RUSSIAN OLIVE AS A FUNCTIONAL FOOD INGREDIENT–POST HARVEST PROCESSING DEVELOPMENT AND OPTIMIZATION

BY

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ABSTRACT

Elaeagnus angustifolia (Russian olive), a member of the Elaeagnaceae family, is a plant that is broadly cultivated for horticultural and environmental purposes. The fruits, leaves and flowers of this tree are rich in various bioactive compounds, and different studies have proved their significant antioxidant activity and various therapeutic properties such as cardioprotective, gastroprotective, anti-inflammatory, antitumour, anti-arthritic, wound healing and hypolipidemic activity. Therefore, Russian olive, with its antioxidant and therapeutic properties can have a good potential for application in the food industry to respond to increasing consumers' demand for functional food products with health benefits. However, despite its beneficial properties, the application of this plant as a valuable food ingredient in the food industry has not been studied extensively by researchers. Therefore, the overall aim of this study was to develop Russian olive-based products and optimize the related post-harvest processes for valorizing this plant in the functional food sector. The research results are presented in Chapters IV to VII.

Chapter IV of this thesis focused on using microwave-assisted extraction for producing antioxidant-rich extracts from Russian olive leaves and flowers, which can be used as food ingredients with effective and safe antioxidant properties. In addition, the most abundant phenolic compounds in the extracts were detected. Response surface methodology (RSM) was used to determine the effects of microwave-assisted extraction parameters on the quality of produced extracts, and the extraction process was optimized for maximum recovery of antioxidant compounds such as phenolics and flavonoids in the extract. The optimal process parameters were found to be using 2 M citric acid, solid to solvent ratio of 7.5 (w/v), ethanol concentration of 66.4 and 59.8%, and temperatures of 97.5 and 97.4 °C for MAE of Russian olive flowers and leaves, respectively. Finally, using HPLC analysis, the extracts from MAE with that of ultrasound-assisted

extraction (UAE) and conventional extraction (CE) were compared. The results showed better efficiency of MAE compared to UAE for extraction of rutin and luteolin; however, isorhamnetin was only detected in the conventionally extracted samples.

Also, through the present research study, the utilization of Russian olive fruit, as the substrate in the formulation of water kefir beverage was proposed. Production of Russian olive water kefir necessitates optimization of different processing stages. Therefore, microbiological, physicochemical, and technological aspects of Russian olive water kefir production with regards to fermentation (Chapter V), drying (Chapter VI) and storage (Chapter VII) were investigated.

Chapter V focused on developing a new functional food with enhanced bioactive properties using Russian olive fruit, named Russian olive water kefir (RWK). The water kefir fermentation process was designed using a central composite design (CCD). Subsequently, response surface methodology (RSM) was used to evaluate the effect of the fermentation process parameters including Russian olive concentration, fermentation time and temperature on bioactive properties of the developed RWK product. The optimized fermentation conditions for the maximized number of water kefir microorganisms, total phenolic contents (TPC) and antioxidant properties in RWK were determined to be 24 hours of fermentation at the temperature of 31.2ºC and using 30 % Russian olive juice concentration. Under these selected fermentation conditions, the number of viable water kefir microorganisms was 7.20, 7.06, and 7.17 $\log_{10}$ CFU/mL for AAB, LAB, and yeasts, respectively, and the values for TPC, DPPH and FRAP antioxidant activity in RWK were 98.32 (µg GAE/mL), 0.096 (µmol Trolox Eq/mL) and 0.22 (µmol FSHE/mL).

In Chapter VI, processing RWK into a powder was performed to improve storage stability and to reduce transportation and storage associated costs of this product for its commercialization. The bioactive compounds and water kefir microorganisms in RWK were encapsulated with carrier

materials and using spray drying as an encapsulation method. The effects of spray drying conditions on the quality of the produced powder were assessed. The optimized spray drying process conditions for the minimum reduction of water kefir microorganism and the maximum retention of antioxidant properties in the produced RWK powder were observed at an inlet air temperature of 120ºC, 35 % feed flow rate, and using 7% drying aid concentration. At the end, the microbial and physicochemical analyses of the spray-dried RWK powder were performed. The results showed spray drying as a promising encapsulation method for preserving the quality of RWK, showing good viability of water kefir microorganisms and level of antioxidant activity, which is closely related but lower than those measured in the freeze-dried samples.

Chapter VII evaluated the storage stability of Russian olive water kefir (RWK) powder, to determine the maximum shelf life of this new functional product. The effects of the storage conditions on the quality of the spray-dried RWK powder were evaluated at different time intervals and the results were compared with freeze-dried RWK samples. In addition, the *in vitro* gastrointestinal resistance of the Russian olive water kefir microorganisms present in the encapsulated RWK samples was evaluated. Overall, During 90 days of storage, degradation of bioactive compounds in RWK powder showed first-order kinetics. Temperature showed no significant effect on the stability of these compounds, whereas cold storage was shown to significantly improve the survival of the water kefir microorganisms in encapsulated RWK samples. In cold storage, spray-dried RWK samples had prolonged storage stability, which was closely related to the freeze-dried samples within the first two months of storage, but lower over the third month. Overall, the results showed good retention of the bioactive properties of spray-dried samples for a minimum of 90 days under cold storage. Also, encapsulated Russian olive water kefir microorganisms of spray-dried RWK showed satisfactory level of survival in transit

through simulated gastrointestinal conditions. Overall, throughout the chapters of this study, the extraction of bioactive compounds from Russian olive leaves and flowers, and development of a functional food based on Russian olive fruit was studied. The results proved the potential of Russian olive fruits, leaves and flowers for being used as functional ingredients in the food industry. The developed and optimized processes in this study (extraction, fermentation, encapsulation) are expected to facilitate the future development of Russian olive-based functional products.

RÉSUMÉ

Elaeagnus angustifolia (olivier de Russie), membre de la famille des Elaeagnacées, est une plante largement cultivée à des fins horticoles et environnementales. Les fruits, les feuilles et les fleurs de cet arbre sont riches en divers composés bioactifs, et différentes études ont prouvé leur importante activité antioxydante et diverses propriétés thérapeutiques telles que cardioprotectrice, gastroprotectrice, anti-inflammatoire, antitumorale, anti- arthritique, cicatrisante et hypolipémiante. Par conséquent, l'olivier de Russie, avec ses propriétés antioxydantes et thérapeutiques, peut avoir un bon potentiel d'application dans l'industrie des aliments fonctionnels pour répondre à la demande croissante des consommateurs pour des produits alimentaires fonctionnels bénéfiques pour la santé. Cependant, malgré ses propriétés bénéfiques, l'application de cette plante en tant qu'ingrédient alimentaire dans l'industrie alimentaire n'a pas été étudiée de manière approfondie par les chercheurs. Par conséquent, l'objectif global de cette étude était de développer des ingrédients à base de l'olivier de Russie et d'optimiser les processus post-récolte associés pour valoriser cette plante dans le secteur des aliments fonctionnels. Les résultats de la recherche sont présentés dans les chapitres IV à VII.

Le chapitre IV de cette thèse s'est concentrée sur l'utilisation de l'extraction assistée par micro-ondes pour produire des extraits riches en antioxydants à partir de feuilles et de fleurs de l'olivier de Russie, qui peuvent être utilisés comme ingrédients alimentaires avec des propriétés antioxydantes efficaces et sûres, et les composés phénoliques les plus abondants dans les extraits ont été détectés. La méthode des surfaces de réponse (RSM) a été utilisée pour déterminer les effets des paramètres d'extraction assistée par micro-ondes sur la qualité des extraits produits, et le processus d'extraction a été optimisé pour une récupération maximale des composés antioxydants tels que les composés phénoliques et les flavonoïdes dans l'extrait. Les paramètres de processus

optimaux se sont avérés l'utilisation de l'acide citrique 2 M, un rapport solide à solvant de 7,5 (w/v), une concentration en éthanol de 66,4 et 59,8% et une température de 97,5 et 97,4 ° C pour l'extraction des fleurs et des feuilles de l'olivier de Russie, respectivement. Enfin, en utilisant l'analyse HPLC, nous avons comparé les extraits de MAE avec ceux de l'extraction par ultrasons et de l'extraction conventionnelle. Les résultats ont démontré la meilleure efficacité de l'extraction MAE comparée aux ultrasons pour l'extraction de la rutine et lutéoline; cependant, l'isorhamnetine n'a été détectée qu'avec l'extraction conventionnelle.

En outre, dans le cadre de la présente étude, l'utilisation du fruit de l'olivier de Russie comme substrat dans la formulation d'une boisson au kéfir a été proposée. La production de kéfir à base d'eau nécessite l'optimisation des différentes étapes de traitement. Par conséquent, les aspects microbiologiques, physico-chimiques et technologiques de la production de kéfir à base d'eau et de fruits de l'olivier de Russie ont été étudiés en ce qui concerne la fermentation (chapitre V), le séchage (chapitre VI) et l'entreposage (chapitre VII).

Le chapitre V s'est concentré sur le développement d'un nouvel aliment fonctionnel doté de propriétés bioactives améliorées à l'aide des fruits de l'olivier de Russie, appelé kéfir d'eau d'olivier de Russie (RWK). Le processus de fermentation du kéfir d'eau a été conçu à l'aide d'un plan central composite (CCD). Par la suite, la méthode des surfaces de réponse (RSM) a été utilisée pour évaluer l'effet des paramètres du processus de fermentation, notamment la concentration en olives russes, le temps de fermentation et la température sur les propriétés bioactives du produit RWK développé. Enfin, les conditions de fermentation optimisées pour le nombre maximal de micro-organismes de kéfir d'eau, la teneur en phénols totaux (TPC) et les propriétés antioxydantes du RWK ont été déterminées à 24 heures de fermentation à la température de 31,2 ° C et en utilisant une concentration de 30 % de jus d'olives russes. Dans ces conditions de fermentation

sélectionnées, le nombre de micro-organismes viables de kéfir d'eau était de 7,20, 7,06 et 7,17 $\log_{10}$ CFU/mL pour AAB, LAB et levures, respectivement, et les valeurs pour l'activité antioxydante TPC, DPPH et FRAP dans RWK étaient de 98,32 (µg GAE/mL), 0,096 (µmol Trolox Eq/mL) et 0,22 (µmol FSHE/mL).

Au chapitre VI, la transformation du RWK en poudre a été réalisée afin d'améliorer la stabilité lors de l'entreposage et réduire les coûts associés au transport de ce produit pour sa commercialisation. Les composés bioactifs et les micro-organismes de kéfir dans la poudre de RWK ont été encapsulés avec des matériaux de support et en utilisant le séchage par atomisation comme méthode d'encapsulation. Les effets des conditions de séchage par atomisation sur la qualité de la poudre produite ont été évalués. Les conditions optimisées du processus de séchage par atomisation pour une réduction minimale des micro-organismes de kéfir et une rétention maximale des propriétés antioxydantes dans la poudre de RWK produite ont été observées à une température d'air d'entrée de 120 °C, un débit d'alimentation de 35 % et une concentration d'aide au séchage de 7 %. Enfin, les analyses microbiennes et physico-chimiques de la poudre de RWK séchée par atomisation ont été effectuées et les résultats ont montré que le séchage par atomisation est une méthode d'encapsulation prometteuse pour préserver la qualité de RWK, démontrant une bonne viabilité des microorganismes et une bonne activité antioxydante en comparaison à la lyophilisation.

Le chapitre VII a évalué la stabilité d'entreposage de la poudre de kéfir d'eau d'olives russes (RWK), afin de déterminer la durée de conservation maximale de ce nouveau produit fonctionnel. Les effets des conditions de d'entreposage sur la qualité de la poudre RWK séchée par atomisation ont été évalués à différents intervalles de temps et les résultats ont été comparés avec des échantillons de RWK lyophilisés. De plus, la résistance gastro-intestinale *in vitro* des micro-

organismes de kéfir présents dans les échantillons de RWK encapsulés a été évaluée. Dans l'ensemble, pendant 90 jours d'entreposage, la dégradation des composés bioactifs dans la poudre RWK a montré une cinétique de premier ordre. La température n'a montré aucun effet significatif sur la stabilité de ces composés, alors que l'entreposage au froid s'est avéré améliorer considérablement la survie des micro-organismes de kéfir dans les échantillons de RWK encapsulés. Avec l'entreposage au froid, les échantillons RWK séchés par atomisation avaient une stabilité prolongée, qui était semblable aux échantillons lyophilisés au cours des deux premiers mois, mais plus faible au cours du troisième mois. Dans l'ensemble, les résultats ont montré une bonne rétention des propriétés bioactives des échantillons séchés par atomisation pendant au moins 90 jours sous entreposage au froid. En outre, des micro-organismes de kéfir d'eau d'olives de Russie encapsulés par atomisation ont montré une survie élevée lors du transit dans des conditions gastro-intestinales simulées. En conclusion, l'extraction de composés bioactifs à partir de feuilles et de fleurs d'olivier russes et le développement d'un aliment fonctionnel à base d'olives russes ont été étudiés avec succès. Les résultats ont prouvé le potentiel des fruits, des feuilles et des fleurs des olives russes comme ingrédients fonctionnels utiles à l'industrie alimentaire. Les processus développés et optimisés dans cette étude (extraction, fermentation, encapsulation) devraient faciliter le développement futur de produits fonctionnels à base de la biomasse de l'Olivier de Russie.

