging activity of pomegranate juice was found as 24.10 µmol TE/mL and was in the same range with literature [154]. In this study, while antioxidant capacity of shalgam juices was similar to plum juices (4.6 µmol

TE/mL) black carrot juices showed similar antioxidant capacity to cherry and raspberry juices (10.0 and 13.4 μmol TE/mL, respectively) as determined by DPPH assay [155].

FRAP method measures the color change when ferric salt Fe (III)$(TPTZ)_2Cl_3$ reduced to Fe (II)$(TPTZ)_2Cl_3$ by antioxidants at acidic pH at 593 nm. The antioxidant capacity of shalgam juice samples in FRAP assay was found between 1.91 μmol TE/mL and 3.61 μmol TE/mL. The highest antioxidant capacity among the samples was found in home-made regular shalgam juice (E1), with 3.61μmol TE/mL and it was significantly higher when compared to that of lower FRAP values. The lowest antioxidant capacity was found in samples Aa2 (2.01 μmol TE/mL) and D2 (1.91 μmol TE/mL), which was significantly higher when compared to shalgam juices with high FRAP values such as E1. Black carrot juices, obtained from Company A and supermarket showed significantly higher ($p<0.05$) antioxidant capacity than all shalgam juices with 7.48 μmol TE/mL and 3.72 μmol TE/mL, respectively. Similar to ABTS and DPPH radical scavenging activity assays, pomegranate juice's antioxidant capacity in FRAP assay was found within the range of previous studies (as 13.52 μmol TE/mL) [154]. Comparably, the study of Herken & Guzel [100] showed that the antioxidant capacity of commercially available juices (apple, apricot, orange and peach) in FRAP assay were 2.0 μmol TE/mL, 2.2 μmol TE/mL, 2.9 μmol TE/mL and 2.4 μmol TE/mL, respectively. On the other hand, FRAP values of black carrot juice were close to sourcherry and strawberry juices (4.6 μmol TE/mL and 4.1 μmol TE/mL, respectively) [100] except that one of the black carrot juice analyzed in our study, which was obtained from Adana) showed higher antioxidant capacity then black carrot juice obtained from Istanbul.

Total Phenolic Content (TPC) of juices was determined by the Folin-Ciocalteu assay at 760 nm using gallic acid standard curves. TPC of shalgam juices was found in the range of 387.24 and 754.44 μg GAE/mL with no significant difference among shalgam juices irrespective of the heat process and the content (regular or hot) of different companies ($p<0.05$), except E1 and D2. Similar to antioxidant capacity results, E1, home-made regular shalgam juice and D2, commercial hot shalgam juice has significantly higher and lower TPC compared to other shalgam juices. Total phenolic content of black carrot was found as 1785.40 μg GAE/mL and similar to antioxidant assays, is significantly higher than all shalgam juices. Shalgam juice had a similar total phenolic content with apple and

orange juices, which were 339 and 755 μg GAE/mL, respectively [97]. The total phenolic content of pomegranate juice was found as 2498.05 mg GAE/mL. This value is in the range of literature (2566 mg/L) [154].

In literature, although there are no studies on the analysis of antioxidant capacities of commercially available shalgam juices, the studies on black carrot and shalgam juice extracts are present [114-116, 136]. Different than our study, the antioxidant capacity and total phenolic contents of ethanol extracts instead of final product of shalgam juice, black carrot and black carrot concentrate was determined by Öztan [136] with DPPH, ABTS, FRAP and Folin Ciocalteu methods and expressed as TE/g (DPPH), mM TEAC (ABTS), ascorbic acid equivalent/mL (FRAP) and GAE/g (TPC). The highest TPC among samples was found in black carrot concentrate extract (15500 μg GAE/g) followed by black carrot and shalgam juice extracts (1078 μg GAE/g and 1052 μg GAE/g, respectively). Similarly, in DPPH (294.64 μmol TE/g) and ABTS (3mM TEAC) assays, the antioxidant capacity of black carrot concentrate extract had the highest antioxidant capacity among samples [136].

The antioxidant capacity of ethanol and water extracts of black carrot was determined also with the oxidation of β-carotene and found as 73.0% and 61.8%, respectively [113]. In another study, lipophilic and hydrophilic extracts of freeze dried black carrots were also used for antioxidant capacity determination with ABTS and DPPH methods and the hydrophilic extracts of black carrots showed highest antioxidant capacity among colored varieties of carrots with 0.28 and 0.13 μmol TE/mg values in ABTS and DPPH assays, respectively [116]. Black carrot's antioxidant capacity was found to be affected by the process, enzyme treatment and drying method [114, 115]. Total phenolic content and antioxidant capacity of heated and with pectinase enzyme treated black carrot juices were determined and TPC of black carrot juices, used as control, was found as 3000 μg GAE/mL while antioxidant capacity was found as 23 μmol TE/mL in FRAP assay, which was higher than our findings. Antioxidant capacity of dried samples were measured using DPPH method and the concentration to decrease the 50% of the radical of raw carrot was found as 30.23 mg sample/mg DPPH while the concentration needed for dried samples varied from 61.44 to 7.80 mg sample/mg DPPH [114].

The comparison between the methods of antioxidant capacity determination cannot be made directly [91]. However, in all methods tested in this study, the antioxidant capacities of E1 was found significantly higher when compared to that of lowest values such as D2. The antioxidant capacities and total phenolic contents of shalgam juices are similar to fruit juices such as apple and orange juices, which are considered as healthy beverages. Therefore, it can be said that shalgam juice has an indicative antioxidant potential *in vitro*. However, further *in vivo* analysis and bioavailability studies are required.

4.3. ENUMERATION AND IDENTIFICATION OF LACTIC ACID BACTERIA IN SHALGAM JUICE

4.3.1. Enumeration of Microorganisms

Enumeration and identification studies were carried out using the non-heat treated regular shalgam juice of Company A, Aa1. Lactic acid bacteria as *Lactobacillus spp.*, *Lactococcus & Streotococcus spp.*, total aerobic microorganisms and yeasts & molds were enumerated for both dough and shalgam juice by using classical culturing techniques (Table 4.5).

Table 4.5. The *Lactobacillus spp.*, *Lactococcus* and *Streptococcus spp.*, aerobic microorganisms and yeast and molds contents (Log CFU/mL) of dough and shalgam juice

	Dough (Log CFU/mL)	**Shalgam (Log CFU/mL)**
Lactobacillus spp.	8.65 ± 0.14	4.09 ± 0.08
Lactococcus & Streptococcus spp.	8.69 ± 0.11	2.90 ± 0.01
Yeasts and molds	8.82 ± 0.14	5.05 ± 0.03
Total Aerobic Microorganisms	7.74 ± 0.07	2.89 ± 0.11

While the population of *Lactobacillus spp.* in shalgam juice enumerated on MRS agar was 4.12 Log CFU/mL, *Lactococcus & Streptococcus spp*. enumerated on M17 agar were 2.90 Log CFU/mL in final product. In previous studies, the amount of lactic acid bacteria present in shalgam juice were determined by only cultivating on MRS agar and the population changed between 4.00-8.95 Log CFU/mL in commercial and lab-scale

produced products [29, 122, 130-132]. In commercially available shalgam juices, the LAB count in final product was found between 4.00-7.97 Log CFU/mL [122, 128, 130]. In a controlled production study examining the growth and occurrence of LAB during shalgam juice fermentation in commercial and lab-scale production, it was observed that LAB levels were increased during fermentation until the amount of 9.01 Log CFU/mL, and then decreased at the end of the fermentation (7.34-8.23 Log CFU/mL) [29]. The microflora of shalgam juice depends on the amount of the black carrot and the type and origin of sourdough used in the production and different fermentation techniques during the process [131, 132]. Güneş [131] studied the effect of black carrot amount on shalgam juice quality and observed that the LAB count increased with the increase in the amount of black carrot and the highest LAB count was obtained when 20% black carrot was used (8.95 Log CFU/mL). In another study, the LAB counts in shalgam juices produced with 3, 6, and 9 cm black carrots were examined and found that the size of the black carrot was not effective on the microbial population of shalgam juice (7.46-7.49 Log CFU/mL) [132]. While the addition of starter cultures such as *Lb. plantarum, Lb. fermentum* and *Lb. paracasei subsp. paracasei* strains had increased the LAB counts during fermentation, shalgam juice production with a one-step fermentation (without a prior dough fermentation) has shown lower LAB counts and the final LAB population was found in the range of 7.43-7.74 Log CFU/mL [106].

Similar to results found in literature (7.1-8.9 Log CFU/g) [29, 131, 132], the amount of *Lactobacillus spp.* and *Lactococcus & Streptococcus spp*. in dough (provided after first fermentation step of Company A) were higher than shalgam juice and found as 8.65 Log CFU/g and 8.70 Log CFU/g, respectively. The decrease in the population of LAB in shalgam juice compared to sourdough can be explained by the decline of the medium pH level because of the increase in organic acids during fermentation. When the growth pattern of LAB in shalgam juice fermentation was examined, it was reported that LAB counts increased in the beginning of the fermentation then a small decrease could be observed at the end of the fermentation [29, 131, 132].

Total mesophilic aerobic bacteria was also determined and found as 2.89 and 7.74 Log CFU/mL in shalgam juice and dough, respectively. In literature total mesophilic aerobic bacteria counts of shalgam juice and dough was found as 3.25-8.57 Log CFU/mL and

7.17-9.03 Log CFU/g, respectively [111, 112, 117, 130, 131]. Similar to LAB counts, there was a significant decrease in total aerobic bacteria counts at the end of the fermentation ($p<0.05$) because of the decrease in pH. The changes in the population can also be explained by the temperature and seasonal variations of raw materials [37].

Yeasts and molds in shalgam juice originated from the raw materials or environment. Total yeast and mold counts of shalgam juice were determined as 5.05 Log CFU/mL in shalgam juice. This result is similar to the literature values which are in the range of 4.4-8.15 Log CFU/mL [122, 130-132]. In dough, the total yeast and mold count was found as 8.82 Log CFU/g, which is in the literature range of 7.19-8.95 Log CFU/g [131, 132] and significantly higher than shalgam juice ($p<0.05$). Sodium benzoate (0.02%), a sodium salt of benzoic acid is used in shalgam juice to control the spoilage and growth of yeasts and molds [122]. Benzoic acid inhibits the yeast growth, therefore the decline in total yeasts and mold counts in final product can be explained by the addition of sodium benzoate to final product and also decrease in pH during fermentation [22, 122].