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CONTRIBUTION OF AUTHORS

This is a manuscript-based thesis prepared following the thesis preparation guidelines by the Graduate and Postdoctoral Studies of McGill University. Chapter I of this thesis comprises an introduction and Chapters II and III cover a comprehensive literature review of the topic. Chapters IV to VII consist of four research manuscripts (published/under revision/review) and Chapter VIII focuses on the conclusions of this study and potential future studies. The four research manuscripts are as follows:

Darvishzadeh, P., Orsat, V., Microwave-assisted extraction of antioxidant compounds from Russian olive leaves and flowers: Optimization, HPLC characterization and comparison with other methods. Under review in the Journal of Applied Research on Medicinal and Aromatic Plants. [Chapter IV]

Darvishzadeh, P., Orsat, V., & Martinez, J. L. (2021). Process Optimization for Development of a Novel Water Kefir Drink with High Antioxidant Activity and Potential Probiotic Properties from Russian Olive Fruit (Elaeagnus angustifolia). Food and Bioprocess Technology, 14(2), 248-260. [Chapter V]

Darvishzadeh, P., Orsat, V., & Faucher, S. P. (2021). Encapsulation of Russian Olive Water Kefir as an Innovative Functional Drink with High Antioxidant Activity. Plant Foods for Human Nutrition, 1-9. [Chapter VI]

Darvishzadeh, P., Orsat, V., Storage stability and in vitro digestion of microencapsulated Russian olive water kefir using spray drying. (Under review in the Journal of Food and Bioprocess Technology) [Chapter VII]

The work reported here was designed and performed by Pariya Darvishzadehboroojeni, who also conducted the preliminary data analysis and composed the manuscripts under the supervision and guidance of Dr. Valérie Orsat (Supervisor). Dr. Valérie Orsat provided scientific advice for the project and reviewed and edited the manuscripts that were published. The majority of the research and analytical experiments were carried out in the post-harvest laboratories at the Department of Bioresource Engineering, Macdonald Campus, McGill University, Montreal, Canada. The microwave-assisted and ultrasound-assisted extraction processes, and high-performance liquid chromatography (HPLC) analysis were performed in Dr. Vijaya Raghavan's laboratory at the Department of Bioresource Engineering, Macdonald Campus, McGill University, Montreal, Canada. Experiments in Chapter V of this study for the development of Russian olive water kefir were performed at the Fermentation core at the Technical University of Denmark (DTU). Dr. Jose Luis Martinez, Department of Biotechnology and Biomedicine, Technical University of Denmark (DTU), and Dr. Orsat provided technical and scientific advice for these experiments. They reviewed and edited the manuscript on the development of RWK and provided suggestions for the final version of the text (Chapter V). Dr. Faucher, Department of Natural Resource Sciences, McGill University, provided bioreactors for performing part of experiments in Chapter VI.

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ABBREVIATIONS

RWK	Russian olive water kefir
DPPH	1,1-diphenyl-2-picrylhydrazyl
FRAP	Ferric reducing ability of plasma
TPC	Total phenolic content
TFC	Total flavonoid content
LAB	Lactic acid bacteria
AAB	Acetic acid bacteria
RSM	Response surface methodology
FDA	Food and Drug Administration
ISO	International Organization for Standardization
MAE	Microwave-assisted extraction
UAE	Ultrasound-assisted extraction
CE	Conventional extraction
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
HPLC	High-performance liquid chromatography
QTOF-MS	Quadrupole time of flight mass spectrometer analysis
CCRD	Central composite rotatable design
FCCD	Face centered central composite design
ANOVA	Analysis of variance
FC	Folin ciocalteu
GAE	Gallic acid equivalents
QE	Quercetin equivalents
FSHE	$FeSO_4.7H_2O$ equivalent
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene

CHAPTER I

INTRODUCTION

The Russian olive (*Elaeagnus angustifolia*) is a tree up to 7 m high, closely related to silverberry, autumn olive and buffalo berry (Edwards 2011). It is a member of the Elaeagnaceae family, which is mainly grown in temperate and subtropical areas of Asian countries, in Europe and some regions of North America and produces edible fruits (Agriculture 1948; Ayaz and Bertoft 2001). In North America, *Elaeagnus angustifolia* tree, known as the Russian-olive or oleaster, can stand temperatures from -45 to 46 °C and extreme winds, has been cultivated for snow- and windbreaks, landscaping, providing pollen for honeybees and horticultural purposes such as soil fertilization. In Canada, in all the southern mainland provinces except in Saskatchewan, the Russian olive tree is planted. Also, due to its tolerance to dry and alkaline soils, the Russian olive tree is frequently used in ornamental planting in Nova Scotia. It is considered as a nitrogen-fixing plant through establishing a symbiotic relationship with the bacteria *Frankia* spp. (Hamilton and Carpenter 1976; Zouhar 2005; Lesica and Miles 2001). Also, the plant has an important role in bio-monitoring of toxic elements in the environment and can act as a bio-fertilizer agent in distressed fields (Hamidpour et al. 2016; Agriculture 1948). Because of these advantages, since 1948 and as recently as 2002, planting of this plant had been sponsored by federally funded programs in the Canadian prairies and it continues to be used as an ornamental choice all over Canada (Collette and Pither 2015).

Due to the prevalence of the Russian olive tree for the mentioned environmental and ornamental purposes, applications of Russian olive leaves, flowers and fruits as food ingredients could valorize further this tree. On the other hand, *E. angustifolia* has a wide variety of active phytochemicals and over the years it has been shown to have different therapeutic effects including anti-

inflammatory, anti-cancer, anti-arthritic, wound healing and anti-diarrhea activities (Farzaei et al. 2015). In addition, many studies have reported significant antioxidant activity of Russian olive leaves, flowers and fruits (Caliskan, Elmastas, & Gokce, 2010; Li, Qi, & Yang, 2012; Mehrabani, 2013; Okmen & Turkcan, 2014). However, despite the various health benefits and great potential of Russian olive to be used as a valuable food ingredient, it has not been broadly used in the food industry (Farzaei et al. 2015). Therefore, this study aims to help develop the industrial application of Russian olive as a potential ingredient with health benefits to respond to increasing consumers' demand for healthy functional products. It is based on using Russian olive leaves and flowers for producing functional ingredients such as antioxidant-rich extracts and using Russian olive fruits for developing functional foods such as water kefir.

1.1 Ph.D. research hypothesis

This study is based on the hypothesis that Russian olive (fruits, leaves and flowers) with various bioactive compounds, antioxidant activity and several health-benefitting properties could be used as functional ingredients while valorizing this frequently planted tree. It consists of two main research hypotheses:

1) Russian olive leaves and flowers could be good sources of antioxidant compounds. Antioxidants are compounds that inhibit oxidation reactions that cause cell damage and degradation of fatty foods. They can prevent undesirable colour changes and rancidity in food products and are broadly used as potential inhibitors of lipid peroxidation in the food industry. The application of antioxidants from synthetic sources such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in foods has been limited due to their potential toxic properties and side effects on human health. Therefore, natural antioxidants from plant materials have great potential for replacing synthetic antioxidants and perhaps avoiding their side effects (Wang et al. 2014). To

minimize the application of synthetic antioxidants and their side effects identifying natural sources of antioxidants such as Russian olive would be advantageous for their application in the food industry, and to use against a variety of oxidative stress related diseases. Investigation of the considerable antioxidant activity of Russian olive by other authors and by our preliminary test results proves the potential of this plant to be used as a valuable food ingredient with antioxidant properties. Therefore, we hypothesize that an optimized process condition is required for maximum retention of antioxidant compounds during the extraction process, to produce extracts from leaves and flowers of Russian olive which can be used as an ingredient with effective antioxidant properties for development of functional food products (Chapter IV).

2) There are several positive effects of kefir products on human health, among which, antioxidant, anti-inflammatory, anti-ulcerogenic, and antimicrobial activities are common with Russian olive fruit (Rodrigues et al. 2005, 2016; Alsayadi et al. 2013; Alsayadi et al. 2014; Farzaei et al. 2015). Therefore, by using Russian olive for producing a non-dairy kefir, a novel fermented beverage can be developed, in which particular health benefits of kefir can be enhanced. It is also hypothesized that Russian olive fruit, with prebiotic activity, high contents of total dietary fiber, minerals, vitamins, sugar and proteins (Dulger et al. 2015), can serve as the supporting medium for the production of a non-dairy kefir product. To the best of our knowledge, no study has developed a none-dairy kefir (water kefir) product using Russian olive fruit as the substrate. The present study focuses on formulating Russian olive water kefir from Russian olive fruit and optimization of the process conditions during fermentation (Chapter V) and dehydration (Chapter VI), and monitoring the encapsulated RWK product's quality during the storage period (Chapter VII).

1.2 Overall objectives

The overall objective of this study is to valorize Russian olive fruit, leaves and flowers through developing and optimizing processes that facilitate the application of this plant as functional ingredients in the food industry.

1.3 Specific objectives

1) To investigate the potential antioxidant activity of Russian olive leaves, flowers and fruit.
2) To develop and optimize the microwave-assisted extraction for obtaining antioxidant-rich extracts from Russian olive leaves and flowers for their application in functional foods.
3) To formulate water kefir beverage using Russian olive and introducing a novel non-dairy probiotic-containing beverage with high antioxidant activity and potential therapeutic properties. Also, to optimize the Russian olive water kefir fermentation process for maximizing the bioactive properties of this product.
4) To produce Russian olive water kefir powder for improving stability and potential commercialization of RWK and facilitating the future application of the developed powder in different products.
5) To optimize encapsulation of bioactive compounds in Russian olive water kefir for protecting its functional properties and improving its storage stability.
6) To monitor the quality of spray-dried powder of Russian olive water kefir during storage and evaluate its shelf life.

CHAPTER II

REVIEW OF LITERATURE AND PRELIMINARY SCREENING RESULTS – PART I

POTENTIAL ANTIOXIDANT ACTIVITY AND THERAPEUTIC PROPERTIES OF RUSSIAN OLIVE

2.1 Russian olive properties

2.1.1 Composition

E. angustifolia has a wide variety of active phytochemicals such as flavone glycosides, esters, ketones, steroids, phenolic acids, phenols, phenyl ethers, terpenes, β-carboline alkaloids, pyrimidines and polysaccharides. A summary of the phytochemical compounds found in different parts of the Russian olive by Farzaei et al. (2015) is provided in Table 2.1.

Table 2.1 *Phytochemical constituents of Elaeagnus angustifolia provided by Farzaei et al. (2015).*

Chemical category	Compound	Plant part
Amino acid	Aspartic acid, threonine, serine, glutamine, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine,	Fruit
	phenylalanine, histidine, lysine, arginine, tryptophan, cysteine and cysteinic acid	
β -carboline alkaloid	2-methyl-1,2,3,4-tetrahydro- β -carboline 1,2-dihyroharmaline Dihydroharmane 3,3-dimethyl-1,3-dihydro-indol-2-one Harmane Harmol N-methyl-1,2,3,4-tetrahydro-β-carboline N-methyltetrahydroharmol Tetrahydroharmane Tetrahydroharmol 2,3,4,9-tetrahydro-1 -methyl-1 H-pyrido[3, 4-b]indole	Bark
Carbohydrate	Fructose, galactose, glucose, mannose, rhamnose, sucrose, xylose and galacturonic acid Polysaccharide of Elaeagnus angustifolia-1 and 2 (PEA -1 and PAE-2)	Fruit palp
Cardiac glycoside	Gitoxigenin	Bark

Ester	2 -phenyl-ethyl benzoate 2 -phenyl-ethyl isovalerate	Flower/ essential oil
Fatty acids	Linoleic acid Palmitic acid	Seed oil Bark
Flavonoid	Epigallocatechin gallate	Bark
	(—)-epicatechin (+)-catechin Isorhamnetin	Leaf
	Isorhamnetin 3-0-β-D-galactopyranoside Isorhamnetin 3-0-β-D-galactopyranoside-4'-0-β-D-glucopyranoside Isorhamnetin-3-0-galactopyranoside Isorhamnetin-3-0-β-D-galactopyranoside Isorhamnetin 3-0-(6-0-E-coumaroyl)-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside (elaeagnoside A) Isorhamnetin 3-0-(6-0-E-feruoyl)-β-D- glucopyranosyl-(1 →2)[α-L-rhamnopyranosyl-(1→)6)]-β-D-galactopyranoside (elaeagnoside B) Isorhamnetin 3-0-(6-0-E-sinapoyl)-β-D-glucopyranosyl- (1→2)[α -L rhamnopyranosyl-(1→)6)]-β-D-galactopyranoside (elaeagnoside C) Isorhamnetin 3-0-(6-0-benzoyl)-β-D-glycopyranosyl-(1→2)[α-L-rhamnopyranosyl-(1→)6)]-β-D-galactopyranoside (elaeagnoside D) Isorhamnetin 3-0 α -L-rhamnopyranosyl-(1→)6)-β-D-glycopyranosyl-(1→2)[6-0-2-methyle,3-hydroxy propanoyl]-β-D-galactopyranoside (elaeagnoside E) Isorhamnetin 3-0-(6-0-E-feruloyl)-β-D-glucopyranosyl-(1→)2)-β-D-galactopyranoside-7-0-β-D-glucopyranoside (elaeagnoside F) Isorhamnetin 3-0-[β-D-glycopyranosyl-(1→2) [α-L-rhamnopyranosyl-(1→)6)]-β-D-galactopyranoside]-7-0-β-D galactopyranoside (elaeagnoside G) Isorhamnetin 3-0-(6-0-E-feruloyl)-β-D-glucopyranosyl-(1→)2)-β-D galactopyranoside Isorhamnetin 3-0-α-L-rhamnopyranosyl-(1→)6)-β-D-galactopyranoside Isorhamnetin 3-0-β-D-glucopyranosyl-(1→)2)-β-D-galactopyranoside Isorhamnetin 3-0-β-D-glucopyranosyl-(1→2)[α-L-rhamnopyranosyl-(1→6)]-β-D galactopyranoside Kaempferol Kaempferol 3-0-β-D-galactopyranoside Kaempferol-3-0-(6-0-E-coumaroyl)-β-D-g lucopyranoside Kaempferol-3-0-(6-0-Z-coumaroyl)-β-D-glucopyranoside Quercetin Quercetin 3,4'-0-β-D-diglucoside Quercetin 3-0-β-D-galactopyranoside-4'-0-β-D-glucopyranoside Quercetol Rutin	Fruit
Ketone	Acetophenone	Flower/ essential oil
Phenol	Coniferyl alcohol	Bark
Phenolic acid	4-hydroxybenzoic acid 4-hydroxycinnamic acid Benzoic acid [2]	Fruit
	Caffeic acid	Young branch
	Chlorogenic acid [23	Leaf
	Ethyl cinnamate	Flower/ essential oil

	Ferulic acid	fruit
	Gentisic acid	Young branch
	E)-isoeugenol	Bark
	Neochlorogenic acid	Leaf [
	p-Coumaric acid	Fruits Young branch
	Protocatechuic acid	Fruit
	Sinapic acid	Young branch
	Vanillic acid	Fruit
Phenyl ether	Anethole	Flower/ essential oil
	3-tert-butyl-4-hydroxyanisole	Bark
Pyrimidine	Thymine	Bark
Steroid	β-sitosterol	Fruit
	β-sitosterol acetate	Bark
	Stigmasterol	
Terpene	Isocaryophyllene	Bark
	Limonene	Flower/ essential oil
	Nerolidol	
	Squalene	
	Ursolic acid	Leaf surface lipids
	α-amyrin	
	β-amyrin	
Vitamins	Ascorbic acid (vitamin C) and vitamins A (or provitamin A, β-carotene), E, and K	Fruit
	α-tocopherol	Leaf surface lipids

Also, the nutritional constituents in *E. angustifolia* including its highest micro-minerals are summarized in Table 2.2. This table presents the proximate composition of Russian olive fruit, in which Dulger et al. (2015) reported 3.74-4.65% protein content, 20.67% to 30.65% total dietary fiber content and 1.87-2.57% ash content for peeled and unpeeled oleaster. Also, they investigated the high levels of micro minerals such as Fe, Cu, and B in flours obtained from oleaster, with the higher level when the flour is obtained from unpeeled fruit compared to peeled fruit. Cansev et al. (2011) reported slightly different values, which can be observed in Table 2.2. Moreover, our preliminary results on the proximate composition of Russian olive fruit are presented in Table 2.3.

Table 2.2 *Summary of the chemical composition of Russian olive (Oleaster), (Peeled and unpeeled flour) adopted from (Cansev et al. 2011; Dulger et al. 2015; Sahan et al. 2015).*

Study	Protein	Fat	Moisture	Ash	Total Dietary Fiber
A. Cansev et al. (2011)	3.60 - 5.78 (%)	0.4 - 0.6 (%)	26.33 - 26.63 (%)	1.14 - 1.30 (%)	
Dulger et al. (2015), Sahan et al. (2015), Sahan et al. (2012)	Peeled: 3.74 - 4.51 (g/100g, db) Unpeeled: 4.49 - 4.65 (g/100g, db)	NA	Peeled: 18.99 - 19.78 (g/100g) Unpeeled: 18.43 - 20.20 (g/100g)	Peeled: 1.87 - 2.46 (g/100g) Unpeeled: 1.87 - 2.57 (g/100g)	Peeled: 20.67 - 23.55 (g/100g, db) Unpeeled: 25.44 - 30.65 (g/100g, db)
Major Element Content:	**K**	**Mg**	**Na**	**Ca**	**P**
A. Cansev et al., (2011)	795.83 - 909.53 (mg/100g)	20.32 - 23.81 (mg/100g)	151.81 - 192.17 (mg/100g)	36.18 - 42.27 (mg/100g)	60.20 - 67.31 (mg/100g)
Major Element Content	**Fe**	**Cu**	**B**	**Zn**	**Mn**
Dulger Alt et al. (2015)	Unpeeled: 17.53 (mg/kg)	Peeled and Unpeeled: 2.37 - 6.11 (mg/kg)	Peeled and Unpeeled: 5.99 - 8.58 (mg/kg)	Unpeeled: 1.90 - 5.50 (mg/kg)	Peeled and Unpeeled: 2.46 - 4.51 (mg/kg)

Table 2.3 *Preliminary data on proximate composition of Russian olive fruit.*

Russian olive compartments	Moisture content (%)	Ash content (%)	Protein content (%)	Fat content (%)	Calories (Cal/g)
Seed+ Exocarp +Mesocarp	13.5	2.26	6.75	1.49	4208.24
Exocarp+ Mesocarp	11.8	2.29	5.73	0.87	4230.55
Mesocarp	9.8	2.38	6.38	0.97	4356.51

2.1.2 Ethnopharmacological uses (folk medicine) and scientifically proven therapeutic properties

E. angustifolia is traditionally used for a variety of therapeutic effects. In Iranian traditional medicine, this plant is known for its healing properties on arthritis, inflammation and is used as an astringent, carminative, and antitussive agent. The fruit of this plant is also used to be part of remedies for osteoporosis, diarrhea, stomach pain and as a female aphrodisiac. The aerial parts of *E. angustifolia* have exhibited therapeutic effects on jaundice, aphthous and swelling of the joints. In Turkey, the fruit is used for its therapeutic properties on diarrhea and kidney disorders. People in Pakistan use the whole plant as a remedy for skin infections, headaches, and heartburn. *E.*

angustifolia is also used in traditional medicine practiced by various ethnic groups against asthenia of the stomach and spleen and as a remedy for dyspepsia, dysentery, enteritis and diseases of the genitourinary tract around the world (Farzaei et al. 2015).

In addition to mentioned ethnopharmacological uses of different parts of this plant, scientific studies have also proved the effectiveness of this plant in reducing wound healing time and relieving pain in rheumatoid arthritis. Additionally, recent studies have reported *E. angustifolia* to have a gastrointestinal effect, wound healing, anti-inflammatory, anti-cancer, antioxidant, anti-diarrhea and antibacterial activities, which will be discussed in detail in the following paragraphs (Lotfi et al. 2016; Motevalian et al. 2017; Muzhapaer et al. 2007; Hamidpour et al. 2017).

2.1.2.1 Analgesic and anti-inflammatory effect

In a study by Tafti et al. (2015) *Elaeagnus angustifolia* fruit extract showed to have an analgesic effect and was found to be effective for improving symptoms from ulcerative colitis. In addition, a clinical trial by Taheri et al. (2010) proved the anti-inflammatory effect of a 19% *Elaeagnus angustifolia* topical gel.

2.1.2.2 Gastrointestinal effect

The ethanolic extract from leaves and flowers of *E. angustifolia* was proved to have a dose-dependent spasmolytic effect on muscle cells of the small intestine in guinea pigs, and the terpenoids and flavonoids were proposed to be among the active compounds. In addition, in an animal model of indomethacin-induced gastric ulcers, gastric ulcer index was considerably decreased by different doses of 400 and 800 mg/kg of hydroalcoholic extract of leaves and fruits of *E. angustifolia*. Also, methanolic extracts of Russian olive fruits have shown significant inhibitory effects against ethanol-induced gastric ulcers (Farzaei et al. 2015).

2.1.2.3 Wound healing activity

It has been observed that the rate of wound contraction is improved by applying the aqueous extract of the fruit of *E. angustifolia* on 10, 12 and 15 days after wounding. Similarly, after 10 and 15 days, the hydroxyproline content and histological score in the wounded tissue were improved. Also, in an animal model of cutaneous excision, the aqueous extract of the fruit improved the re-epithelialization process and the collagen content (Farzaei et al. 2015).

2.1.2.4 Antibacterial activity

Methanolic extract of Russian olive leaves has shown antibacterial activity against mastitis-inducing bacteria including five clinical strains of coagulase-negative *Staphylococci* and two of *Staphylococcus aureus* strains. Antibacterial tests on a variety of gram-positive and gram-negative bacteria including food pathogens have shown *E. angustifolia* leaves methanol extract to be most effective against *Yersinia enterocolitica* among tested microorganisms by inhibition zone of 16 mm (Okmen and Turkcan 2013). Also, Bucur et al. (2006) have confirmed the antibacterial effect of the extract of flowering tops of Russian olive on *Klebsiella pneumonia, Streptococcus pyogenes, Staphylococcus aureus* and *Escherichia. coli*. Fungicidal activity of leaves against *Botrytis cinerea* and *Alternaria solani* was shown by Bahraminejad et al. (2015). In addition, Khan et al. (2016) have shown fungicidal effects of different parts of *E. angustifolia* on *Aspergillus flavis, Aspergillus fumigatus* and *Aspergillus niger* (Farzaei et al. 2015).

2.1.2.5 Antioxidant activity

So far, many studies have reported the significant antioxidant activity of Russian olive leaves, flowers and fruits (Caliskan, Elmastas, & Gokce, 2010; Li, Qi, & Yang, 2012; Mehrabani, 2013; Okmen & Turkcan, 2014). The antioxidative properties of the plants are mainly attributed to their phenolic compounds. These compounds are secondary metabolites, which perform a variety of

functions for the plants, such as responding to wounding, pathogens, UV radiation and other ecological and physiological pressures (Khoddami et al. 2013). Phenolic compounds contain one or more aromatic rings connected with hydroxyl groups, which can potentially donate hydrogen and act as an antioxidant. In fruits of Russian olive, Kusova et al. (1988) isolated the following phenolic compounds: isorhamnetin 3-O-β-D-galactopyranoside, caffeic acid and isorhamnetin using chromatography. Ayaz and Bertoft (2001) identified seven phenolic acids through HPLC examinations including benzoic acid, 4-hydroxybenzoic acid, vanillic acid, 4-hydroxycinnamic acid, protocatechuic acid, ferulic acid and caffeic acid. Among these phenolic acids, caffeic acid (32 mg/100 g dry weight) and 4-hydroxybenzoic acid (45.8 mg/100 g dry weight) were detected to be the most abundant; but ferulic acid and benzoic acid were identified as the least abundant (2.3 and 11.6 mg/100 g dry weight, respectively). Moreover, based on the phytochemical studies on extracts of Russian olive, flavonoids, which are one of the largest classes of phenolic compounds having an important role in biological systems, have been detected. These compounds are a group of low molecular weight polyphenolic substances with strong antioxidant activities which are categorized based on their chemical structures into flavonols, flavones, flavanones, flavanols (catechins), dihydroflavonols, isoflavones, anthocyanidins, and chalcones. Flavonoids widely exist in fruits and vegetables and their antioxidant activity results from their phenolic hydroxyl groups, and they can minimize free radicals (Okmen and Turkcan 2014; Dubick & Omaye 2006). Wang et al. (2012) isolated nine flavonoids including luteolin, (-)-epicatechin, (+)-gallocatechin, kaempferol, (+)-catechin, quercetin, isorhamnetin, isorhamnetin-3-O-β-D—galactopyranoside and (-)-epigallocatechin from Russian olive. In addition, they identified four flavonoid glycosides including quercetin 3,4'-O-β-D-diglucoside, quercetin 3-O-β-D-galactopyranoside- 4'-O- β-D-glucopyranoside, isorhamnetin-3-O-β-D-galactopyranoside and

isorhamnetin 3-O-β-D-Galactopyranoside-4'-O-β-D-glucopyranoside. They also indicated a dose-dependent antioxidant activities of these compounds.

In order to confirm the antioxidant activity of *E. angustifolia*, as reported by other studies, we evaluated the *in vitro* antioxidant activity of Russian olive leaves, flowers and fruits through 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing ability of plasma (FRAP) assays. We also measured the total phenolic content (TPC) and total flavonoid content (TFC) of the fruits, leaves and flowers of this plant. The extracts of Russian olive were prepared using organic solvent extraction following a procedure by Caliskan et al. (2010) with slight modifications and using 50, 70 and 90% methanol and ethanol in aqueous solutions. Results are shown in Figures 2.1-2.4, presenting the significant antioxidant activity of Russian olive.

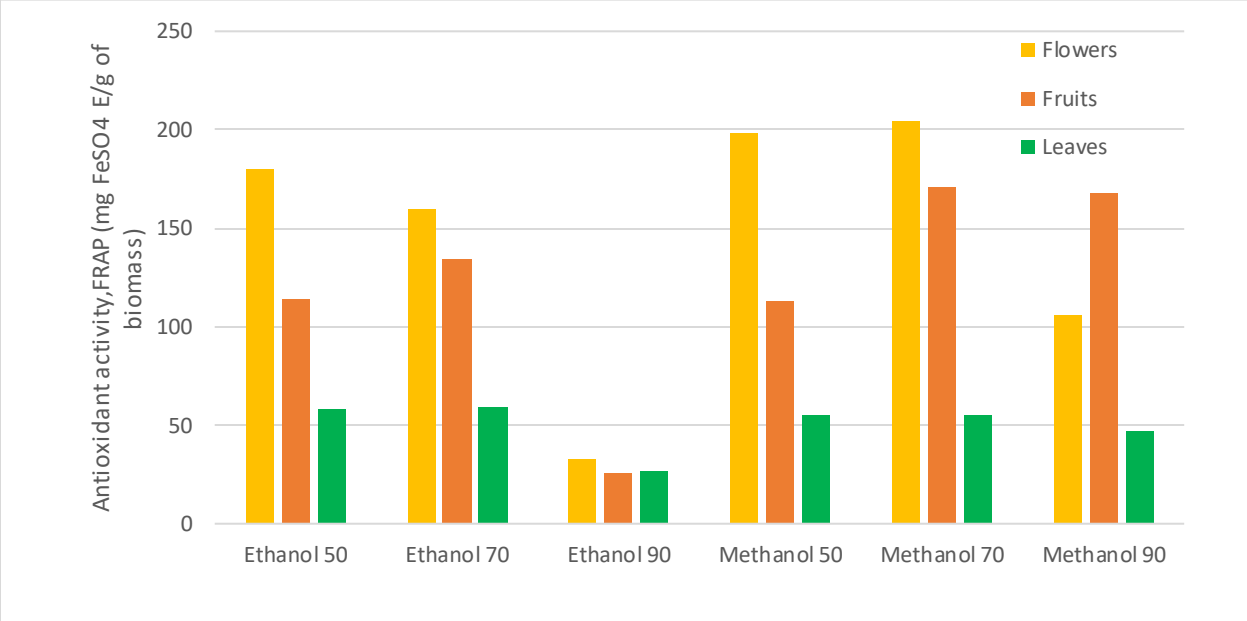


Figure 2.1 *Antioxidant activity of leaves, flowers, and fruits of E. angustifolia by FRAP assay.*