4.3.2. Identification of LAB in Shalgam Juice

Flavor, aroma and texture attributes of shalgam juice, as a lactic acid fermented beverage are affected by the properties of microflora of the product. The lactic acid bacteria in shalgam juice fermentation mostly comes from the bulgur dough as well as other sources such as ingredients and the tanks used in production [106]. The microflora of shalgam juice was determined in previous studies by conventional methods based on morphological, physiological and biochemical characteristics of bacteria [29, 106, 128, 130, 135]. In this study, identification of LAB in shalgam juice was carried out using species-specific PCR to get more accurate information about LAB of shalgam juice since identification based on molecular methods are found to be more accurate, especially for closely related species [51]. The PCR results for amplification of total DNA extracted from shalgam juice with species-specific primers showed bands between 100 and 200 bp which were the expected amplified product sizes of DNA (Figure 4.3) [143].

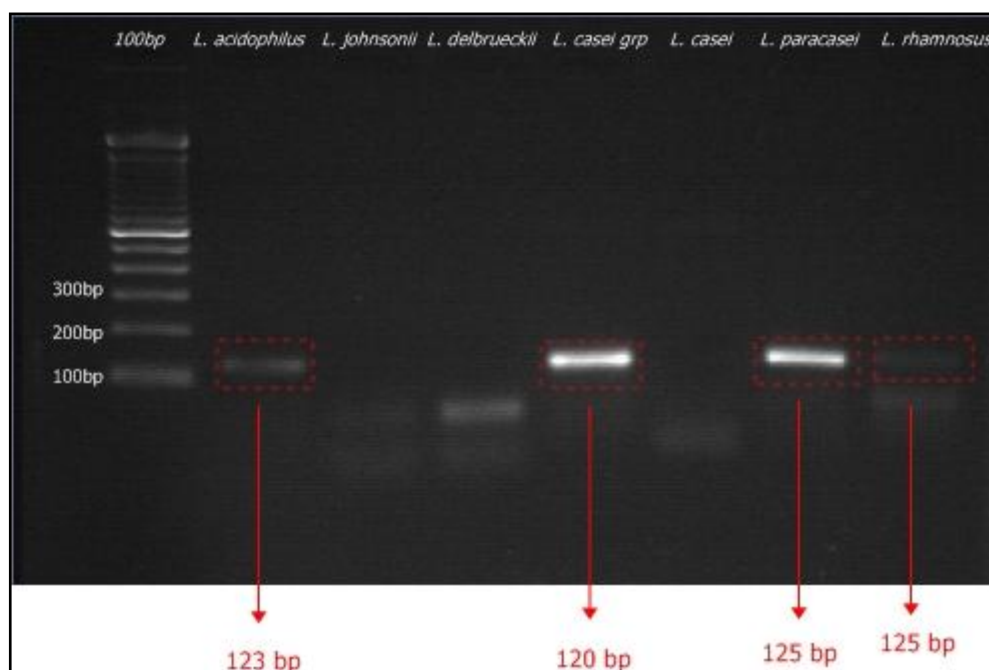


Figure 4.3. The bands of PCR products from the amplification of total DNA directly extracted from shalgam juice with species-specific primers were found between 100-200 bp

The amplified target DNAs were compared by published sequences of LAB species from the NCBI nucleotide sequence database with the BLAST search program (Table 4.6). As seen in Table 4.6, a total of 21 *Lactobacillus* species and subspecies were identified from shalgam juice with 88-97% homology with the sequences which were retrieved from Genbank accession numbers. Homology between the identified species and the sequences in the database should be above 70% for precise identification [68].The results of our study showed 88-97% similarities with previously identified species in literature. Among identified species, while *Lactobacillus plantarum subsp. plantarum* ATCC 14917, *Lb. casei* ATCC 334, *Lb. brevis* and *Lb. helveticus* were detected from the isolated colonies of shalgam juice and of dough, the rest of the species were identified directly from shalgam juice. Other than *Lb. plantarum, Lb. brevis, Lb. paracasei subsp. paracasei* and *Lb. delbrueckii,* which had previously been phenotypically identified in shalgam juice, the present study was first to genotypically (using 16S rDNA) identify all the species and subspecies shown in Table 4.6 [29, 128, 130, 135].

Table 4.6. Percentage (%) similarity of the partial 16S rRNA sequences of the LAB to their closest relatives available in the NCBI nucleotide sequence database

Bacterial species	**Homology (%)**	**GenBank Accession no. of strain**	**Reference**
Lactobacillus plantarum and subsp.			
Lb. plantarum subsp. plantarum ST-III chromosome	96	NC_014554.1	[156]
Lb. plantarum JDM1	96	NC_012984.1	[157]
Lb. plantarum subsp.	92	AJ965482	-
Lb. plantarum subsp. argentoratensis	92	AJ640078	[158]
Lactobacillus casei group			
Lb. casei ATCC 334	97	NC_008526.1	[159]
Lb. casei	93	D16552	[160]
Lb. casei subsp. casei	93	AF469172	[161]
Lb. paracasei subsp. paracasei	93	D79212	[160]
Lb. paracasei subsp. tolerans	93	AB181950	[162]
Lactobacillus brevis			
Lb. brevis ATCC 367	94	NC_008497.1	[159]
Lb. brevis	93	M58810	-
Lb. parabrevis	93	AM158249	[65]
Lactobacillus delbrueckii group			
Lactobacillus delbrueckii subsp. lactis	92	AY050173	[163]
Lactobacillus delbrueckii subsp. delbrueckii	92	AY773949	[163, 164]
Lactobacillus delbrueckii subsp. indicus	92	AY421720	[166]
Lactobacillus acidophilus group			
Lactobacillus acidophilus	95	AY773947	[61]
Lactobacillus gasseri	92	AF519171	[158, 166]
Lactobacillus helveticus	94	AM113779	[168]
Lactobacillus helveticus DSM 20075 contig00259	88	ACLM01000202.1	-
Lactobacillus reuteri	93	L23507	-
Lactobacillus sharpeae	91	M58831	-

Identified species were classified and also put into a phylogenetic tree according to the homology results obtained from the BLAST search as seen in Figure 4.4. ClustalW software was used to this sequence tree presentation based upon the alignments.

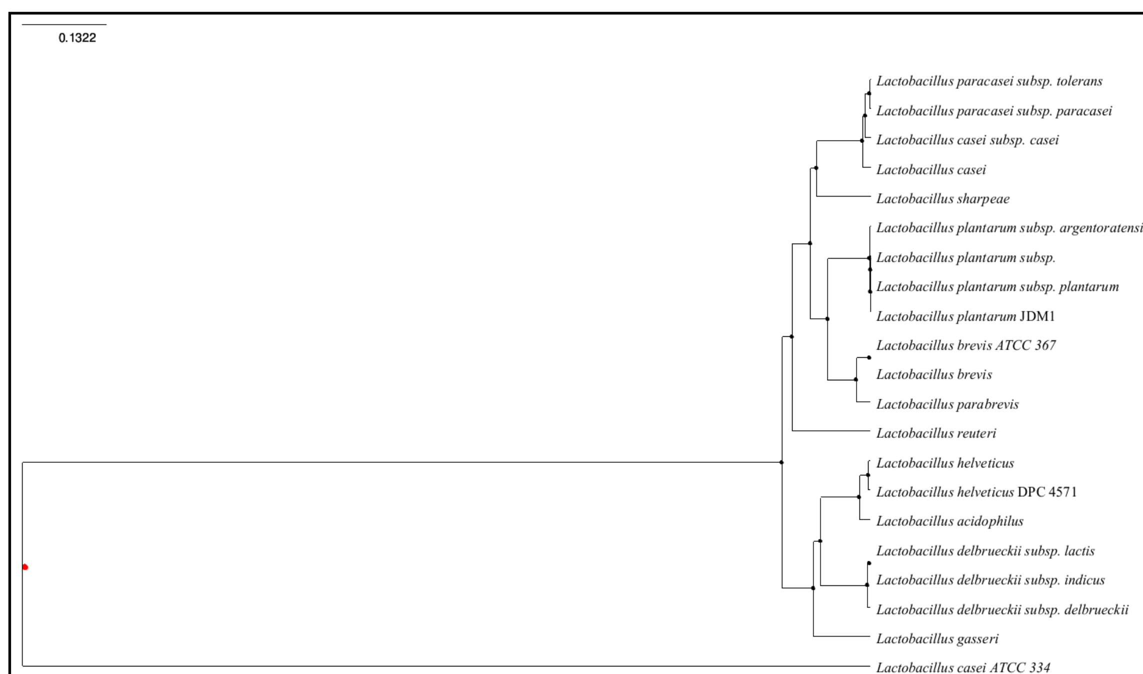


Figure 4.4. Phylogenetic tree of species of *Lactobacillus* and related taxa

Lactobacillus plantarum and its different strains (*Lb. plantarum JDM1*) and subsp. (*Lb. plantarum subsp. plantarum, Lb. plantarum subsp. argentoratensis*) were identified both directly from shalgam juice and isolated colonies. It had 92-96% homology (Table 4.6) with previously described *Lb. plantarum* isolated from kimchi and vegetables when compared sequences in the database [156]. In literature, *Lb. plantarum* was reported as the predominant lactic acid bacteria at the beginning, middle and end of the shalgam juice fermentation with a concentration of 7.19-8.17 Log CFU/mL using commercially available kits (API) based on the phenoytpical properties of LAB [29, 128, 130]. Similarly, *Lb. plantarum* species was also identified by Erginkaya and Hammes [135] in lab scale produced shalgam juices with biochemical and phenotypical methods. However, other subspecies and strains such as *Lb. plantarum subsp. plantarum, Lb. plantarum subsp. argentoratensis,* were only identified with molecular methods used in this study suggesting that species-specific PCR based on 16S rDNA method provided more detailed information

on microflora in subspecies level. *Lb. plantarum* species are facultative heterofermentative and pH resistant lactic acid bacteria species found in dairy, meat, sourdough and vegetable fermentations and products such as cheeses, sausages, olives and wines [30, 42, 169]. In fermented vegetable juices such as beet, cabbage, carrot and tomato juices *Lb. plantarum* strains are used as starter cultures [14, 15, 16]. *Lb. plantarum* strains are considered as safe and used in commercial probiotic products due to their beneficial health effects such as cholesterol reducing, natural immune response improving properties and protective effects against intestinal infection in numerous studies [42, 169].

Lactobacillus casei group, *Lb. casei*, *Lb. casei subsp. casei, Lb. paracasei subsp. paracasei, Lb. paracasei subsp. tolerans* were also identified from both shalgam juice and isolated colonies using species-specific PCR based on 16S rDNA (Table 4.6 and Figure 4.4). Among these, while *Lb. casei* ATCC 334 was shown 97% homology with previously described strain [159] isolated from fermented vegetables, other *Lb. casei* group LAB had 93% homology [160, 161, 162]. On the other hand, in literature, among the *Lactobacillus casei* group, only *Lb. paracasei subsp. paracasei* was determined as one of the predominant LAB species in commercially available and lab scale produced shalgam juice during fermentation using commercial identification kits [29, 53, 128, 130, 170] *Lb. casei* group are found in many habitats, including dairy products, sourdough, mouth and intestines [171] and used as starter cultures in dairy products, commercially available probiotic products and fermented vegetable juices [14, 16, 39, 170]. Health benefits of *Lb. casei* are well documented as a probiotic. Some of the effects includes antitumor, immunostimulatory and antimicrobial activities [39].

Other species identified directly from shalgam juice and the isolated colonies, were *Lactobacillus brevis*. It had 94 and 93% homology with two different strains, of which genome sequences were reported in literature found in fermented plant products [30, 159]. *Lb. brevis* is an obligate heterofermentative LAB, which was determined as one of the predominant lactic acid bacteria in commercially available shalgam juices during and at the end of the fermentation in previous studies in which identification was made with methods based on phenotypical and biochemical properties of microorganisms [29, 130, 135]. *Lb. brevis* is also used in the production of fermented tomato juice as a starter culture and identified in fermented fruit juices such as hardaliye [20, 22]. *Lb. parabrevis* is a newly

proposed taxon of *Lb. brevis* (Figure 4.4) which is facultatively heteromermentative LAB and was also identified in shalgam juice with 93% homology with previously described *Lb. parabrevis* isolated from farmhouse red Chesshire cheese [65]. *Lb. brevis* species can be found mostly in sourdough fermentation [171] suggesting that *Lb. brevis* found in shalgam juice might be coming from dough. The same strain may also cause spoilage in vegetable products [171]. Although *Lb. brevis* is not used as probiotic in commercial products, it's proposed as probiotic due to its beneficial effects such as immune system stimulation, antagonistic effects towards potentially harmful microorganisms and adhesion properties to intestine [44, 45]. However, *Lb. brevis* was not suitable as being the only strain in fermentation since use of only some *Lb. brevis* strains was not enough to acidify the product [45]. However, it was suggested that *Lb. brevis* can be used as a supplementary strain in fermented products along with other *Lactobacillus* strains.

Lactobacillus delbrueckii group, *Lb. delbrueckii subsp. lactis, Lb. delbrueckii subsp. delbrueckii, Lb. delbrueckii subsp. indicus* were identified from shalgam juice directly but not in isolated colonies. They showed 92% similarity with species identified previously [163, 164, 165]. Among these species, only *Lb. delbrueckii subsp. delbrueckii* was found in literature, in the beginning of the fermentation in the commercially available shalgam juices however, it was not present in later steps of fermentation and in the final product most probably because of the decrease in the pH [130]. Phenotypical methods are based on the cultivation, only viable microorganisms can be identified. In our study, identification was done with DNA-based methods which can provide information on microflora even the species are not viable. *Lb. delbrueckii* group bacteria are obligate homofermentative LAB and ferment glucose strictly to lactic acid. [171]. *Lb. delbrueckii subsp. lactis* is found in starter cultures in cheese production and *Lb. delbrueckii subsp. delbrueckii* is related to sourdough fermentation [171, 172].

Lb. acidophilus and Lb. gasseri were two of the bacteria identified in shalgam juice directly. They are classified as *Lactobacillus acidophilus* group bacteria and are closely related (Figure 4.4) [171]. Therefore, they cannot be differentiated with phenotypic methods but molecular methods can be used to identify them [171]. *Lb. acidophilus* found in shalgam juice had 95% homology with strains isolated from probiotic dairy products, yogurt and milk powder, in literature [61] and *Lb. gasseri* had 92% similarity with

previously described *Lb. gasseri* [159, 167] isolated from vegetable fermentation. *Lb. acidophilus* group bacteria are obligate homofermentative LAB and can be found in dough and dairy products [39, 172]. *Lb. acidophilus* is used in acidophilus milk and considered a probiotic strain. Probiotic properties of *Lb. acidophilus* includes antagonistic effects against enteropathogenic bacteria, inhibition of carcinogenic products by short chain fatty acids, antimutagenic effects and immunomodulation [39].

Lactobacillus helveticus was also identified from shalgam juice with 94 and 88% homology with two different strains, which were previously isolated from cheese [168]. *Lb. helveticus* is closely related to *L. acidophilus* group (Figure 4.4). This species can be found mostly in dairy products and dough and is an obligate homofermentative LAB species [171, 172]. *Lb. helveticus* is found to be immunomodulatory and antimutagenic due to its peptide compounds released in fermented milk products [173, 174].

Lactobacillus reuteri, an obligate heterofermentative LAB, was identified in shalgam juice and had 93% homology with *Lb. reuteri*, which was retrieved from Genbank accession number. It has gained attention due to the probiotic functions of its product, reutocyclin [171]. In previous shalgam juice studies, while *Lb. reuteri* was not identified in shalgam juice *Lb. fermentum* was found [29, 130, 135]. The reason for this might be even they're not closely related in genome level, it is hard to distinguish *Lb. reuteri* and *Lb. fermentum* from each other using phenotypic methods [171]. *Lb. reuteri* is mostly found in gastrointestinal tract of humans but it also has been identified as a predominant LAB in sourdough as well as cereal products [172]. The most important feature of *Lb. reuteri* is reutocyclin, an antibiotic substance produced during fermentation, which is effective against some of the gram negative bacteria and some lactic acid bacteria species, therefore proposed as a preservative in bread production [175].

Lactobacillus sharpeae, an obligate heterofermentative LAB species, was identified from shalgam juice directly and it has 91% homology with previously reported *Lb. sharpeae* in Genbank. This species can be found in meat products, however its properties are not widely known [176].

Some of the LAB in shalgam juice (*Lb. pentosus, Lb. buchneri, Lactococcus lactis subsp. lactis* and *Leuconostoc mesenteroides subsp. mesenteroides/dextranicum*) couldn't be identified in our study using molecular methods, although they were detected in a very recent study [29, 130]. Species-specific PCR is a reliable method to distinguish closely related lactic acid bacteria however only previously designated species can be amplified with the species-specific primers and primers for species with no gene sequence information cannot be developed [170]. Therefore, to identify other species, new primers targeting wanted species should be designed according to the recent publication. Also, some species can’t be identified due to the reduced PCR sensitivity due to complex food matrix [67]. Use of other techniques such as plasmid profiling, the analyses of fragment length polymorphisms (RFLP, ribotyping), pulse-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), and different culture-independent strategies (DGGE, TGGE) might be useful for more detailed analysis [177].

4.4. CYTOTOXIC EFFECT OF SHALGAM JUICE

Phenolic compounds, especially anthocyanins are known to have anti-proliferative effects against colorectal carcinoma cells mostly due to their antioxidant capacities [178-181]. Anti-proliferative effects of phenolics are mostly investigated by exposing cells to individual phenolics, however in vivo systems are complex and the food matrix can affect the structure and properties of phenolics [181]. Therefore, investigating effects of whole phenolics of a product are more reliable than individual phenolics [181].

In previous studies, anthocyanin rich extracts of black carrots inhibited the proliferation of colorectal carcinoma cells (HT-29) in a dose dependent manner. Highest concentration of reconstituted, lyophilised anthocyanin rich black carrot extract (2000µg/mL) was shown to have 80% inhibition in HT-29 colorectal carcinoma cell lines. However, there is no study on the effect of shalgam juice in colorectal carcinoma cell lines [117].

To determine the potential anti-proliferative effects of shalgam juice, colorectal carcinoma (Caco-2) cells were treated with lyophilised whole shalgam juice rather than individual anthocyanins. Black carrot was also used as control since it was shown to have anti-proliferative effect on colorectal carcinoma cells in previous studies [117]. As seen in

Figure 4.5, the viability of Caco-2 cells was inhibited by shalgam juice in a dose-dependent manner (50-6400 µg/mL) with 55.5-91.4% cell viability. Even in low concentrations (50 µg/mL), the viability decreased 8.6% compared to untreated cells. In the highest concentration (6400 µg/mL), cells treated with shalgam juice showed 55.5 % viability.

The anti-proliferative effect of shalgam juice on cells was higher than black carrot's (Figure 4.5) and shown 61.9% viability at the highest concentration (6400 µg/mL). The reason might be related with the chemical (salt, organic acids, sodium benzoate) and microbiological composition (LAB) of shalgam juice.

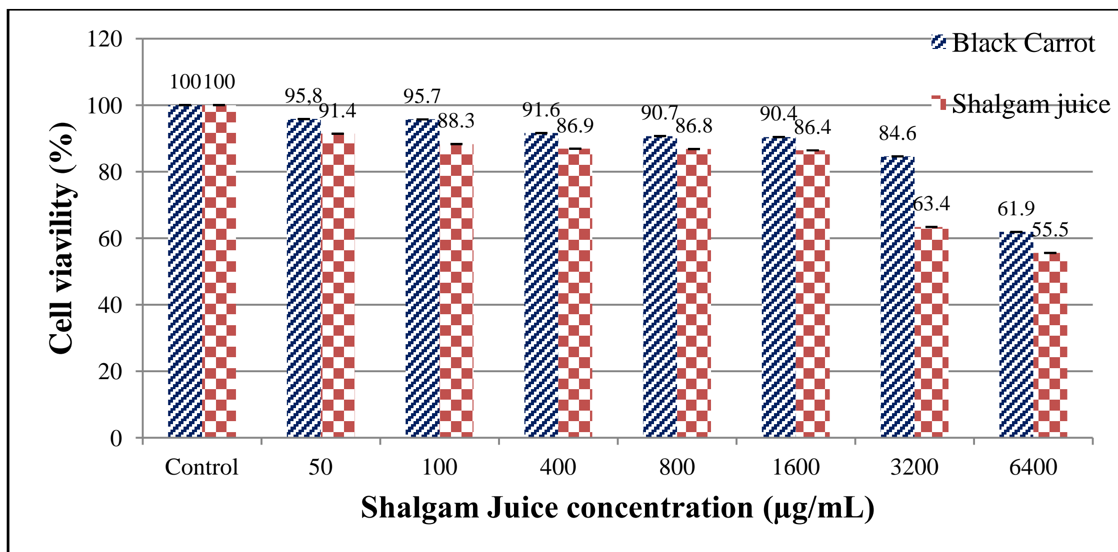


Figure 4.5. Cell viability in passage 32 Caco-2 cells incubated with shalgam juice and black carrot

As mentioned in previous sections, as a lactic acid fermented vegetable juice, organic acids such as lactic acid and acetic acid are found in high concentrations (4.19-8.90 g/L lactic acid, 1.29-2.65 g/L acetic acid, 1.25-1.10 g/L citric acid) in shalgam juice. The presence of organic acids decreases the pH and affects the composition of shalgam juice. Cytotoxic effects of organic acids produced by anaerobic intestinal bacteria, including some of the organic acids found in shalgam juice such as lactic, acetic and succinic acid, were investigated in epithelial cells [182]. It was found that organic acids can be cytotoxic in several epithelial cell lines including colon epithelial cells and can cause apoptosis. Among organic acids, lactic acid and acetic acid has shown lower cytotoxic effect compared to

other acids [182]. Since lactic acid and acetic acid are predominant acids in shalgam juice, they may have antiproliferative activity in Caco-2 cell lines.