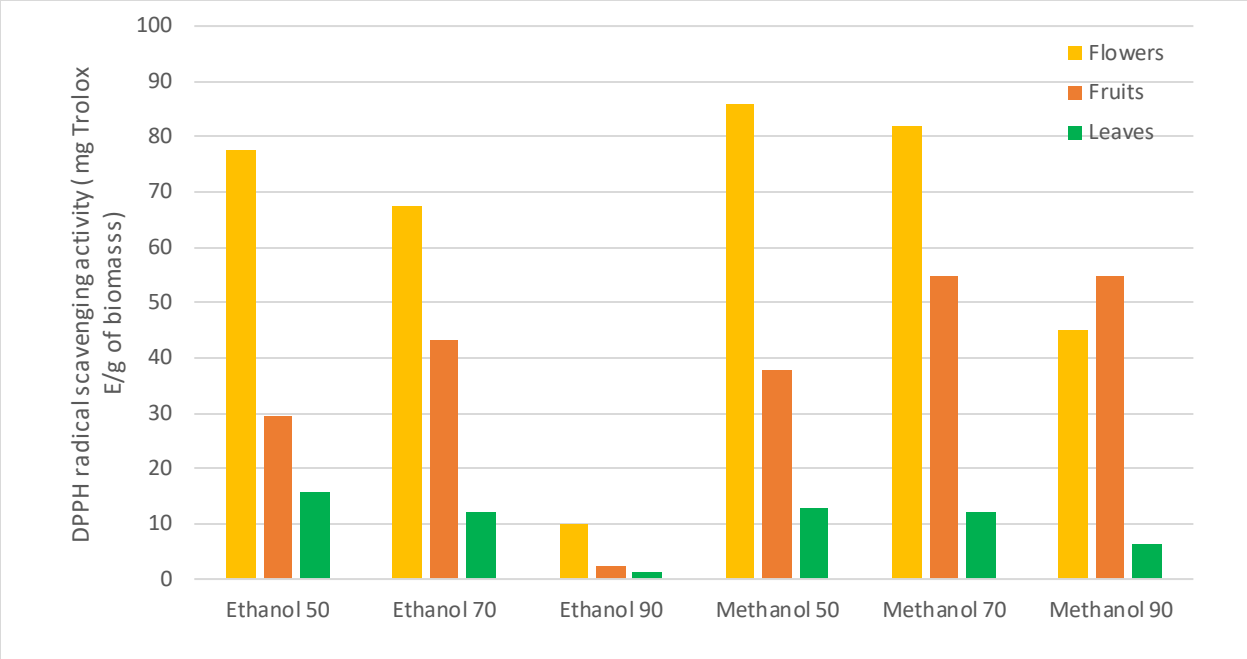


Figure 2.2 *DPPH radical scavenging activity of leaves, flowers, and fruits of E. angustifolia.*

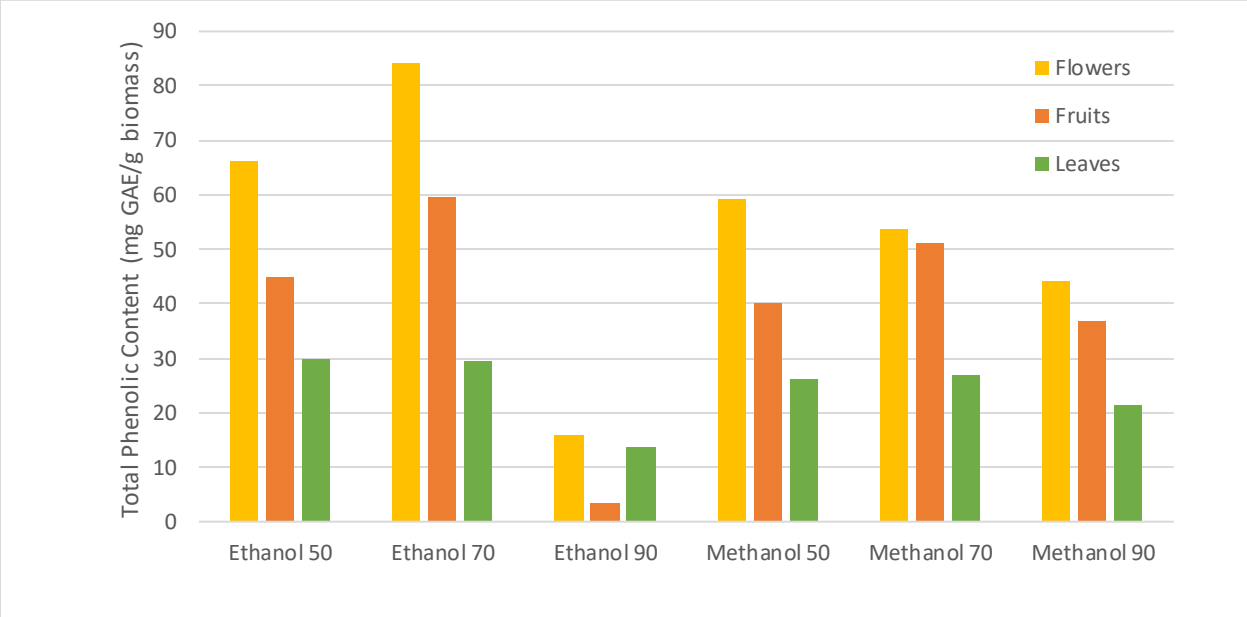


Figure 2.3 *Total phenolic content of leaves, flowers, and fruits of E. angustifolia.*

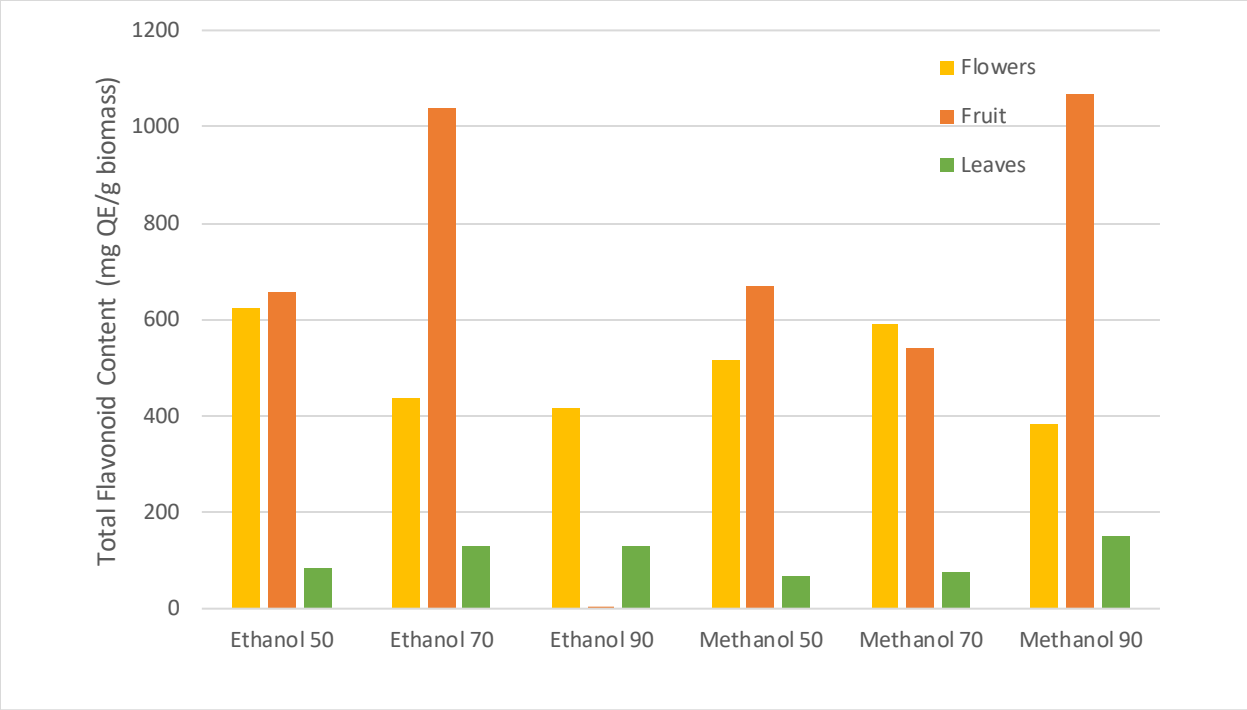


Figure 2.4 *Total flavonoid content of leaves, flowers, and fruits of E. angustifolia.*

Following the screening of the antioxidant activity of the plant, fine dried powders of the flowers and leaves from Iran and Canada and fruits from Canada were extracted by 70 percent aqueous solution of ethanol or methanol. Subsequently, the extracts were used for identification and quantification of polyphenols using an Agilent series 1290 UPLC instrument coupled with an Agilent 6545 quadrupole time-of-flight mass spectrometer (QTOFMS) in electrospray ionization (Dual AJS ESI) mode. A volume of 2 µL of each extract was injected on a Poroshell 120 Bonus-RP C18 column (2.1 mm x 100 mm, 2.7 µm, Agilent Technologies) at a column temperature of 35 °C. A gradient elution was achieved using mobile phase water (solvent A) and acetonitrile (solvent B), both containing 0.1% (v/v) formic acid at a flow rate of 0.6 ml/min with the following gradient conditions: 0 min, 50% B; 5 min, 0% B; 20 min, 100%; 25 min, 100% B; 20.5 min, 0% B; 30min, 0% B; with a 1 min equilibration time. Tables 2.4, 2.5 and 2.6 show the phenolic compounds of the different parts of Russian olive cultivated in Canada including fruit, flower and leaves.

Additionally, they provide information on differences in the phenolic compounds of leaves and flowers cultivated in Canada versus the ones cultivated in Iran and extracted using ethanol versus using methanol. For example, theobromine, isorhamnetin 3-O-glucoside, (+)-cyanidin, 6-gingerol and isorhamnetin were detected in Russian olive fruit. We also identified a series of polyphenol compounds in Russian olive leaves including (+)-cyanidin, (+)-ideain, 4-Caffeoylquinic acid, 6-gingerol, isorhamnetin, luteolin, p-coumaric acid and rutin. In addition, similar compounds in Russian olive flowers were identified as follows: phloridzin, (+)-ideain, astragalin, luteolin, (+)-cyanidin, eleutheroside B, ginkgolide C, isorhamnetin 3-O-glucoside, procyanidin dimer B1, (+)-catechin, 6-gingerol, 4-caffeoylquinic acid, rutin and (+)-catechin.

Table 2.4 *Phenolic compounds of Russian olive fruit from Canada, extracted by 70% aqueous solution of ethanol (EtOH 70%) or methanol (MeOH 70%), identified by high-resolution quadrupole time-of-flight mass spectrometry.*

Fruit Canada (MeOH 70%)	**Fruit Canada (EtOH 70%)**
Theobromine	Isorhamnetin 3-O-glucoside
Isorhamnetin 3-O-glucoside	Isorhamnetin
(+)-Cyanidin -1H	(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H
Isorhamnetin	Nordihydrocapsaicin
6-Gingerol	(+)-Cyanidin 3-O-xylosyl-rutinoside -1H
Dihydrocaffeic acid 3-O-glucuronide	(+)-Malvidin 3-O-(6"-caffeoyl-glucoside) -1H
m-Coumaric acid	
(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H	
Nordihydrocapsaicin	
(+)-Cyanidin 3-O-xylosyl-rutinoside -1H	
(+)-Malvidin 3-O-(6"-caffeoyl-glucoside) -1H	
Carnosic acid; Salvin	
Ganoderic acid F	

Table 2.5 *Phenolic compounds of Russian olive leaves from Canada and Iran, extracted by 70% aqueous solution of ethanol (EtOH 70%) or methanol (MeOH 70%), identified by high-resolution quadrupole time-of-flight mass spectrometry.*

Leaves Canada (MeOH 70%)	**Leaves Canada (EtOH 70%)**	**Leaves Iran (MeOH 70%)**	**Leaves Iran (EtOH 70%)**
p-Coumaric acid	p-Coumaric acid	Eleutheroside B	Eleutheroside B

6-Gingerol	(+)-Cyanidin -1H	6-Gingerol	Luteolin
Luteolin	Luteolin	Luteolin	(+)-Cyanidin -1H
(+)-Cyanidin -1H	4-Caffeoylquinic acid	(+)-Cyanidin -1H	4-Caffeoylquinic acid
4-Caffeoylquinic acid	Rutin	4-Caffeoylquinic acid	Ginkgolide C
Rutin	(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H	(+)-Ideain -1H	(+)-Ideain -1H
Isorhamnetin	Isorhamnetin	Isorhamnetin 3-O-glucoside	Theobromine
(+)-Ideain -1H	m-Coumaric acid		Isorhamnetin 3-O-glucoside
Coumarin	Dihydrocaffeic acid 3-O-glucuronide	(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H	(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H
Dihydrocaffeic acid 3-O-glucuronide	Nordihydrocapsaicin	Astragalin MID	Astragalin MID
(+)-Cyanidin 3-O-glucosyl-rutinoside -1H	(+)-Cyanidin 3-O-glucosyl-rutinoside -1H	Ganoderic acid F	Dihydrocaffeic acid 3-O-glucuronide
Nordihydrocapsaicin	(+)-Cyanidin 3,5-O-diglucoside -1H	Coumarin	Ferulic acid 4-O-glucoside
(+)-Cyanidin 3,5-O-diglucoside -1H	Hydroxytyrosol 4-O-glucoside	Ferulic acid 4-O-glucoside	Coumarin
Hydroxytyrosol 4-O-glucoside	Ginkgolide J	Dihydrocaffeic acid 3-O-glucuronide	(+)-Cyanidin 3,5-O-diglucoside -1H
Ginkgolide J		(+)-Cyanidin 3,5-O-diglucoside -1H	Ginkgolide J
		Ginkgolide J	Nordihydrocapsaicin
		Nordihydrocapsaicin	Isorhamnetin 3-O-rutinoside
		Isorhamnetin 3-O-rutinoside	Phlorin
		(+)-Cyanidin 3-O-glucosyl-rutinoside -1H	(+)-Cyanidin 3-O-glucosyl-rutinoside -1H
		(+)-Cyanidin 3-O-(6"-caffeoyl-glucoside) -1H	Apigenin 7-glucoside - Cosmosiin
			(+)-Cyanidin 3-O-(6"-caffeoyl-glucoside) -1H
			Dihydrocapsaicin

Table 2.6 *Phenolic compounds of Russian olive flowers from Canada and Iran extracted by 70% aqueous solution of ethanol (EtOH 70%) or methanol (MeOH 70%), identified by high-resolution quadrupole time-of-flight mass spectrometry.*

Flowers Canada (MeOH 70%)	Flowers Canada (EtOH 70%)	Flowers Iran (MeOH 70%)	Flowers Iran (EtOH 70%)
Eleutheroside B	Eleutheroside B	Eleutheroside B	Eleutheroside B
6-Gingerol	Phloridzin	Phloridzin	(+)-Cyanidin -1H
Phloridzin	Luteolin	6-Gingerol	Luteolin
(+)-Cyanidin -1H	(+)-Cyanidin -1H	(+)-Cyanidin -1H	Kaempferol
Luteolin	Ginkgolide C	Luteolin	Ginkgolide C
Ginkgolide C	4-Caffeoylquinic acid	Kaempferol	4-Caffeoylquinic acid
4-Caffeoylquinic acid	(+)-Catechin	Ginkgolide C	(+)-Ideain -1H
(+)-Catechin	Astragalin	4-Caffeoylquinic acid	(+)-Catechin
Astragalin	Procyanidin dimer B1	(+)-Ideain -1H	Theobromine
Procyanidin dimer B1	Rutin	(+)-Catechin	(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H
(+)-Ideain -1H	(+)-Ideain -1H	Theobromine	5-5'-Dehydrodiferulic acid