Other reason of anti-proliferative effect of shalgam juice can be high concentration (2%) of salt. Although salt itself is not carcinogenic or mutagenic, it can be cocarcinogenic or comutagenic when carcinogens and mutagens are present [37]. However, comutagenicity of salt with raw materials was not observed in fermented products such as kimchi, a fermented vegetable product [37]. This was also supported with our findings (data not shown) suggesting that salt had no inhibitory activity in colorectal carcinoma cells [183].

Sodium benzoate is a chemical preservative used in shalgam juice production to prevent the spoilage by inhibiting the growth of yeasts and molds [122]. However, the concentration (0.02%) used in shalgam juice production is under the limit of FDA regulations (0.1%).The previous studies showed that 0.03-0.12% of sodium benzoate did not have any cytotoxic or carcinogenic effects in rat studies and human lymphocyte cells [184, 185].

On the other hand, presence of lactic acid bacteria might have anti-proliferative effect on Caco-2 cells. *Lb. casei* and *Lb. acidophilus* strains, which were also found in shalgam juice, were shown to have anti-proliferative effect on colorectal carcinoma cells (HT-29) by inhibiting the cell growth 21-28% when they were added to cell lines at the concentration of 10^8 CFU/mL [38]. Also, in another study, when LAB were co-cultured with other probiotic cultures (*Bifidobacterium spp*.) and glucose, They reduced the proliferation of colorectal carcinoma cells more [186]. Although shalgam juice as it is now, does not meet the requirements to be considered as probiotic product (less than 10^6 viable bacteria) in the final product, presence of LAB in shalgam juice may still confer some possible health benefits [39]

Similar to shalgam juice, fruit juices such as grape-orange-apricot juice which were subjected to gastrointestinal digestion, inhibited the growth of Caco-2 cells 53.4% at 24 h when they were added at the concentration of 7.5% (V/V) to the culture media [181]. Similarly, fresh and digested fruit juices such as pineapple and red fruit juice containing raspberries, cherry, red grape, blackberry and blackcurrant inhibited the growth of Caco-2

cells when they were added to growth medium at 4% concentration. Red fruit juice, which contains anthocyanins, was shown to have higher anti-proliferative activity than pineapple juice [187].

Overall, although shalgam juice was found to be cytotoxic in Caco-2 cells, the mechanism behind anti-proliferative action is still unknown. The anti-proliferative effect may be caused by anthocyanins or lactic acid bacteria and their end products (organic acids) or by a combined effect of both. However the source of the effect still needs to be investigated.

5. CONCLUSION

In this study, chemical and biological characterization of shalgam juice were done by determining its antioxidant capacity, identifying its microflora using species-specific PCR based on 16S rDNA and checking its anti-proliferative effects on Caco-2 cell lines in vitro conditions to determine its preliminary health benefits which can help to understand the bioactivity of the shalgam juice.

The results of our study showed that shalgam juice had similar in vitro antioxidant capacity to apple and orange juices. The antioxidant capacity and total phenolic content of 11 different shalgam juices sold in the market were not significantly different from each other except that home-made regular shalgam juices and sample D2. The differences were attributed to the storage conditions, production methods or region of the black carrot used in production.

Also, identification of lactic acid bacteria of shalgam juice with species-specific PCR was provided valuable information about the microflora of the product. Some of the identified LAB was previously reported as probiotics due to their functional properties suggesting that shalgam juice may have probiotic potential in addition to its nutritional properties.

Cytotoxicity studies showed that shalgam juice had anti-proliferative effect on Caco-2 cells in vitro even though it was not clear whether the effect comes from its anthocyanins contents or microflora having probiotic properties.

In conclusion, although this study provided detailed valuable information about the antioxidant and anti-proliferative effects and microbial composition of shalgam juice *in vitro*, the mechanism behind possible health benefits of shalgam juice is still not clear and needs to be studied in future studies, both *in vitro* and *in vivo*.

REFERENCES

1. Caplice, E. and Fitzgerald, G. F., “Food fermentations: role of microorganisms in food production and preservation.” *International Journal of Food Microbiology*, Vol. 50, pp. 131–149, 1999.

2. Kabak, B. and Dobson, A. D. W., “An introduction to the traditional fermented foods and beverages of Turkey.” *Critical Reviews in Food Science and Nutrition*, Vol. 51 (3), pp. 248–260, 2011.

3. Prado, F. C., Parada, J. L., Pandey, A. and Soccol, C. R. “Trends in non-dairy probiotic beverages.” *Food Research International*, Vol. 41 (2), pp. 111–123, 2008.

4. Tamang, J. P. “Animal-Based Fermented Foods of Asia,” in Hui, Y. H. and Evranuz, E. O. (eds.), *Handbook of Animal-Based Fermented Food and Beverage Technology*, pp. 61–72, CRC Press, Florida, 2012.

5. Abdelgadir, W., Nielsen, D. S., Hamad, S. and Jakobsen, M. “A traditional Sudanese fermented camel’s milk product, Gariss, as a habitat of Streptococcus infantarius subsp. infantarius.” *International Journal of Food Microbiology*, Vol. 127(3), pp. 215–219, 2008.

6. Kebede, A., Viljoen, B. C., Gadaga, T. H., Narvhus, J. A. and Lourens-Hattingh, A. “The effect of container type on the growth of yeast and lactic acid bacteria during production of Sethemi, South African spontaneously fermented milk.” *Food Research International*, Vol. 40(1), pp. 33–38, 2007.

7. Rahman, N., Xiaohong, C., Meiqin, F. and Mingsheng, D. “Characterization of the dominant microflora in naturally fermented camel milk shubat.” *World Journal of Microbiology and Biotechnology*, Vol. 25(11), pp. 1941–1946, 2009.

8. Blandino, A., Al-Aseeri, M. E., Pandiella, S. S., Cantero, D. and Webb, C. “Cereal-based fermented foods and beverages.” *Food Research International*, Vol. 36(6), pp. 527–543, 2003.

9. Soccol, C. R., Lindner, J. D. D., Yamaguishi, C. T., Spier, M. R. “Probiotic Nondairy Beverages,” in Hui, Y. H. (ed.), *Handbook of Plant-Based Fermented Food and Beverage Technology*, pp. 707–728, CRC Press, Florida, 2012.

10. Von Mollendorff, W. J. *Characterization of bacteriocins produced by lactic acid bacteria from fermented beverages and optimization of starter cultures*, M.S. Thesis, 2009.

11. Gotcheva, V., Pandiella, S. S., Angelov, A., Roshkova, Z. and Webb, C. “Monitoring the fermentation of the traditional Bulgarian beverage boza.” *International Journal of Food Science & Technology*, Vol. 36(2), pp. 129–134, 2001.

12. Luckow, T. and Delahunty, C. “Which juice is ‘healthier’? A consumer study of probiotic non-dairy juice drinks.” *Food Quality and Preference*, Vol. 15(7-8), pp. 751–759, 2004.

13. Rakin, M., Sekulić, M. V. and Mojović, L. “Health Benefits of Fermented Vegetable Juices,” in Hui, Y. H. . and Evranuz, E. Ö. (eds.), *Handbook of Plant-Based Fermented Food and Beverage Technology*, CRC Press, Florida, pp. 385–406, 2012.

14. Yoon, K. Y., Woodams, E. E. and Hang, Yong D. “Production of probiotic cabbage juice by lactic acid bacteria.” *Bioresource technology*, Vol. 97(12), pp. 1427–1430, 2006.

15. Yoon, K. Y., Woodams, E. E. and Hang, Y. D. “Probiotication of tomato juice by lactic acid bacteria.” *Journal of microbiology*, Vol. 42(4), pp. 315–318, 2004.

16. Yoon, K. Y., Woodams, E. E. and Hang, Y. D. “Fermentation of beet juice by beneficial lactic acid bacteria.” *LWT - Food Science and Technology*, Vol. 38(1), pp. 73–75, 2005.

17. Klewicka, E., Motyl, I. and Libudzisz, Z. “Fermentation of beet juice by bacteria of genus Lactobacillus sp.” *European Food Research and Technology*, Vol. 218(2), pp. 178–183, 2004.

18. Rakin, M., Vukasinovic, M., Siler-Marinkovic, S. and Maksimovic, M. “Contribution of lactic acid fermentation to improved nutritive quality vegetable juices enriched with brewer’s yeast autolysate.” *Food Chemistry*, Vol. 100(2), pp. 599–602, 2007.

19. Sheehan, V. M., Ross, P. and Fitzgerald, G. F. “Assessing the acid tolerance and the technological robustness of probiotic cultures for fortification in fruit juices.” *Innovative Food Science & Emerging Technologies*, Vol. 8(2), pp. 279–284, 2007.

20. Tsen, J., Lin, Y., Huang, H. and King, V. A. “Studies on the fermentation of tomato juice by using.” *Journal of Food Processing and Preservation*, Vol. 32, pp. 178–189, 2008.

21. Di Cagno, R., Surico, R. F., Paradiso, A., De Angelis, M., “Effect of autochthonous lactic acid bacteria starters on health-promoting and sensory properties of tomato juices.” *International Journal of Food Microbiology*, Vol. 128(3), pp. 473–83, 2009.

22. Arici, M. and Coskun, F. “Hardaliye: fermented grape juice as a traditional Turkish beverage.” *Food Microbiology*, Vol. 18(4), pp. 417–421, 2001.

23. Berry, S. K., Manan, J. K., Joshi, G. J., Saxena, A. K. and Kalra, C. L. “Preparation and evaluation of ready-to-serve (RTS) black carrot beverage (Kanji).” *Journal of food science and technology*, Vol. 26(6), pp. 327–328, 1989.

24. Erten, H., Tanguler, H. and Canbaş, A. “A Traditional Turkish Lactic Acid Fermented Beverage: Shalgam (Salgam).” *Food Reviews International*, Vol. 24(3), pp. 352–359, 2008.

25. Mousavi, Z. E., Mousavi, S. M., Razavi, S. H., Emam-Djomeh, Z. and Kiani, H. “Fermentation of pomegranate juice by probiotic lactic acid bacteria.” *World Journal of Microbiology and Biotechnology*, Vol. 27(1), pp. 123–128, 2010.

26. Demir, N., Acar, J. and Bahçeci, K. S. “Effects of storage on quality of carrot juices produced with lactofermentation and acidification.” *European Food Research and Technology*, Vol. 218(5), pp. 465–468, 2004.

27. Nazzaro, F., Fratianni, F., Sada, A. and Orlando, P. “Synbiotic potential of carrot juice supplemented with Lactobacillus spp . and inulin or fructooligosaccharides.”, *Journal of the Science of Food and Agriculture*, Vol. 8, pp. 2271–2276, 2008.

28. Barath, A., Halasz, A., Nemeth, E. and Zalan, Z. “Selection of LAB strains for fermented red beet juice production.” *European Food Research and Technology*, Vol. 218(2), pp. 184–187, 2004.

29. Tanguler, H. and Erten, H. “Occurrence and growth of lactic acid bacteria species during the fermentation of shalgam (salgam), a traditional Turkish fermented beverage.” *LWT - Food Science and Technology*, Vol. 46(1), pp. 36–41, 2012.

30. Axelsson, L. “Lactic Acid Bacteria: Classification and Physiology,” in Salminen, S. and von Wright, A. (eds.), *Lactic Acid Bacteria: Microbiological and Functional Aspects*, CRC Press, 2004.

31. Grajek, W., Olejnik, A. and Sip, A. “Probiotics, prebiotics and antioxidants as functional foods.” *Acta biochimica Polonica*, Vol. 52(3), pp. 665–71, 2005.

32. Sip, A. and Grajek, W. “Chapter 8. Probiotics and Prebiotics,” in Smith, J. and Charter, E. (eds.), *Functional Food Product Development*, Blackwell, Oxford, 2010.

33. Lourens-Hattingh, A. and Viljoen, B. C. “Yogurt as probiotic carrier food.” *International Dairy Journal*, Vol. 11(1-2), pp. 1–17, 2001.

34. Lye, H., Kuan, C., Ewe, J., Fung, W. and Liong, M. “The improvement of hypertension by probiotics: effects on cholesterol, diabetes, renin, and phytoestrogens.” *International journal of molecular sciences*, Vol. 10(9), pp. 3755–3775, 2009.

35. Wollowski, I., Rechkemmer, G. and Pool-Zobel, B. L. “Protective role of probiotics and prebiotics in colon cancer.” *The American Journal of Clinical Nutrition*, Vol. 73, p. 451S–455S, 2001.

36. Sanders, M. E. “Probiotics : Considerations for Human Health.” *Nutrition Reviews*, Vol. 61(3), pp. 91–99, 2003.

37. Park, K. and Kim, B. “Lactic Acid Bacteria in Vegetable Fermentations,” in Salminen, S., von Wright, A., Ouwehand, A. (eds.), *Lactic Acid Bacteria Microbiological and Functional Aspects*, CRC Press, 2011.

38. Choi, S. S., Kim, Y., Han, K. S., You, S., Oh, S., Kim, S. H. “Effects of Lactobacillus strains on cancer cell proliferation and oxidative stress in vitro” *Letters in Applied Microbiology*, Vol. 42(5), pp. 452–458, 2006.

39. Shah, N. P. “Functional cultures and health benefits.” *International Dairy Journal*, Vol. 17(11), pp. 1262–1277, 2007.

40. McCracken, V. J., Chun, T., Baldeón, M. E., Ahrné, S., Molin, G., Mackie, R. I., Gaskins, H. R. “TNF-alpha Sensitizes HT-29 Colonic Epithelial Cells to Intestinal Lactobacilli.” *Experimental Biology and Medicine*, Vol. 227(8), pp. 665–670, 2002.

41. Michail, S. and Abernathy, F. “Lactobacillus plantarum inhibits the intestinal epithelial migration of neutrophils induced by enteropathogenic Escherichia coli.” *Journal of Pediatric Gastroenterology and Nutrition*, Vol. 36(3), pp. 385–391, 2003.

42. De Vries, M. C., Vaughan, E. E., Kleerebezem, M. and De Vos, W. M. “Lactobacillus plantarum—survival, functional and potential probiotic properties in the human intestinal tract.” *International Dairy Journal*, Vol. 16(9), pp. 1018–1028, 2006.

43. Naruszewicz, M., Johansson, M., Zapolska-Downar, D. and Bukowska, H. “Effect of Lactobacillus plantarum 299v on cardiovascular disease risk factors in smokers.” *The American Journal of Clinical Nutrition*, Vol. 76, pp. 1249–1255, 2002.

44. Kishi, A., Uno, K., Matsubara, Y., And, C. O. and K., T. “Effect of the oral administration of Lactobacillus brevis subsp. coagulans on interferon-alpha producing capacity in humans.” *Journal of American College of Nutrition*, Vol. 15(6), pp. 408–412, 1996.

45. Rönkä, E., Malinen, E., Saarela, M., Rinta-Koski, M., Aarnikunnas, J., Palva, A. “Probiotic and milk technological properties of Lactobacillus brevis.” *International Journal of Food Microbiology*, Vol. 83(1), pp. 63–74, 2003.

46. Guglielmotti, D. M., Marcó, M. B., Golowczyc, M., Reinheimer, J. A. and Quiberoni, A. D. L. “Probiotic potential of Lactobacillus delbrueckii strains and their phage resistant mutants.” *International Dairy Journal*, Vol. 17(8), pp. 916–925, 2007.

47. Saide, J. A. O. and Gilliland, S. E. “Antioxidative activity of lactobacilli measured by oxygen radical absorbance capacity.” *Journal of Dairy Science*, Vol. 88(4), pp. 1352–1357, 2005.

48. Cenci, G., Rossi, J., Trotta, F. and Caldini, G. "Lactic acid bacteria isolated from dairy products inhibit genotoxic effect of 4-nitroquinoline-1-oxide in SOS-chromotest." *Systematic and Applied Microbiology*, Vol. 25(4), pp. 483–490, 2002.

49. Verdenelli, M. C., Ghelfi, F., Silvi, S., Orpianesi, C., Cecchini, C., Cresci, A. "Probiotic properties of Lactobacillus rhamnosus and Lactobacillus paracasei isolated from human faeces." *European Journal of Nutrition*, Vol. 48(6), pp. 355–363, 2009.

50. Holzapfel, W. H., Haberer, P., Geisen, R. and Schillinger, U. "Taxonomy and important features of probiotic microorganisms in food and nutrition." *The American Journal of Clinical Nutrition*, Vol. 73, pp. 365–373, 2001.

51. Vaughan, E. E., Heilig, H. G. H. J., Zoetendal, E. G., Satokari, R., Collins, J. K., Akkermans, A.D. L., de Vos, W. M "Molecular approaches to study probiotic bacteria." *Trends in Food Science & Technology*, Vol.10, pp. 400–404, .1999.

52. Mohania, D., Nagpal, R., Kumar, M., Bhardwaj, A., Yadav, M., Jain, S., Marotta, F., Singh, V., Parkash, O., Yadav, H. "Molecular approaches for identification and characterization of lactic acid bacteria." *Journal of Digestive Diseases*, Vol. 9(4), pp. 190–198, 2008.

53. Klein, G., Pack, A., Bonaparte, C. and Reuter, G. "Taxonomy and physiology of probiotic lactic acid bacteria." *International Journal of Food Microbiology*, Vol.41(2), pp. 103–125, 1998.

54. Sánchez, I., Seseña, S. and Palop, L. L. "Polyphasic study of the genetic diversity of lactobacilli associated with 'Almagro' eggplants spontaneous fermentation, based on combined numerical analysis of randomly amplified polymorphic DNA and pulsed-field gel electrophoresis patterns." *Journal of Applied Microbiology*, Vol. 97(2), pp. 446–458, 2004.

55. Claisse, O., Renouf, V. and Lonvaud-Funel, A. “Differentiation of wine lactic acid bacteria species based on RFLP analysis of a partial sequence of rpoB gene.” *Journal of microbiological methods*, Vol. 69(2), pp. 387–390, 2007.

56. Zhong, W., Millsap, K., Bialkowska-Hobrzanska, H., Zhong, W. E. I. and Reid, G. “Differentiation of Lactobacillus Species by Molecular Typing Differentiation of Lactobacillus Species by Molecular Typing.”, *Applied and Environmental Microbiology*, Vol. 64(7), pp. 2418-2423, 1998.

57. Roy, D. and Sirois, S. “Molecular differentiation of Bifidobacterium species with amplified ribosomal DNA restriction analysis and alignment of short regions of the ldh gene.”, *FEMS Microbiology Letters*, Vol. 191, pp. 17–24, 2000.

58. Rodas, A. M., Ferrer, S. and Pardo, I. “16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine.” *Systematic and Applied Microbiology*, Vol. 26(3), pp. 412–422, 2003.

59. Gevers, D., Huys, G. and Swings, J., “Applicability of rep-PCR fingerprinting for identification of Lactobacillus species.” , *FEMS Microbiology Letters*, Vol. 205, pp. 31–36, 2001.

60. Ogier, J., Son, O., Gruss, A., Tailliez, P. and Delacroix-Buchet, A. “Identification of the Bacterial Microflora in Dairy Products by Temporal Temperature Gradient Gel Electrophoresis.”, Applied and Environmental Microbiology, Vol. 68(8), pp. 3691–3701, 2002.

61. Kao, Y., Liu, Y. and Shyu, Y. “Identification of Lactobacillus spp. in probiotic products by real-time PCR and melting curve analysis.” *Food Research International*, Vol. 40(1), pp. 71–79, 2007.

62. Woese, C. R. “Bacterial Evolution.” *Microbiology Reviews*, Vol. 51(2), pp. 221–271, 1987.

63. Pál, K., Szén, O., Kiss, A. and Naár, Z., “Comparison and evaluation of molecular methods used for identification and discrimination of lactic acid bacteria.” *Journal of the Science of Food and Agriculture*, Vol. 92(9), pp. 1931–1936, 2012.