Rutin	Theobromine	(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H	Pyrogallol ; Pyrogallic acid
Isorhamnetin 3-O-glucoside	Isorhamnetin 3-O-glucoside	5-5'-Dehydrodiferulic acid	Ferulic acid 4-O-glucoside
(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H	(+)-Catechin	Pyrogallol ; Pyrogallic acid	Astragalin MID
5-5'-Dehydrodiferulic acid	(+)-Cyanidin 3-O-(6"-p-coumaroyl-glucoside) -1H	Ferulic acid 4-O-glucoside	Coumarin
Ferulic acid 4-O-glucoside	5-5'-Dehydrodiferulic acid	Astragalin MID	Ganoderic acid F
Pyrogallol ; Pyrogallic acid	Ferulic acid 4-O-glucoside	Coumarin	Ginkgolide J
Coumarin	Pyrogallol ; Pyrogallic acid	Dihydrocaffeic acid 3-O-glucuronide	(+)-Cyanidin 3-O-glucosyl-rutinoside -1H
Ginkgolide J	Dihydrocaffeic acid 3-O-glucuronide	Ganoderic acid F	Dihydrocaffeic acid 3-O-glucuronide
Ganoderic acid F	Ginkgolide J	Ginkgolide J	Nordihydrocapsaicin
p-Coumaric acid ethyl ester	m-Coumaric acid	(+)-Cyanidin 3,5-O-diglucoside -1H	(+)-Cyanidin 3,5-O-diglucoside -1H
Ginkgolide J	Coumarin	Caffeic acid 3-O-glucuronide	Apigenin 7-O-glucuronide
Apigenin 7-glucoside - Cosmosiin	Resveratrol 3-O-glucuronide (cis)	Apigenin 7-glucoside - Cosmosiin	Caffeic acid 3-O-glucuronide
(+)-Cyanidin 3-O-glucosyl-rutinoside -1H	Ganoderic acid F	(+)-Delphinidin -1H	Apigenin 7-glucoside - Cosmosiin
Nordihydrocapsaicin	p-Coumaric acid ethyl ester	(+)-Cyanidin 3-O-(6"-caffeoyl-glucoside) -1H	(+)-Cyanidin 3-O-(6"-caffeoyl-glucoside) -1H
(+)-Cyanidin 3-O-(6"-caffeoyl-glucoside) -1H	Ginkgolide J	(+)-cyanidin 3-O-diglucoside-5-O-glucoside -1H	(+)-Delphinidin -1H
(+)-Cyanidin 3-O-arabinoside -1H	Nordihydrocapsaicin	(-)-4'-O-Methylepigallocatechin	Naringin-rutinoside-4'-O-glucoside
Isorhamnetin 3-O-rutinoside	Apigenin 7-glucoside - Cosmosiin	Malvin	(+)-Catechin 3-O-glucose
Resveratrol 3-O-glucuronide (cis)	(+)-Cyanidin 3-O-(6"-caffeoyl-glucoside) -1H	Naringin-rutinoside-4'-O-glucoside	(+)-cyanidin 3-O-diglucoside-5-O-glucoside -1H
(+)-Cyanidin 3,5-O-diglucoside -1H	(+)-Cyanidin 3-O-arabinoside -1H		
	Resveratrol 3-O-glucuronide (cis)		
	Isorhamnetin 3-O-rutinoside		
	(+)-Cyanidin 3,5-O-diglucoside -1H		
	Naringin-rutinoside-4'-O-glucoside		
	(+)-Delphinidin 3-O-galactoside -1H		
	Dihydrocapsaicin		

Our results confirmed reported studies indicating the potential of *E. angustifolia* as a natural source of phenolic compounds with strong antioxidant activities. Therefore, our hypothesis for the

potential application of Russian olive extracts as antioxidant-rich food additives in different food applications was shown to be promising and microwave-assisted extraction of Russian olive leaves and flowers will be further developed and optimized.

CONNECTING TEXT

The first part of the literature review (Chapter II) emphasized on the potential use of Russian olive leaves and flowers for developing functional food products. Results from other studies and our preliminary tests confirmed Russian leaves and flowers as a natural source of phenolic compounds with strong antioxidant activities. It was concluded that for providing Russian olive extracts, to be used as antioxidant-rich food additives in different food applications, an efficient microwave-assisted extraction method needs to be further developed and optimized.

The aim of the second part of the literature review, as presented in Chapter III, is to investigate the potential of Russian olive fruit to be used as a functional ingredient for the development of Russian olive-based functional food products such as water kefir. This chapter focuses on evaluating postharvest processes such as water kefir fermentation and encapsulation, which can facilitate the successful commercialization of the developed Russian olive-based water kefir.

CHAPTER III

REVIEW OF LITERATURE AND PRELIMINARY SCREENING RESULTS – PART II

POTENTIAL OF RUSSIAN OLIVE FRUIT TO BE USED AS FUNCTIONAL INGREDIENT FOR DEVELOPMENT OF RUSSIAN OLIVE WATER KEFIR (RWK)

3.1 An introduction to kefir and water kefir

The use of lactic acid fermentation for preserving and improving the quality and flavour of milk products has a long history worldwide. A variety of lactic acid bacteria including *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc,* and *Streptococcus* have been used as the main agents for the production of fermented dairy products. Alternatively, kefir grains can be applied as starter cultures for fermentation of dairy matrices, which results in a product called kefir (Fiorda et al. 2017). These grains contain a symbiotic culture of yeast species, lactic acid and acetic acid bacteria. Kefir is widely produced in Eastern Europe and Russia and is a well-known beverage in many countries such as Sweden, Germany, Brazil, and Iran (Alsayadi et al. 2013). The word kefir is commonly referred to a dairy beverage that is fermented by the white cauliflower-shaped kefir grains and by the action of the group of microorganisms they contain (Pidoux 1989). This fermented milk beverage, which is described to be a viscous pourable liquid, acidic, slightly alcoholic and foamy, is believed to contain a variety of functional compounds that are providing several health benefits including antioxidant, anti-hyperglycemic, anti-hyperlipidemic, anti-inflammatory, anti-ulcerogenic and antimicrobial activities (Alsayadi et al. 2013; Alsayadi et al. 2014b; Rodrigues et al. 2016; Rodrigues et al. 2005). A variety of milk sources such as cow, goat,

buffalo and sheep have been used for the production of kefir. Also, kefir is produced from other sources such as milk whey, cheese whey, soy whey, soy milk, rice milk, lactose-rich wastes or from carbohydrate solutions (Alsayadi et al. 2013).

A growing number of non-dairy consumers are showing an increasing interest in non-dairy probiotics (Corona et al. 2016). To provide kefir's beneficial health effects for vegan people, and consumers with lactose intolerance or an allergy to milk-derived products, brown sugar can be used to produce non-dairy kefir, called water kefir or sugary kefir (Marsh et al. 2013). Production of water kefir, which is a carbonated and slightly alcoholic drink with yellowish colour, is induced by translucent water kefir grains (Neve and Heller 2002; A. Gulitz et al. 2011). Initially, in 1889, Beijerinck, who has published the first scientific report on sugary kefir, has associated sugary kefir grains to the ginger beer plants brought by British soldiers from the Caucasus after the Crimean War. Whereas in 1899, a similar system originated from Mexico named "Tibi" was described by Lutz. Therefore, it seems probable that sugary kefir has multiple sources, and its true origins remain unknown. However, for the first time, the name of sugary kefir grains was assigned by Vayssier in France in 1978 to distinguish them from milk kefir grains. "Tibico", "Tibi,", "African bees", "California bees", "Japanese Beer Seeds", "Balm of Gilead", "ale nuts" and "Bebees" are also among other names used for sugary water kefir (Fiorda et al. 2017; Pidoux 1989). However, despite the fact that there is more than one origin for this grain, principally they all share a similar complex microbial community (Neve and Heller 2002).

3.2 Manufacturing of water kefir beverage (water kefir fermentation)

Figure 3.1 represents the process flowchart for the production of sugary kefir beverage using brown sugar as the substrate as reported by Fiorda et al. (2017). This process consists of a series of simple steps including pasteurization and subsequent cooling of the substrate, addition of kefir

grains followed by incubation for a period of 24 hours at 25-30 ^{0}C. At the end of the fermentation process, grains are separated, washed and dried for the following fermentation processes. In order to establish an industrialized process for sugary kefir production, the development of manufacturing scale equipment is required. In addition, different challenges in the fermentation process need to be addressed. These challenges include transportation of the starter microbiota, stabilizing their growth and ensuring stability and consistency of sugary kefir microbial consortium in different batches. Following the historical evolution of locally produced fermented products can indicate the desired direction for developing technology for the production of sugary kefir. This includes several steps such as using selected strains of sugary kefir grains as a starter culture, and consequently design and optimization of the fermentation vessel's functionality. The next step is to develop a well-controlled and industrialized process of sugary kefir production (Fiorda et al. 2017).

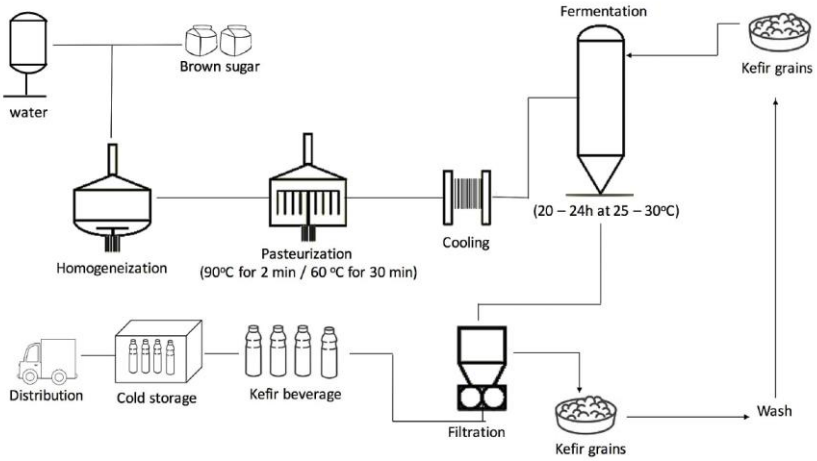


Figure 3.1 *Process flow diagram for the production of sugary kefir beverages by Fiorda et al. (2017).*

Fiorda et al. (2017) demonstrated that over a typical sugary kefir fermentation process, the major fermentation end products include ethanol, lactic acid and acetic acid. In addition, a variety of beneficial metabolites such as esters, mannitol, glycerol and other organic acids will be released. Acetic acid bacteria convert glucose to gluconic acid and fructose into acetic acid. On the other

hand, the presence of ethanol facilitates the growth of acetic acid bacteria and thereby the production of acetic acid. Acetic acid stimulates the yeast cells to convert sugar and produce ethanol via the glycolysis metabolic pathway (Figure 3.2). Both ethanol and acetic acid have been reported to have antimicrobial activity against pathogenic bacteria which can explain the antimicrobial activity of sugary kefir (Ayed et al. 2017).

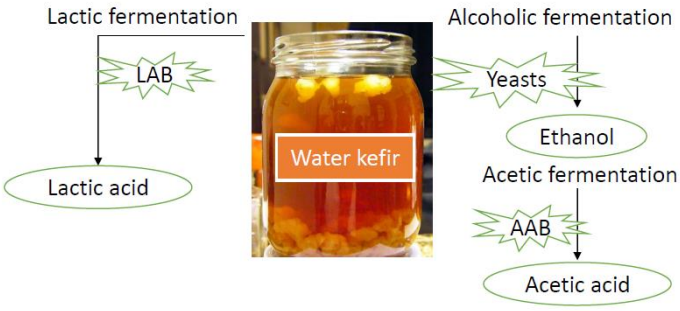


Figure 3.2 *Microbial metabolic activities during sugary kefir fermentation. LAB (Lactic Acid Bacteria); AAB (Acetic Acid Bacteria) adapted from Fiorda et al. (2017).*

During the kefir fermentation, other microbial interactions can exist which make the study of the microbial association of kefir fermentation complicated and therefore, the mechanism of symbiogenesis interaction of microorganisms of kefir grain is not well known. However, as a fermentation process, other forms of microbial interactions can occur during this process. As an example, during the sourdough and wine fermentation process, growth stimulation factors for LAB such as vitamins and nutrients will be released by yeast as a result of autolysis while in the wine fermentation process, the growth of yeasts is inhibited by some LAB metabolites. Similarly, these microbial interactions are likely to exist during the water kefir fermentation process (Fiorda et al. 2017).

3.3 Water kefir grains

Water kefir grains determine the characteristics of the resulting water kefir fermentation process (Martínez-Torres et al. 2017). These grains have a unique starter culture community, which is

known to be a stable association of lactic acid bacteria, yeasts and acetic acid bacteria, firmly embedded in a water-soluble matrix of dextran, a glucose polymer that can retain the water over the process of fermentation (Gulitz et al. 2011; Magalhães et al. 2010; Fiorda et al. 2017; Rodrigues et al. 2005; Neve and Heller 2002). In milk kefir, a glucogalactan in the kefir grains is synthetized mainly by *Lactobacillus kefiranofaciens*; whereas, in water kefir, a 1–6 glucose dextran is produced by *Lactobacillus hilgardii* (Martínez-Torres et al. 2017). Water kefir grains and milk kefir grains are comparable in terms of their structure, fermentation end products and associated microbiota. However, due to different sources of carbon provided by milk or sugar solutions for microbial species, their growth, frequency and composition vary. These differences will further affect the granulation of the grains as well as the final concentration of the by-products (Fiorda et al. 2017). Figure 3.3 by Fiorda et al. (2017) provides a comparison of sugary kefir microbiota versus kefir consortium. Hsieh et al. (2012) compared the fermentation of sugary kefir grains in brown sugar with different kinds of milk. According to their study, a significant change in the microbial composition of sugary kefir grains and their beverages occur when sugary kefir grains are fermented in different culture medium. They reported that sugary kefir grains may contain a variety of different microorganisms which will change based on an adaption to the source of carbon and energy available during the stage of granulation and growth. Similarly, Martínez-Torres et al. (2017) showed that despite fermentation of water kefir grains in milk, no growth of water kefir grains was detected, because *Lactobacillus hilgardii*, the key microorganism for the biosynthesis of the grains, does not produce gelling polysaccharides from consuming lactose.

3.4 Water kefir grains resistance

According to Schneedorf (2012), gelatinous grains can protect the yeasts and bacteria inside the polysaccharide matrix against chemical and physical stresses, such as UV exposure and

antibacterial administration, making them more resistant than free strains in solution. A high level of resistance of kefir microorganisms allows them a relative recovery of regular growth after exposure to stress conditions. Consequently, it helps them to remain active during long storage periods. Similarly, Koutinas et al. (2005) reported high resistance of kefir to contamination under semi-industrial scale conditions. Schneedorf (2012) also found that kefir grains have an adaptive potential for bacteriocins secretion to suppress the growth of *Saccharomyces aureus* when the filtered kefir samples were stimulated with *S. aureus* for 20 days. However, improper processing such as excessive washing of the kefir grains may alter the microbiota of the kefir grains and consequently affect the final product's quality. As an example, Cetinkaya and Elal Mus (2012) reported contamination of kefir with *Escherichia coli* and *S. aureus* due to poor hygienic conditions during the production process. To assure the safety of the fermented product, fermentation needs to be conducted under strictly controlled conditions.

3.5 Water kefir associated microbiota and metabolites

Interest in microbiology of the water kefir dates back to 1892 by Dr. Ward who was a researcher on probiotics. Over the last 30 years, a variety of studies have been conducted to describe the microbial diversity of sugar kefir and in particular compared to milk kefir, which is summarized in Table 3.1 and Figure 3.3, provided by Fiorda et al. (2017).

Table 3.1 *Microbial composition of sugary and milk kefir grains by Fiorda et al. (2017).*

Microbial group	Genus	Sugary kefir	Milk kefir	References
Bacteria	*Acetobacter*	*A. fabarium*, *A. orientalis*, *A. lovaniensis*.	*A. fabarium*, *A. orientalis*, *A. lovaniensis*, *Acetobacter aceti*, *A. rasens*.	Laureys et al. (2016); Gulitz et al. (2013); Gulitz et al. (2011); Garofalo et al. (2015); Magalhães et al. (2010).
	Lactobacillus	*L. brevis*, *L. buchneri*, *L. casei* subsp. *casei*, *L. casei* subsp. *rhamnosus*, *L. diolivorans*, *L. fermentum*, *L. harbinensis*, *L. hilgardii*, *L. hordeii*, *L. kefiranofaciens*, *L. kefiri*, *L. lactis*, *L. mali*, *L. nagelli*, *L. paracasei*, *L. parafarraginis*, *L. perolens*, *L. plantarum*, *L. satsumensis*.	*L. acidophilus*, *L. brevis*, *L. buchneri*, *L. casei* subsp. *pseudoplantarum*, *L. delbrueckii*, *L. fermentum*, *L. helveticus*, *L. kefiranofaciens*, *L. kefiri*, *L. otakiensis*, *L. paracasei*, *L. parabuchneri*, *L. plantarum*, *L. rhamnosus*, *L. sake*, *L. sunkii*.	Moinas et al. (1980); Pidoux (1989); Galli et al. (1995); Garrote et al. (2001); Simova et al. (2002); Witthuhn et al. (2005); Chen et al. (2008); Magalhães et al. (2010); Sabir et al. (2010); Gulitz et al. (2011); Kesmen and Kacmaz (2011); Gulitz et al. (2013); Garofalo et al. (2015); Zaniratiet al. (2015); Fiorda et al. (2016a); Laureys et al. (2016).
	Leuconostoc	*L. citreum*, *L. mesenteroides*.	*L. mesenteroides*.	Garrote et al. (2001); Magalhães et al. (2010); Sabir et al.(2010); Waldherr et al. (2010); Gulitz et al. (2011); Kesmen and Kacmaz (2011); Gulitz et al. (2013); Fiorda et al. (2016a) (2016a)
	Lactococcus	ND.	*L. cremoris*, *L. lactis*, *L. raffinolactis*.	Yuksekdag et al. (2004); Kesmen and Kacmaz (2011); Magalhães et al. (2011a); Sabir et al. (2010); Garofalo et al.(2015).
	Pediococcus	ND.	*P. acidilactici*, *P. dextrinicus*, *P. pentosaceus*.	Sabir et al. (2010).
	Streptococcus	ND.	*S. durans*, *S. thermophilu*.	Simova et al. (2002); Yuksekdag et al. (2004); Chen et al. (2008); Kesmen and Kacmaz (2011); Garofalo et al. (2015).
	Other species	*Lysinibacillus sphaericus*, *Oenococcus kitaharae*, *Bifidobacterium psychraerophilum*.	ND.	Gulitz et al. (2013); Fiorda et al. (2016a); Zanirati et al.(2015).
Yeast	*Candida*	ND.	*C. iconspicua*, *C. kefir*, *C. krusei*, *C. lambica*, *C. maris*, *C. humilis*.	Simova et al. (2002); Witthuhn et al. (2005); Garofalo et al.(2015).
	Saccharomyces	*S. cerevisiae*	*S. cerevisiae*, *S. turicensis*.	Simova et al. (2002); Wang et al. (2008); Magalhães et al.(2010); Puerari et al. (2012); Gulitz et al. (2013); Garofalo
	Pichia	*P. membranifaciens*, *P. kudriavzevii*	*P. fermentans*.	Wang et al. (2008); Fiorda et al. (2016a).
	Lanchancea	*L. fermentati*, *L. meyercii*.	*L. meyercii*.	Magalhães et al. (2011a); Magalhães et al. (2010); Gulitz et al. (2011); Fiorda et al. (2016a).
	Kluyveromyces	*K. lactis*, *K. marxianus*.	*K. lactis*.	Garrote et al. (2001); Wang et al. (2008); Magalhães et al. (2010); Magalhães et al. (2011a); Puerari et al. (2012).
	Kazachstania	*K. aerobia*, *K. unispora*.	*K. unispora*, *K. servazzii*, *K. aerobia*, *K. solicola*	Magalhães et al. (2010); Puerari et al. (2012); Garofalo et al.(2015).
	Hanseniaspora	*H. valbyensis*, *H. uvarum*.	*H. guillermondi*.	Gulitz et al. (2011); Garofalo et al. (2015); Fiorda et al.(2016a).
	Other species	*Zygotorulaspora florentina*, *Issatchenkia orientalis*, *Zygosaccharomyces fermentati*, *Dekkera bruxellensis*.	*Cryptococcus humicolus*, *Geotrichum candidium*, *Zygosaccharomyces fermentati*.	Witthuhn et al. (2005); Gulitz et al. (2011); Fiorda et al. (2016a), Laureys et al. (2016).

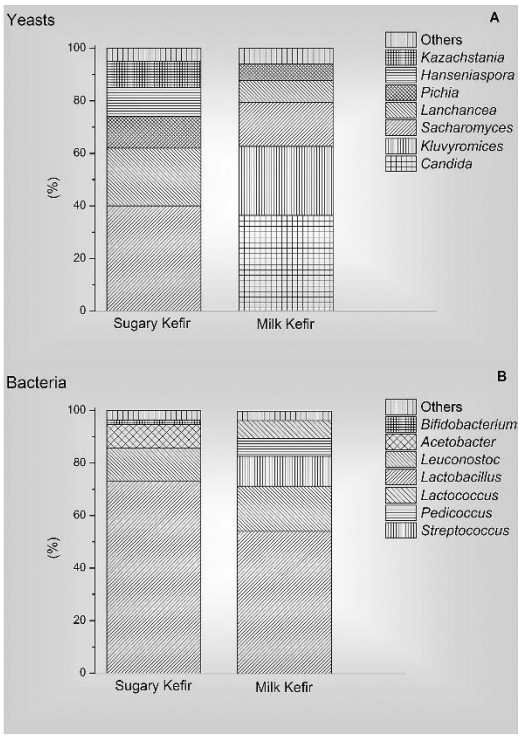


Figure 3.3 *Predominant microbial groups and their frequency in milk kefir versus water kefir reported by Fiorda et al. (2017).*

In most of the studies on sugary kefir, a stable consortium of mainly LAB, AAB, and yeasts is reported. For example, Franzetti et al. (1998) found the yeasts and Lactic acid bacteria as the dominant microbial forms in sugar kefir drinks (and the latter primarily responsible for the synthesis of the sugar kefir grains). They also reported a negligible presence of acetic acid bacteria. According to their study, depending on a variety of factors in the fermentation process including the preparation conditions and metabolic activity of the strains, the quantity and compositions vary. However, they reported lactic acid and ethanol as substances that are produced in large quantities as a result of the metabolite activity of the bacteria and the yeasts. Recent studies have also applied a variety of molecular techniques, such as ARDRA, metagenomic and DGGE for understanding the microbiology of sugary kefir fermentation which has led to key advances in this area (Fiorda et al. 2017). Magalhães et al. (2010) evaluated the microbial composition of Brazilian sugary kefir by using phenotypic and genotypic methods and identified a total of 129 yeasts and 289 bacteria.

They reported *Lactobacillus paracasei* as the major isolated bacteria (23.8%). Subsequently, *Acetobacter lovaniensis, Lactobacillus parabuchneri, Lactobacillus kefir* and *Lactococcus lactis* were identified as main components accounting for (16.31%), (11.71%), (10.03%) and (10.03%) of the bacteria isolated respectively. In addition, they identified *Saccharomyces cerevisiae* (54.26%) and *Kluyveromyces lactis* (20.15%) as the most predominant yeasts in this beverage. Marsh et al. (2013) performed sequencing-based analysis of the bacterial population of water kefir beverages obtained from Canada, the UK and the United States. They revealed that *Zymomonas*, an ethanol-producing bacterium, is the dominant bacteria fraction of all samples of water kefir. In addition to Lactic acid bacteria and acetic acid bacteria, they reported that the fungal component of the water kefir grains is comprised of the genera *Lachancea, Dekkera, Torulaspora, Saccharomyces, Zygosaccharomyces,* and *Hanseniaspora*.

Pidoux (1989) used scanning electron microscopy for observing sugary kefir grain and indicated that on the periphery of the grains, bacteria are covered by filamentous yeasts. In addition, they attributed the strain of *Lactobacillus hilgardii* as the key microorganism for the biosynthesis of the grains by producing a gelling polysaccharide. *L. hilgardii,* which has almost identical physiological characteristics to *L. brevis,* has shown gel-forming properties in pure culture. It was suggested that *L. mesenteroides* ssp. *dextranicum* and *L. casei* ssp. *casei* also produce polysaccharides in sucrose solution, which with the dextrans of L. *hilgardii*, these polysaccharides could be condensed to help to retain a great number of associated bacteria inside the grains (Pidoux 1989). Similarly, Gulitz et al. (2011) and Laureys and Vuyst (2014a) have indicated the main role of *L. hilgardii* in the production of polysaccharides that form the water kefir grains.

Regarding the water kefir fermentation metabolites, Laureys and Vuyst (2014a) reported that the major fraction of fermentation metabolites are produced within the first 72 hours of the process,

in which pH is declined from 4.26 to 3.45. These metabolites include mainly ethanol and lactic acid and low concentrations of glycerol, acetic acid, and mannitol. They indicated the prevalence of a variety of volatile aroma compounds such as, ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, that correlate with the end product aroma. As previously mentioned, the yeast-bacteria community in sugary kefir share their metabolites as energy sources and growth simulation factors to survive due to their symbiotic relationships (Fiorda et al. 2017). In this regard, Martínez-Torres et al. (2017) have proposed an outline of the carbon flow from sucrose to metabolite products during 96 h of water kefir fermentation (Figure 3.4). They identified the most metabolically active species during water kefir fermentation. Accordingly, they proposed *Lactobacillus hilgardii*, *Saccharomyces cerevisiae* and *Acetobacter tropicalis* as a minimal and efficient consortium for water kefir production and as main contributors of Lactic acid, alcohol and acetic acid production respectively. Identification of the simplified consortium could be beneficial for the investigation of the microbial interaction and expression patterns for the development of this beverage. Also, they reported increasing alcohol content at the initial stage and accumulation of lactic acid and acetic acid only after 24 hours. In addition, they reported a continuation of water kefir fermentation for 8 days resulting in the entire oxidation of ethanol to acetic acid, accumulation of acetic acid and production of water kefir vinegar as a result. Therefore, optimization of fermentation time is crucially important with respect to the type of desired beverage.

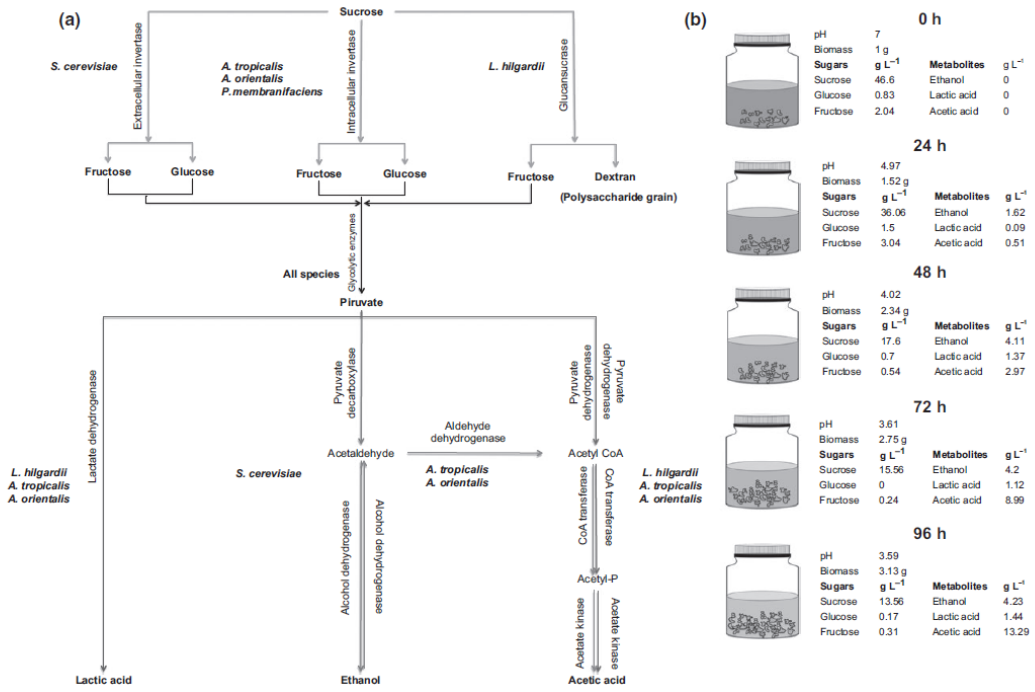


Figure 3.4 *(a) Hypothetical scheme of carbon during a water kefir fermentation, (b) The substrate consumption and metabolites production over a water kefir fermentation progress from 0 to 96 h reported by Martínez-Torres et al. (2017).*

3.6 Development of Russian olive water kefir

Due to the associated health benefits of water kefir and its specific sensory properties, there is an increasing trend in consumption of this product specifically in North America, Asia, Europe and Latin America. Among them, Japan, Malaysia, Thailand, Brazil, Argentina, Peru, Chile, Mexico, United States of America, Canada, Russia, France, Turkey, Netherlands, the United Kingdom and Sweden are the countries with the highest consumption of water kefir beverage as a fermented beverage (Fiorda et al. 2017; Anar 2000). However, studies on kefir fermentation using alternative matrices are limited in comparison with the use of dairy matrices. On the other hand, fruit juices are considered as an appropriate medium for microbial growth due to their suitable content in water, minerals, vitamins, sugar and proteins. In addition, consumption of fruits and vegetables is strongly recommended by many governments for reducing the risk of certain diseases and over the past years, diverse methods of fruit processing have been studied for developing new products and

introducing them to the market to widen the choice for their consumption. Fermentation with probiotics is one of these processes, which can provide numerous health benefits to consumers. Therefore, the development of non-dairy kefir using fruits as the fermentation substrate, which can deliver similar health benefits to kefir, is advantageous. Different fruits and vegetables have been used as non-dairy sources for producing water kefir (Fiorda et al. 2016b; Puerari et al. 2012; Corona et al. 2016; Randazzo et al. 2016; Cui et al. 2013). However, there is no reported study on the development of a water-kefir beverage based on Russian olive, which has high antioxidant activity and various therapeutic properties. Application of Russian olive fruit as an alternative non-dairy substrate for developing water kefir as a probiotic-containing functional drink with potential health benefits provides not only a new way of Russian olive consumption, but also creates diversification in developing a new functional beverage for people with certain limitations in consuming dairy probiotic-containing products.