64. Tabasco, R., Paarup, T., Janer, C., Peláez, C. and Requena, T., “Selective enumeration and identification of mixed cultures of Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus, L. acidophilus, L. paracasei subsp. paracasei and Bifidobacterium lactis in fermented milk.”, *International Dairy Journal*, Vol. 17(9), pp. 1107–1114, 2007.

65. Vancanneyt, M., Naser, S. M., Engelbeen, K., De Wachter, M., Van der Meulen, R., Cleenwerck, I., Hoste, B., De Vuyst, L., Swings, J., “Reclassification of Lactobacillus brevis strains LMG 11494 and LMG 11984 as Lactobacillus parabrevis sp. nov.”, *International Journal of Systematic and Evolutionary Microbiology*, Vol. 56, pp. 1553–1557, 2006.

66. Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K., Alatossava, T., “Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers.” *Applied and Environmental Microbiology*, Vol. 66(1), pp. 297–303, 2000.

67. Aymerich, T., Martín, B., Garriga, M. and Hugas, M., “Microbial Quality and Direct PCR Identification of Lactic Acid Bacteria and Nonpathogenic Staphylococci from Artisanal Low-Acid Sausages”, *Applied and Environmental Microbiology*, Vol. 69(8), 2003.

68. Matsuki, T., Watanabe, K. and Tanaka, R., “Genus- and Species-Specific PCR Primers for the Detection and Identification of Bifidobacteria.”, *Current. Issues in Intestestinal Microbiology*, Vol.4, pp. 61–69, 2003.

69. Liu, R. H., “Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals.”, *The American Journal of Clinical Nutrition*, Vol.78, p. 517S–520S, 2003.

70. Scalbert, A., Manach, C., Morand, C., Rémésy, C. and Jiménez, L., “Dietary polyphenols and the prevention of diseases.” *Critical Reviews in Food Science and Nutrition*, Vol. 45(4), pp. 287–306, 2005.

71. Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jiménez, L., “Polyphenols: food sources and bioavailability.”, *The American Journal of Clinical Nutrition*, Vol. 79(5), pp. 727–747, 2004.

72. Bravo, L., “Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance.”, *Nutrition reviews*, Vol. 56(11), pp. 317–333, 1998.

73. Crozier, A., Jaganath, I. B. and Clifford, M. N., “Dietary phenolics: chemistry, bioavailability and effects on health.”, *Natural Product Reports*, Vol. 26(8), pp. 1001–1043, 2009.

74. Lila, M. A., “Anthocyanins and Human Health: An In Vitro Investigative Approach.”, *Journal of Biomedicine & Biotechnology*, Vol. 2004(5), pp. 306–313, 2004.

75. Giusti, M. M. and Wrolstad, R. E., “Acylated anthocyanins from edible sources and their applications in food systems.”, *Biochemical Engineering Journal*, Vol. 14(3), pp. 217–225, 2003.

76. Wang, H., Cao, G. and Prior, R. L., “Oxygen Radical Absorbing Capacity of Anthocyanins.”, *Journal of Agricultural and Food Chemistry*, Vol. 45, pp. 304–309, 1997.

77. Francis, F. J. and Markakis, P. C., “Food colorants : Anthocyanins.”, *Critical Reviews in Food Science and Nutrition*, Vol. 28(4), pp. 273–314, 1989.

78. Wang, L. and Stoner, G. D., “Anthocyanins and their role in cancer prevention.”, *Cancer Letters*, Vol. 269(2), pp. 281–290, 2008.

79. Kammerer, D., Carle, R. and Schieber, A., “Quantification of anthocyanins in black carrot extracts (Daucus carota ssp. sativus var. atrorubens Alef.) and evaluation of their color properties.”, *European Food Research and Technology*, Vol. 219(5), pp. 479–486, 2004.

80. Stintzing, F. C., Stintzing, A. S., Carle, R., Frei, B. and Wrolstad, R. E., “Color and antioxidant properties of cyanidin-based anthocyanin pigments.”, *Journal of Agricultural and Food Chemistry*, Vol. 50(21), pp. 6172–6181, 2002.

81. Stintzing, F. C. and Carle, R., “Functional properties of anthocyanins and betalains in plants, food, and in human nutrition.”, *Trends in Food Science & Technology*, Vol. 15(1), pp. 19–38, 2004.

82. Kang, S., Seeram, N. P., Nair, M. G. and Bourquin, L. D., “Tart cherry anthocyanins inhibit tumor development in ApcMin mice and reduce proliferation of human colon cancer cells.”, *Cancer Letters*, Vol. 194(1), pp. 13–19, 2003.

83. Yan, X., Murphy, B. T., Hammond, G. B., Vinson, J. A. and Neto, C. C., “Antioxidant activities and antitumor screening of extracts from cranberry fruit (Vaccinium macrocarpon).”, *Journal of Agricultural and Food chemistry*, Vol. 50(21), pp. 5844–5849, 2002.

84. Seeram, N. P., Adams, L. S., Zhang, Y., Lee, R., Sand, D., Scheuller, H. S., Heber, D., “Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro.”, *Journal of Agricultural and Food Chemistry*, Vol. 54(25), pp. 9329–9339, 2006.

85. Wang, J. and Mazza, G., “Effects of anthocyanins and other phenolic compounds on the production of tumor necrosis factor alpha in LPS/IFN-gamma-activated RAW

264.7 macrophages.”, *Journal of Agricultural and Food Chemistry*, Vol. 50(15), pp. 4183–4189, 2002.

86. Bodet, C., Chandad, F. and Grenier, D., “Anti-inflammatory activity of a high-molecular-weight cranberry fraction on macrophages stimulated by lipopolysaccharides from periodontopathogens.”, *Journal of Dental Research*, Vol. 85(3), pp. 235–239, 2006.

87. Reed, J., “Cardiovascular Health Cranberry Flavonoids, Atherosclerosis and Cardiovascular Health.”, *Critical Reviews in Food Science and Nutrition*, Vol. 42(3), pp. 301–316, 2002.

88. Hagiwara, A., Yoshino, H., Ichihara, T., Kawabe, M. and Tamano, S., “Prevention by natural food anthocyanins , purple sweet potato color and red cabbage color, of 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyrıdıne (PhIP)-associated colorectal carcinogeness.”, *The Journal of Toxicological Sciences*, Vol. 27(1), pp. 57–68, 2002.

89. Dröge, W., “Free radicals in the physiological control of cell function.”, *Physiological Reviews*, Vol. 82(1), pp. 47–95, 2002.

90. Espín, J. C., Soler-Rivas, C., Wichers, H. J. and García-Viguera, C., “Anthocyanin-based natural colorants: a new source of antiradical activity for foodstuff.”, *Journal of Agricultural and Food Chemistry*, Vol. 48(5), pp. 1588–1592, 2000.

91. Prior, R. L., Wu, X. and Schaich, K., “Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements.”, *Journal of Agricultural and Food Chemistry*, Vol. 53(10), pp. 4290–4302, 2005.

92. Huang, D., Ou, B. and Prior, R. L., “The chemistry behind antioxidant capacity assays.”, *Journal of Agricultural and Food Chemistry*, Vol. 53(6), pp. 1841–1856, 2005.

93. Cao, G., Alessio, H. M. and Cutler, R. G., "Oxygen-radical absorbance capacity assay for antioxidants.", *Free Radical Biology and Medicine*, Vol. 14(3), pp. 303–311, 1993.

94. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. "Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorizarion Assay.", *Free Radical Biology & Medicine*, Vol. 26(9/10), pp. 1231–1237, 1999.

95. Brand-Williams, W., Cuvelier, M. E. and Berset, C., "Use of a free radical method to evaluate antioxidant activity.", *LWT - Food Science and Technology*, Vol. 28(1), pp. 25–30, 1995.

96. Benzie, I. F. and Strain, J. J., "The ferric reducing ability of plasma (FRAP) as a measure of 'antioxidant power': the FRAP assay.", *Analytical Biochemistry*, Vol. 239(1), pp. 70–76, 1996.

97. Gardner, P. T., White, T. A. C., McPhail, D. B. and Duthie, G. G., "The relative contributions of vitamin C , carotenoids and phenolics to the antioxidant potential of fruit juices.", Food Chemistry, Vol. 68, pp. 471–474, 2000.

98. Halvorsen, B. L., Holte, K., Myhrstad, M. C. W., Barikmo, I., Hvattum, E., Remberg, S. F., Wold, A., Haffner, K., Baugerød, H., Andersen, L. F., Moskaug, J., Ø., Jacobs, D.R., Blomhoff, R., "A Systematic Screening of Total Antioxidants in Dietary Plants.", *Journal of Nutrition*, Vol. 132(3), pp. 461–471, 2002.

99. Seeram, N. P., Aviram, M., Zhang, Y., Henning, S. M., Feng, L., Dreher, M., Heber, D., "Comparison of antioxidant potency of commonly consumed polyphenol-rich beverages in the United States.", *Journal of Agricultural and Food Chemistry*, Vol. 56(4), pp. 1415–1422, 2008.

100. Herken, E. N. and Guzel, S., "Total Antioxidant Capacity and Total Phenol Contents of Selected Commercial Fruit Juices in Turkey.", *International Journal of Food Properties*, Vol. 13(6), pp. 1373–1379, 2010.

101. Fukumoto, L. R. and Mazza, G., “Assessing antioxidant and prooxidant activities of phenolic compounds.”, *Journal of Agricultural and Food Chemistry*, Vol. 48(8), pp. 3597–3604, 2000.

102. Singleton, V. L. and Rossi Jr., J. A., “Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents.”, *American Journal of Enology and Viticulture*, Vol. 16, pp. 144–158, 1965.

103. Canbaş, A., “Şalgam Suyu Üzerine bir Araştırma.”, *Gıda*, Vol. 9(5), pp. 279–286, 1984.

104. Anonymous, “Turkish Standard- Şalgam suyu/ Turnip juice.”, Türk Standartları Enstitüsü, 2003.

105. Bradeen, J. M. and Simon, P. W. "Carrot" in Kole, C. (ed.) *Genome Mapping and Molecular Breeding in Plants - Vegetables*, Springer Berlin Heidelberg, Berlin, 2007.

106. Tanguler, H., *Şalgam Suyu Üretiminde Etkili Olan Laktik Asit Bakterilerinin Belirlenmesi ve Şalgam Suyu Üretim Tekniğinin Geliştirilmesi, Ph.D. Thesis, 2010.*

107. Alasalvar, C., Grigor, J. M., Zhang, D., Quantick, P. C. and Shahidi, F., “Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties.”, *Journal of Agricultural and Food Chemistry*, Vol. 49(3), pp. 1410–1416, 2001.

108. Kammerer, D., Carle, R. and Schieber, A., “Detection of peonidin and pelargonidin glycosides in black carrots (Daucus carota ssp. sativus var. atrorubens Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry.”, *Rapid Communications in Mass Spectrometry, Vol.* 17(21), pp. 2407–2412, 2003.

109. Montilla, E. C., Arzaba, M. R., Hillebrand, S. and Winterhalter, P., “Anthocyanin composition of black carrot (Daucus carota ssp. sativus var. atrorubens Alef.)

cultivars Antonina, Beta Sweet, Deep Purple, and Purple Haze.", *Journal of Agricultural and Food Chemistry*, Vol. 59(7), pp. 3385–3390, 2011.

110. Malien-Aubert, C., Dangles, O. and Amiot, M. J., "Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation.", *Journal of Agricultural and Food Chemistry*, Vol. 49(1), pp. 170–176, 2001.

111. Geleijnse, J. M., Launer, L. J., Van der Kuip, D. A. M., Hofman, A. and Witteman, J. C. M., "Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study.", *The American Journal of Clinical Nutrition*, Vol. 75(5), pp. 880–886, 2002.

112. Kırca, A., Özkan, M. and Cemeroğlu, B., "Effects of temperature, solid content and pH on the stability of black carrot anthocyanins.", *Food Chemistry*, Vol. 101(1), pp. 212–218, 2007.

113. Kaur, C. and Kapoor, H. C., "Anti-oxidant activity and total phenolic content of some Asian vegetables.", *International Journal of Food Science and Technology*, Vol. 37(2), pp. 153–161, 2002.

114. Uyan, S. E., Baysal, T.., Yurdagel, U. and El, S. N., "Effects of drying process on antioxidant activity of purple carrots.", *Die Nahrung/Food*, Vol. 48(1), pp. 57–60, 2004.

115. Khandare, V., Walia, S., Singh, M. and Kaur, C., "Black carrot (Daucus carota ssp. sativus) juice: Processing effects on antioxidant composition and color.", *Food and Bioproducts Processing*, Vol. 89(4), pp. 482–486, 2011.

116. Sun, T., Simon, P. W. and Tanumihardjo, S. A., "Antioxidant phytochemicals and antioxidant capacity of biofortified carrots (Daucus carota L.) of various colors.", *Journal of Agricultural and Food Chemistry*, Vol. 57(10), pp. 4142–4147, 2009.

117. Netzel, M., Netzel, G., Kammerer, D. R., Schieber, A., Carle, R., Simons, L., Bitsch, I., Bitsch, R., Konczak, I. “Cancer cell antiproliferation activity and metabolism of black carrot anthocyanins.”, *Innovative Food Science & Emerging Technologies*, Vol. 8(3), pp. 365–372, 2007.

118. Charron, C. S., Kurilich, A. C., Clevidence, B. A., Simon, P. W., Harrison, D. J., Britz, S. J., Baer, D.J., Novotny, J.A. “Bioavailability of anthocyanins from purple carrot juice: effects of acylation and plant matrix.”, *Journal of Agricultural and Food Chemistry*, Vol. 57(4), pp. 1226–1230, 2009.

119. Doğanay “Personal Communication.”, 2012

120. Rodriguez-Sevilla, M. D., Villanueva-Suarez, M. J. and Redondo-Cuenca, A. “Effects of processing conditions on soluble sugars content of carrot , beetroot and turnip.” *Food Chemistry*, Vol. 66, pp. 81–85, 1999.

121. Nout, M. J. R. and Rombouts, F. M., “Fermentative preservation of plant foods.”, *Journal of Applied Microbiology*, Vol. 73, p. 136s–147s, 1992.

122. Öztürk, O., *Adana Piyasasindaki Şalgam Sularinin Bileşimleri Üzerine Bir Araştirma*, M.S. Report, 2009.

123. Anonymous, “Renklendiriciler ve Tatlandırıcılar Dışındaki Gıda Katkı Maddeleri Tebliği.” *Türk Gıda Kodeksi Yönetmeliği, Tarım ve Köyişleri Bakanlığı*, Tebliğ No:(Resmi Gazete 22/05/2008-26883), 2008.

124. FDA “Code of Federal Regulations, Title 21, Volume 3 [21CFR184.1733], 2012.”

125. Turker, N., Aksay, S. and Ekiz, H. I., “Effect of storage temperature on the stability of anthocyanins of a fermented black carrot (Daucus carota var. L.) beverage: shalgam.”, *Journal of agricultural and food chemistry*, Vol. 52(12), pp. 3807–3813, 2004.

126. Özler, N., *Şalgam Suyu Üretimi Üzerinde Çalışmalar*, M.S. Report, 1995.

127. Deryaoğlu, A. *Salgam Suyu Üretimi ve Bilesimi Uzerinde Bir Arastirma*, M.S. Report, 1990.

128. Arici, M., "Mikrobiologische und chemische Eigenschaften von Salgam.", *Ernährungs*, Vol. 51, pp. 10–11, 2004.

129. Çakır, P., *Ülkemizde Üretilen Şalgam Sularının Bileşimleri ve Gıda Mevzuatına Uygunlukları Üzerine Bir Araştırma.*, M.S. Report, 2011.

130. Tanguler, H. and Erten, H., “Chemical and Microbiological Characteristics of Shalgam (Şalgam): A Traditional Turkish Lactic Acid Fermented Beverage.”, *Journal of Food Quality*, Vol. 35, 298-306, 2012.

131. Güneş, G., *Şalgam suyu üretiminde en uygun siyah havuç (Daucus carota) miktarının belirlenmesi üzerine bir araştırma*., M.S. Report, 2008.

132. Utuş, D. *Şalgam Suyu Üretiminde Kullanilan Siyah Havuç (Daucus Carota) Boyutunun Şalgam Suyu Kalitesi Üzerine Etkisi.*, M.S. Report, 2008.

133. Özler, N. and Kılıç, O., “Şalgam Suyu Üretimi Üzerinde Araştırmalar.”, *Gıda*, Vol. 21(5), pp. 323–330, 1996.

134. Turker, N., Aksay, S., Istanbullu, O. and Artuvan, E., “A Study on the Relation Between Anthocyanin Content and Product Quality : Shalgam as a Model Beverage.”, *Journal of Food Quality*, Vol. 30, pp. 953–969, 2007.

135. Erginkaya, Z. and Hammes, W. P., “Şalgam Suyu Fermentasyonu Sırasında Mikroorganizmaların Gelişimi ve İzole Edilen Laktik Asit Bakterilerinin Tanımlanmaları Üzerine Bir Araştırma.”, *Gıda*, Vol. 17(5), pp. 311–314, 1992.

136. Öztan, T. *Mor Havuç, Konsantresi, Şalgam Suyu, Nar Suyu ve Nar Ekşisi Ürünlerinde Antioksidan Aktivitesi Tayini ve Fenolik Madde Profilinin Belirlenmesi.*, M.S. Report, 2006.

137. A.O.A.C *Official Methods of Analysis of the Association of Official Analytical Chemists* 16th ed. Hekrich, K. (ed.), 1998.

138. DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., "Colorimetric Method for Determination of Sugars and Related Substances.", *Analytical Chemistry*, Vol. 28(3), pp. 350–356, 1956.

139. Andersson, R. and Hedlund, B., "HPLC analysis of organic acids in lactic acid fermented vegetables.", *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, Vol. 176(6), pp. 440–443, 1983.

140. McFeeters, R. F., Thompson, R. L. and Fleming, H. P., "Liquid Chromatographic Analysis of Sugars, Acids and Ethanol in Lactic Acid Vegetable Fermentations.", *Journal of the Association of Official Analytical Chemists*, Vol. 67(4), pp. 710–714, 1984.

141. Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V. and Milner, A., "A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates.", *Clinical Science*, Vol. 84(4), pp. 407–412, 1993.

142. Ainsworth, E. A. and Gillespie, K. M., "Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent.", *Nature Protocols*, Vol. 2(4), pp. 875–877, 2007.

143. Furet, J., Quénée, P. and Tailliez, P., "Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR.", *International Journal of Food Microbiology*, Vol. 97(2), pp. 197–207, 2004.

144. Redzepović, S., Orlić, S., Sikora, S., Majdak, A. and Pretorius, I. S., "Identification and characterization of Saccharomyces cerevisiae and Saccharomyces paradoxus strains isolated from Croatian vineyards.", *Letters in Applied Microbiology*, Vol. 35(4), pp. 305–310, 2002.

145. Guarneri, T., Rossetti, L. and Giraffa, G., "Rapid identification of Lactobacillus brevis using the polymerase chain reaction.", *Letters in Applied Microbiology*, 33, pp. 377–381, 2001.

146. Anonymous , "Basic Cell Culture." [online] , http://www.uvm.edu/~wschaeff/BasicCulture1.html [retrieved 16 October 2012].

147. Maki, M. "Lactic Acid Bacteria in Vegetable Fermentations," in Salminen, S., von Wright, A., and Ouwehand, A. (eds.), *Lactic Acid Bacteria Microbiological and Functional Aspects,* 2004.

148. Shui, G. and Leong, L. P., "Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography.", *Journal of Chromatography A*, Vol. 977, pp. 89–96, 2002.

149. Yalim, S. and Ozdemir, Y., "Effects of preparation procedures on ascorbic acid retention in pickled hot peppers.", *International Journal of Food Sciences and Nutrition*, Vol. 54(4), pp. 291–296, 2003.

150. Gardner, L. K. and Lawrence, G. D., "Benzene Production from Decarboxylation of Benzoic Acid in the Presence of Ascorbic Acid and a Transition-Metal Catalyst.", *Journal of agricultural and food chemistry*, Vol. 41(5), pp. 693–695, 1993.

151. Rodríguez, H., Curiel, J. A., Landete, J. M., de las Rivas, B., de Felipe, L. F., Gómez-cordovés, C., Mancheno, M. J., Muñoz, R., "Food phenolics and lactic acid bacteria.", *International Journal of Food Microbiology*, Vol. 132(2-3), pp. 79–90, 2009.

152. Barthelmebs, L., Divies, C. and Cavin, J., “Knockout of the p -Coumarate Decarboxylase Gene from Lactobacillus plantarum Reveals the Existence of Two Other Inducible Enzymatic Activities Involved in Phenolic Acid Metabolism”, *Applied and Environmental Microbiology*, Vol. 66(8), pp. 3368–3375, 2000.