3.6.1 Potential prebiotic activity of Russian olive for developing Russian olive water kefir

There is an increasing interest in prebiotics, which can be used as food ingredients or as a functional compound in a synbiotic product. A synbiotic is a mixture of probiotics and prebiotics which selectively promotes metabolism, improves survival or stimulates the growth of health-promoting bacteria in the gastrointestinal tract and thus beneficially affects the host. Prebiotics are mostly complex carbohydrates ranging from small sugar alcohols to large polysaccharides, and fructans, inulin and fiber gums are some examples. Prebiotics share common characteristics including not being hydrolyzed and absorbed in the intestine and changing the qualitative composition of intestinal microflora, by acting as a selective substrate for probiotics and promoting the growth of beneficial bacteria (Bevilacqua et al. 2016). Methods for *in vitro* assessment of the prebiotic activity of substrates range from simple batch cultures to multiple-stage continuous

cultures (Vulevic et al. 2004). Palframan et al. (2003) developed a prebiotic index equation, a simple and quick quantitative method to measure prebiotic activity by assessing changes in the microbial population. Further, Vulevic et al. (2004) developed an *in vitro* quantitative assessment of prebiotic activity, which also includes fermentation end products such as short-chain fatty acids in the equation and termed it a measure of prebiotic effect (MPE). In this study, the prebiotic activity of Russian olive was measured to evaluate its capability to support the growth of probiotics, relative to non-probiotics and relative to growth on glucose as a non-prebiotic substrate. Therefore, a quantitative score was established to describe the potential prebiotic activity of Russian olive to support the selective growth of *Lactobacillus acidophilus* (NRRL No. B-4495) and *Bifidobacterium animalis spp. Lactis* (NRRL No. B-41405) compared to an enteric strain of *Escherichia coli* (ATCC No.10798) following a methodology described by Huebner et al. (2007), Mandalari et al. (2008) and Rastall (2010). Russian olive fruit powder was first subjected to a combined model of the gastrointestinal tract, including *in vitro* oral, gastric and intestinal digestion. The resulting fractions were subsequently used as substrates for two probiotic strains and one enteric strain as an alternative to the colonic model, in which fecal batch fermentation is performed. Subsequently, their influence on the metabolic activity of microorganisms of interest was assessed (Mandalari et al. 2008; Huebner et al. 2007; Rastall 2010).

3.6.1.1 Digestion stage

To simulate the gastrointestinal digestion process of Russian olive, the standardized in vitro digestion method developed by Minekus et al. (2014), was employed as follows:

3.6.1.1.1 Oral phase

Initially, to create a paste-like consistency, SSF (Simulated Salivary Fluid) electrolyte stock solution was mixed with Russian olive. Then, human salivary α-amylase was added to achieve a

final concentration of 75U/ml in the mixture followed by adding $CaCl_2$ (to achieve a concentration of 0.75 mM) and the required volume of water for dilution. The final ratio of food to SSF in the mixture was targeted at 50: 50 (w/v) and digestion was carried out for 2 minutes at 37°C.

3.6.1.1.2 Gastric phase

After the oral phase, the pastes were exposed to the gastric phase and SGF (Simulated Gastric Fluid) stock electrolyte solution was added to the pastes at the ratio of 50: 50 (v/v). Porcine pepsin and $CaCl_2$ were added to achieve a concentration of 2000 U/mL and 0.075 mM, respectively in the final digestion mixture followed by adding the required amount of water. pH was adjusted to 3.0 using HCl and digestion was carried out for 2 hours at 37°C.

3.6.1.1.3 Intestinal digestion

Subsequently, the gastric chyme was mixed with SIF (Simulated Intestinal Fluid) electrolyte stock solution for intestinal digestion. A final ratio of gastric chyme to SIF of 50: 50 (v/v) in the mixture was targeted after additions of enzymes (pancreatin from the porcine pancreas), bile, $CaCl_2$ and water. (1 M NaOH) was used to neutralize the mixture to pH 7.0 and intestinal digestion was carried out for 2 hours.

3.6.1.2 In vitro fermentation stage

Studies show that prebiotic activity scores are both strain and substrate-specific. However, clinical studies have proven the beneficial gastrointestinal effects of *Lactobacillus acidophilus*, in combination with *Bifidobacterium animalis spp. Lactis* (De Vrese et al. 2011; Chatterjee et al. 2013). Therefore, these strains were used in evaluating the tests for the prebiotic activity of Russian olive. The prebiotic activity assay was measured based on the changes in population after 24 h or 48 h of growth of the probiotic strains on 1% w/v of Russian olive fruit, or glucose as control and

inulin as a standard prebiotic, relative to the change in the population of an enteric strain, grown under identical conditions. Each test was completed in triplicate and the number of viable colony forming units (CFU)/mL before (P0 and E0) and after incubation for 24 h or 48 h (P24/48 and E24/48) on 1 % (w/v) Russian olive, glucose, and on inulin as the prebiotic standard reference (Px and Ex), was measured following a methodology by (Anprung and Sangthawan 2012). Finally, equation 1 was used to calculate the prebiotic activity score of the samples.

$$\text{Prebiotic activity} = [(\log P_X\, 24 - \log P_X\, 0)/(\log P_G\, 24 - \log P_G\, 0)] - [(\log E_x\, 24 - \log E_x\, 0)/(\log E_G\, 24 - \text{Log}\, E_G\, 0)] \quad \text{Eq.1}$$

3.6.1.3 Results

Neither the Russian olive flour nor inulin showed a promising prebiotic activity score when *Bifidobacterium animalis spp. Lactis* was used as a probiotic strain. However, when *Lactobacillus acidophilus* was tested as the probiotic strain, both Russian olive and inulin showed a considerable prebiotic activity score. The reason is that different probiotic strains have diverse enzymes specific for the digestion of prebiotics in foods (Thuaytong and Anprung 2011). These results are consistent with other studies, which indicate the prebiotic activity scores are dependent on the probiotic bacterial strain tested and the type of prebiotic (Huebner et al. 2007). Results on the prebiotic activity of Russian olive are demonstrated in Figure 3.5.

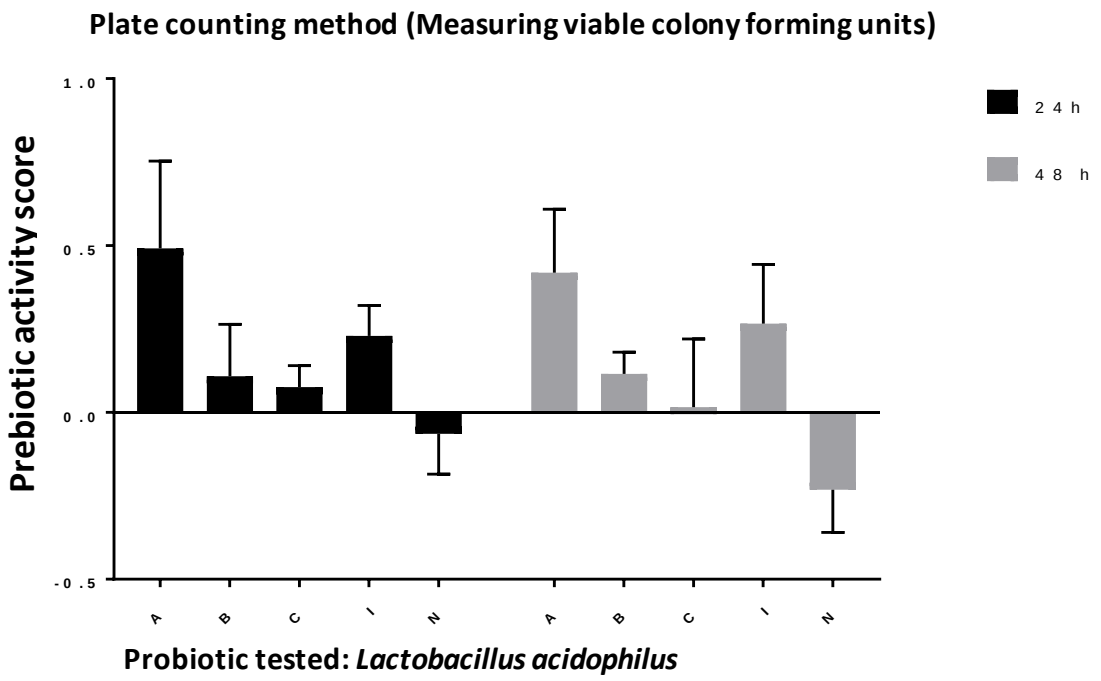


Figure 3.5 *Probiotic activity score by measuring viable colony-forming units. A) Russian olive exocarp, mesocarp and seed, B) Russian olive exocarp and mesocarp, C) Russian olive mesocarp, I) Inulin, N) Control.*

The prebiotic activity scores showed a similar pattern at both 24 h and 48 h. Considering the prebiotic activity score from the colony-forming units (CFU), the highest score was obtained for *Lactobacillus acidophilus* grown on Russian olive consisting of seed, exocarp and mesocarp (sample A), and the lowest score was obtained when it was grown on Russian olive mesocarp (sample C). This study provides a supporting basis for the hypothesis of using Russian olive as a prebiotic in the development of synbiotic products such as Russian olive water kefir, and considering the results, sample B (Russian olive exocarp and mesocarp) will be used in future experiments for the development of Russian olive kefir.

3.6.2 Preparation steps, design and optimization of the water kefir fermentation process

3.6.2.1 Selection of initial inoculum (water kefir grains versus selected microbiota)

Amorphous and translucent water kefir grains, in which related microbiota are embedded, are used as the starter culture to produce water kefir beverages. Different studies have used different sources

of water kefir grains as initial microbiota. Particular microbiota in these grains varies based on the source of the grains and the procedure used for producing the beverage. However, certain species of acetic acid bacteria and lactic acid bacteria, as well as *Saccharomyces cerevisiae* yeasts, have been frequently isolated (Martínez-Torres et al. 2017). The microbial diversity of kefir grains is the prime reason for the wide variation in the quality of the kefir products. According to Sarkar (2008), the application of a suitable combination of yeasts, LAB and AAB rather than kefir grains would result in more uniformity in the quality of the kefir product. For that purpose, Laureys and Vuyst (2014b) analyzed the species diversity in water kefir grains and water kefir beverage and they indicated *Lactobacillus casei/paracasei, Lactobacillus harbinensis, Lactobacillus hilgardii*, *Bifidobacterium psychraerophilum/crudilactis, Saccharomyces cerevisiae*, and *Dekkera bruxellensis* as the most important microbial species in both water kefir beverages and grains.

Therefore, some studies have used a previously identified consortium of microorganisms mainly containing LAB and yeasts as an initial starter culture for the water kefir fermentation process. For instance, Stadie et al. (2013) prepared water kefir using the predominant water kefir microorganisms, isolated and characterized by Gulitz et al. (2011). These selected microorganisms included *Lactobacillus hordei, Lactobacillus nagelii* as predominant bacteria, and *Zygotorulaspora florentina* and *Saccharomyces cerevisiae* as the main representative of yeasts. Similarly, Randazzo et al. (2016) used a commercial water kefir microbial preparation containing LAB (*Lactobacillus, Lactococcus* and *Leuconostoc*) and *Saccharomyces spp*. Therefore, current studies on the predominant microorganisms of water kefir, provide an alternative option of using commercial starter culture isolated from water kefir fermentation, in comparison with the water kefir grains used in our study. Improved uniformity in the quality of the produced RWK product in our study might be achieved by replacing water kefir grains with a selected and controlled starter

culture (or cultures). However, using water kefir grains is advantageous for industrial applications due to the water kefir grain's resistance against extreme conditions, as previously discussed.

3.6.2.2 Fermentation variables

Various factors are affecting the success of a fermentation process. In the production of water kefir, the activity of the microbial population is affected by a variety of factors including, the quality and the ratio of the water kefir grains to the substrate, incubation time and temperature, sanitation conditions and finally storage environments (Randazzo et al. 2016). These factors will be set as independent variables in designing the fermentation process in this study and will be discussed in the following sections.