153. Vivas, N., Lonvaud-Funel, A. and Glories, Y., “Effect of phenolic acids and anthocyanins on growth , viability and malolactic activity of a lactic acid bacterium.”, *Food Microbiology*, Vol. 14, pp. 291–300, 1997.

154. Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M. and Kader, A. A., “Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing.”, *Journal of Agricultural and Food Chemistry*, Vol. 48(10), pp. 4581–4589, 2000.

155. Bermudez-Soto, M. and Tomas-Barberan, F., “Evaluation of commercial red fruit juice concentrates as ingredients for antioxidant functional juices.”, *European Food Research and Technology*, Vol. 219(2), pp. 133–141, 2004.

156. Wang, Y., Chen, C., Ai, L., Zhou, F., Wang, L., Zhang, H., Chen, W., Guo, B., “Complete genome sequence of the probiotic Lactobacillus plantarum ST-III.”, *Journal of Bacteriology*, Vol. 193(1), pp. 313–314, 2011.

157. Zhang, Z., Liu, C., Zhu, Y., Zhong, Y., Zhu, Y., Zheng, H., Zhao, G., Wang, S., Guo, X., “Complete genome sequence of Lactobacillus plantarum JDM1.”, *Journal of Bacteriology*, Vol. 191(15), pp. 5020–5021, 2009.

158. Bringel, F., Castioni, A., Olukoya, D. K., Felis, G. E., Torriani, S., Dellaglio, F., “Lactobacillus plantarum subsp. argentoratensis subsp. nov., isolated from vegetable matrices.”, *International Journal of Systematic and Evolutionary Microbiology*, Vol. 55, pp. 1629–1634, 2005.

159. Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou,

Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D. M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J-H., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D. “Comparative genomics of the lactic acid bacteria.”, *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 103(42), pp. 15611–15616, 2006.

160. Mori, K., Yamazaki, K., Ishiyama, T., Katsumata, M., Kobayashi, K., Kawai, Y., Inoue, N., Shinano, H. “Comparative sequence analyses of the genes coding for 16S rRNA of Lactobacillus casei-related taxa.”, *International Journal of Systematic Bacteriology*, Vol. 47(1), pp. 54–57, 1997.

161. Acedo-Felix, E., Perez-Martinez, G., “Significant differences between Lactobacillus casei subsp. casei ATCC 393T and a commonly used plasmid-cured derivative revealed by a polyphasic study.”, *International Journal of Systematic and Evolutionary Microbiology*, Vol. 53(1), pp. 67–75, 2003.

162. Collins, M. D., Phillips, B. A. and Zanoni, P., “Deoxyribonucleic Acid Homology Studies of Lactobacillus casei, Lactobacillus paracasei sp. nov., subsp. paracasei and subsp. tolerans, and Lactobacillus rhamnosus sp. nov., comb. nov.”, *International Journal of Systematic Bacteriology*, Vol. 39(2), pp. 105–108, 1989.

163. Germond, J.-E., Lapierre, L., Delley, M., Mollet, B., Felis, G. E., Dellaglio, F., “Evolution of the Bacterial Species Lactobacillus delbrueckii: A Partial Genomic Study with Reflections on Prokaryotic Species Concept.”, *Molecular Biology and Evolution*, Vol. 20(1), pp. 93–104, 2003.

164. Falsen, E., Pascual, C., Sjbden, B., Ohlnl, M. and Collins, M. D., “Phenotypic and phylogenetic characterization of a novel Lactobacillus species from human sources :

description of Lactobacillus iners sp. nov.”, *International Journal of Systematic Bacteriology*, Vol. 49, pp. 217-221, 1999.

165. Howey, R. T., Lock, C. M. and Moore, L. V. H., “Subspecies Names Automatically Created by Rule 46.”, *International Journal of Systematic Bacteriology*, Vol. 40(3), pp. 317–319, 1990.

166. Dellaglio, F., Felis, G. E., Castioni, A., Torriani, S. and Germond, J., “Lactobacillus delbrueckii subsp. indicus subsp. nov., isolated from Indian dairy products.”, *International Journal of Systematic and Evolutionary Microbiology*, Vol. 55, pp. 401–404, 2005.

167. Azcarate-Peril, M. A., Altermann, E., Goh, Y. J., Tallon, R., Sanozky-Dawes, R. B., Pfeiler, E. A., O'Flaherty, S., Buck, B. L., Dobson, A., Duong, T., Miller, M. J., Barrangou, R., Klaenhammer, T. R. “Analysis of the genome sequence of Lactobacillus gasseri ATCC 33323 reveals the molecular basis of an autochthonous intestinal organism.” *Applied and Environmental Microbiology*, Vol. 74(15), pp. 4610–4625, 2008.

168. Callanan, M., Kaleta, P., O’Callaghan, J., O’Sullivan, O., Jordan, K., McAuliffe, O., Sangrador-Vegas, A., Slattery, L., Fitzgerald, G. F., Beresford, T., Ross, R. P., “Genome sequence of Lactobacillus helveticus, an organism distinguished by selective gene loss and insertion sequence element expansion.”, *Journal of Bacteriology*, Vol. 190(2), pp. 727–735, 2008.

169. Gobbetti, M., De Angelis, M., Corsetti, A. and Di Cagno, R., “Biochemistry and physiology of sourdough lactic acid bacteria.”, *Trends in Food Science & Technology*, Vol. 16(1-3), pp. 57–69, 2005.

170. Huang, C. and Lee, F., “Development of novel species-specific primers for species identification of the Lactobacillus casei group based on RAPD fingerprints.”, *Journal of the Science of Food and Agriculture*, Vol. 89(11), pp. 1831–1837, 2009.

171. Stiles, M. E. and Holzapfel, W. H., “Lactic acid bacteria of foods and their current taxonomy.”, *International Journal of Food Microbiology*, Vol. 36, pp. 1–29, 1997.

172. De Vuyst, L. and Neysens, P., “The sourdough microflora: biodiversity and metabolic interactions.”, *Trends in Food Science & Technology*, Vol. 16(1-3), pp. 43–56, 2005.

173. Matar, C., Nadathur, S. S., Bakalinsky, A. T. and Goulet, J., “Antimutagenic effects of milk fermented by Lactobacillus helveticus L89 and a protease-deficient derivative.”, *Journal of Dairy Science*, Vol. 80(9), pp. 1965–1970, 1997.

174. LeBlanc, J. G., Matar, C., Valdéz, J. C., LeBlanc, J. and Perdigon, G., “Immunomodulating effects of peptidic fractions issued from milk fermented with Lactobacillus helveticus.”, *Journal of Dairy Science*, Vol. 85(11), pp. 2733–2742, 2002.

175. Gänzle, Michael. G., Höltzel, A., Walter, J., Hammes, W. P. and Ho, A., “Characterization of Reutericyclin Produced by Lactobacillus reuteri LTH2584”, *Applied and Environmental Microbiology*, Vol. 66(10), pp. 4325–4333, 2000.

176. Holley, R. A., Lamoureux, M. and Dussault, F. “Identification of lactic spoilage bacteria from vacuum-packed cooked luncheon meat and induction of repairable injury by mild thermel stress.”, *Lebensmittel - Wissenschaft + Technologie*, Vol. 29(1-2), pp. 114–122, 2000.

177. Ehrmann, M. A. and Vogel, R., “Molecular taxonomy and genetics of sourdough lactic acid bacteria.”, *Trends in Food Science & Technology*, Vol. 16(1-3), pp. 31–42, 2005.

178. Bermúdez-Soto, M. J., Larrosa, M., Garcia-Cantalejo, J. M., Espín, J. C., Tomás-Barberan, F. A., García-Conesa, M. T., “Up-regulation of tumor suppressor carcinoembryonic antigen-related cell adhesion molecule 1 in human colon cancer Caco-2 cells following repetitive exposure to dietary levels of a polyphenol-rich

chokeberry juice.", *The Journal of Nutritional Biochemistry*, Vol. 18(4), pp. 259–271, 2007.

179. Tarozzi, A., Marchesi, A., Cantelli-Forti, G. and Hrelia, P., "Cold-storage affects antioxidant properties of apples in Caco-2 cells.", *The Journal of Nutrition*, Vol. 134(5), pp. 1105–1109, 2004.

180. McDougall, G. J., Ross, H. A., Ikeji, M. and Stewart, D., "Berry extracts exert different antiproliferative effects against cervical and colon cancer cells grown in vitro.", *Journal of Agricultural and Food Chemistry*, Vol. 56(9), pp. 3016–3023, 2008.

181. Cilla, A., González-Sarrías, A., Tomás-Barberán, F. A., Espín, J. C. and Barberá, R., "Availability of polyphenols in fruit beverages subjected to in vitro gastrointestinal digestion and their effects on proliferation, cell-cycle and apoptosis in human colon cancer Caco-2 cells.", *Food Chemistry*, Vol. 114(3), pp. 813–820, 2009.

182. Sakurazawa, T. and Ohkusa, T., "Cytotoxicity of organic acids produced by anaerobic intestinal bacteria on cultured epithelial cells.", *Journal of Gastroenterology*, Vol. 40(6), pp. 600–609, 2005.

183. Ozcan, E., Aydin, K., Baser, G. M., Guclu-Ustundag, O., Korachi, M., Ekinci, F. Y., "Evaluation of shalgam juice antiproliferative activity against a colon cancer cell line.", *New Biotechnology*, 29S(September), pp.115, 2012.

184. Nair, B., "Final report on the safety assessment of Benzyl Alcohol, Benzoic Acid, and Sodium Benzoate.", *International Journal of Toxicology*, Vol. 20(3), pp. 23–50, 2001.

185. Mpountoukas, P., Vantarakis, A., Sivridis, E. and Lialiaris, T., "Cytogenetic study in cultured human lymphocytes treated with three commonly used preservatives." *Food and Chemical Toxicology*, Vol. 46(7), pp. 2390–2393, 2008.

186. Grimoud, J., Durand, H., De Souza, S., Monsan, P., Ouarné, F., Theodorou, V., Roques, C., “In vitro screening of probiotics and synbiotics according to anti-inflammatory and anti-proliferative effects.”, *International Journal of Food Microbiology*, Vol. 144(1), pp. 42–50, 2010.

187. Frontela-Saseta, C., López-Nicolás, R., González-Bermúdez, C. A., Peso-Echarri, P., Ros-Berruezo, G., Martínez-Graciá, C., Canali, R., Virgili, F. “Evaluation of antioxidant activity and antiproliferative effect of fruit juices enriched with Pycnogenol® in colon carcinoma cells. The effect of in vitro gastrointestinal digestion.”, *Phytotherapy Research*, Vol. 25(12), pp. 1870–1875, 2011.