3.6.2.3 Substrate concentration and its ratio to the water kefir grain

In different studies, where microbiology of the water kefir has been evaluated, usually, water kefir is prepared by kefir grains' inoculation of a sugar solution prepared with mineral drinking water. However, in studies, where fruits and vegetables are used as a substrate for the production of water kefir, the sugar can be partially or completely omitted from the desired substrate (Koh et al. 2017; Randazzo et al. 2016). Most commonly, the traditional and scientific production of water kefir use 3-5 % as the ratio of kefir grains to the substrate solution (Fiorda et al. 2016a; Koh et al. 2017; Magalhães et al. 2010; Randazzo et al. 2016). However, in studies where sugar is replaced with other substrates, the substrate concentration can vary significantly. For instance, Fiorda (2016) inoculated kefir grains into honey solutions with concentrations between 28 and 42% w/v in the production of honey kefir. Therefore, based on the literature review, the initial concentrations of the Russian olive will be evaluated in this study to find an optimized substrate concentration, which is specific to the production of Russian olive water kefir.

3.6.2.4 Incubation Time and Temperature

Recommended incubation temperature-time combinations for kefir production vary throughout the literature (Sarkar 2008) as follows: 20°C /20 h by H. Chen et al. (2006), 20°C /48 h by Abraham and De Antoni (1999), 22°C /11 h by Li et al. (2004), 24-27°C /20 h by Klupsch (1985), 22-25°C /8-12 h by Koroleva (1988) and 20°C /24 h by Hsieh et al. (2012). Also, a two-stage fermentation process, which is a combination of 28°C for 5 h and 20°C for 16 h, has been suggested for kefir production by Simova et al. (2006). Regarding the water kefir fermentation process, commonly used incubation time and temperatures are recommended as 25°C /24 h by Magalhães et al. (2010) and Marsh et al. (2013) and 25-30 °C /24 h by Alsayadi et al. (2013). In the most relevant study, Koh et al. (2017) reported 24 h at 32 °C as the optimized conditions for the fermentation of pumpkin water kefir with good overall acceptability and high viability of water kefir microorganisms. Therefore, to optimize the fermentation process for Russian olive water kefir in our study, based on the literature, incubation time and temperature will be investigated in the range of 12-48 hours and 20-32 °C respectively.

3.7 Importance of drying process for Russian olive water kefir

To respond to the increasing consumer interest in the consumption of probiotic products with health benefits such as kefir (Atalar and Dervisoglu 2015), it is important to increase the shelf life and stability of Russian olive water kefir for facilitating its commercial production. This is possible through dehydration processes for processing of Russian olive water kefir into a powder and limiting changes in microbial metabolites that affect the water kefir sensory properties such as taste and flavour during the storage period (Nale et al. 2017). Also, microencapsulation of probiotics by different methods such as spray drying can improve their viability during possible severe food matrices conditions (such as the presence of natural antimicrobial compounds, high

salt or sugars concentrations, low pH and low water activity) during the storage period (Barbosa and Teixeira 2017). Therefore, processing of the Russian olive water kefir into a quality powder is crucial to increase stability and reduce storage and transportation associated costs of this product for improving its commercialization potential (Nale et al. 2017; Atalar and Dervisoglu 2015). The application of different drying techniques for the preservation of microorganisms has a long history, among which, spray drying and freeze drying are more frequent processes used for the preparation of dried probiotic cultures (Reddy 2007). Considering the viability of bacteria, sensory and nutritional properties of produced powders, freeze drying is known as the best drying method; however, its application is limited due to the high cost of this technology. On the other hand, due to several advantages of spray drying over freeze drying such as short drying time, cost-effectiveness, and high rate of moisture removal, it is the most commonly used technology in the powder industry (Atalar and Dervisoglu 2015).

Regarding kefir-related studies, Atalar and Dervisoglu (2015) and Golowczyc et al. (2010) reported that spray drying is a suitable method to preserve micro-organisms isolated from kefir grains. However, as a thermal process, spray drying affects the viability of kefir microorganisms by many factors such as airflow direction, inlet and outlet temperature and atomization type (Atalar and Dervisoglu 2015). This process comprises four important steps namely atomization, contact of sprayed particles and hot air, evaporation of water droplets, and separation of dried product and humid air. Survival of probiotics are affected by various factors before spray drying (strain characteristics, growth medium, growth phase and sub-lethal stress exposure), during spray drying (drying medium and drying parameters) and finally after spray drying such as packaging and storage conditions (Barbosa and Teixeira 2017). In this study, the focus will be on factors affecting the bioactive properties of Russian olive water kefir (survival of water kefir microorganisms and

retention of bioactive compounds) during the spray drying process including drying medium (application of drying aids) and drying parameters (in chapter VI).

3.7.1 Importance of optimization of the drying process for Russian olive water kefir

During the spray drying process of water kefir, it is important to improve the survival rate of the beneficial kefir microorganisms and powder properties using optimization methodologies for determining the best production conditions. In this study, response surface methodology (RSM), which is an optimization method used for the improvement of existing products or formulation of new products, will be used to optimize the spray drying process conditions for the production of Russian olive water kefir powder as a novel water kefir powder. Among drying parameters, outlet air temperature is considered as the main parameter affecting the cell viability of various probiotics (Barbosa and Teixeira 2017). In addition, results from previous studies, including a study by Golowczyc et al. (2010) on probiotic strains isolated from kefir, have confirmed that this parameter is the most important drying parameter affecting the survival of spray-dried cultures. Outlet air temperature depends on other parameters including feed flow rate and inlet air temperature. Therefore, inlet air temperature and feed flow rate will be considered as two important independent variables in our study. Additionally, the application of drying aids to improve the survival rate of microorganisms will be considered as the third independent variable in our study.

3.7.2 Drying aids in spray drying of Russian olive water kefir

In spray drying, the residence time is short and the temperature reached by the particles is low, which makes this technology to be considered as a moderate drying technique. However, many studies have reported decreased survival of probiotics during drying and subsequent storage period (Barbosa and Teixeira 2017). Subsequently, solutions on improving the survival of probiotics during this drying technique are broadly investigated by many studies, and different protective

carriers are added to the drying medium for improving the survival of probiotics over spray drying. In addition, like with other fruit juices, Russian olive juice contains a high concentration of low-molecular-weight sugars, such as glucose, fructose and organic acids (Farzaei et al. 2015), which makes it very sticky during spray drying and consequently making its drying impossible in its pure state. In addition to the drying challenges, caking of the produced powder, which is associated with low glass transition temperature, can also occur during the storage period of the powder. Therefore, the application of drying aids such as maltodextrins or gum Arabic with high molecular weights is required to increase their glass transition temperature and to reduce the thermal plasticity of the Russian olive fruit juice (Barbosa and Teixeira 2017). These carriers are also known to contribute towards higher survival rates of microorganisms during spray drying. In addition, due to the prebiotic properties of these molecules, their application in the beverage formulation also contributes to the production of a synbiotic functional product (Barbosa and Teixeira 2017). Therefore, the application of drying aids, and in particular these two carriers is widely investigated. Considering the application of drying aids in kefir related studies, sensory evaluation of kefir by Nale et al. (2017) revealed that although the moisture content of kefir powder with no encapsulants was in the appropriate range, it had insufficient product yield and solubility as an instant powder, and had high hygroscopicity, which negatively affected its storage stability and flowability. They reported that the addition of encapsulants to the kefir powder before spray drying improved the product yield and solubility to an acceptable level. Also, Golowczyc et al. (2011) evaluated the effect of different protectants on the survival of the probiotic Lactobacillus kefir strains during spray drying and subsequent storage and reported a significant improvement in their viability. However, the concentration of drying aid needs to be optimized. For example, a study on spray drying of a mixed juice by Mestry et al. (2011) showed that to produce a free-

flowing powder and non-caking product, the addition of 5% maltodextrin is not enough and its concentration needs to be in the range of 10-15%. whereas, some studies on spray drying of fruit juices have reported that the use of 10% or greater concentration of maltodextrin has resulted in the loss of the attractive colour of the powders. In addition, a high solid content creates larger particles which results in longer drying time, longer exposure of the probiotics to high temperature, consequently decreasing their survival (Barbosa and Teixeira 2017). Therefore, concluding from similar studies, drying aid concentration around 10 percent seems to be appropriate; however, this study will determine the optimum concentration of drying aids in the successful spray drying of Russian olive water kefir beverage.

To the best of our knowledge, there are no studies on the characterization and optimization of the drying process for Russian olive water kefir and characterization of the produced powder in the literature. Therefore, in this study, the drying process of Russian olive water kefir into powder will be carried out using a spray dryer and the spray drying process parameters such as inlet temperature, feed rate and drying aid concentration will be optimized based on product yield, microbial viability, antioxidant activity and colour of the produced water kefir powder.

3.8 Importance of monitoring storage stability of Russian olive water kefir

In addition to attempts for maximizing the survival of probiotics during the probiotic food production and drying process, it is of crucial importance to maintain the viability of probiotics during packaging and storage. In order to confer the probiotic health benefits, fermented products require to deliver a minimum count of 10^6 CFU/g to the consumers. Different studies in various probiotic-containing products have reported that the concentration of probiotics drops significantly during storage (Baú et al. 2014). Under storage conditions, relative humidity and temperature are the most important factors affecting the viability of probiotics. Many studies on spray-dried

bacteria have associated lower storage temperature with higher survival of probiotics, while the higher temperature is attributed to loss of cell viability due to protein denaturation and lipid oxidation. High humidity during storage affects the glass transition temperature of the powder, induces caking in dried powder and subsequently causes loss of viability of the probiotics. This can be improved by the addition of protective carriers which help dried powders to keep a glassy state and to improve probiotics viability during the period of storage (Barbosa and Teixeira 2017). In addition to mentioned factors in storage conditions, other studies have reported the significant effect of the storage time on the survival of microorganisms and physicochemical properties of kefir powder (Nale et al. 2017; Golowczyc et al. 2010). Different studies have evaluated the ideal storage conditions for kefir considering the stability of probiotics and retention of favourable metabolites for this product during storage (Sarkar 2008). However, despite the close association between milk kefir and water kefir, their ideal storage conditions and product stability within storage are expected to be significantly different. Therefore, ensuring the stability of water kefir microorganisms in Russian olive water kefir during the storage period is important and challenging and will be investigated in this study (in chapter VII).

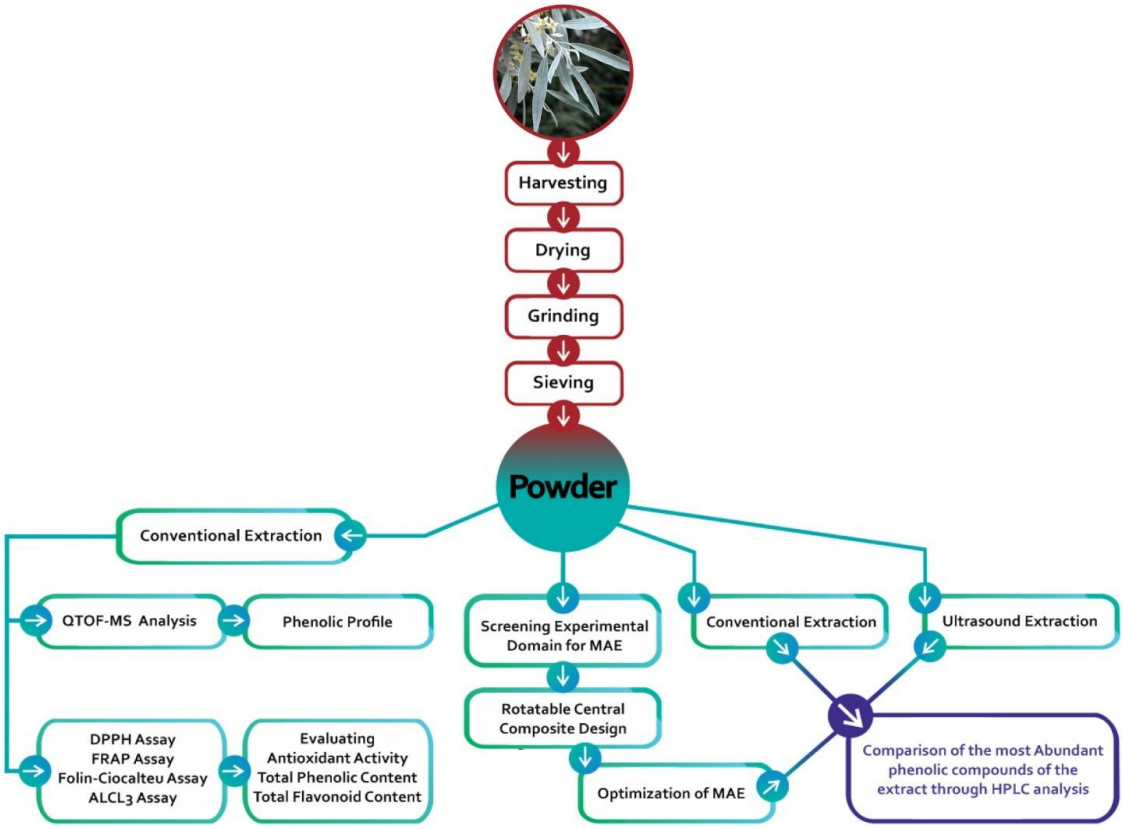
CONNECTING TEXT

It is clear from the literature review and the results from our preliminary tests (Chapter II) that Russian olive leaves and flowers have significant antioxidant activity, and the extraction of these compounds can have potential applications in the functional food sector. Extraction of antioxidant compounds from plants depends on a variety of factors, and optimization of the extraction method for the complete extraction of these compounds is a critical step. However, there is no study on the optimization of microwave-assisted extraction (MAE) of bioactive compounds with antioxidant properties from Russian olive leaves and flowers. MAE is one of the most employed advanced extraction methods, which, compared to conventional systems, is a less time-consuming process and requires a lower amount of solvent (Vieira et al. 2017). Therefore, Chapter IV of this study focuses on obtaining antioxidant-rich extracts from Russian olive leaves and flowers. First, efforts were made to screen the significant factors affecting the efficiency of MAE. Second, the effects of the extraction variables on the quality of the produced Russian olive extracts were evaluated. Finally, optimized microwave-assisted extraction parameters to yield high-quality extracts from Russian olive leaves and flowers were proposed.

CHAPTER IV

MICROWAVE-ASSISTED EXTRACTION (MAE) OF ANTIOXIDANT COMPOUNDS FROM RUSSIAN OLIVE LEAVES AND FLOWERS

Graphical Abstract



4.1 Abstract

Phytochemicals with antioxidant activities are of great interest due to their beneficial health effects and their application in the food industry. Therefore, our preliminary studies produced extracts from Russian olive leaves and flowers and determined their potential antioxidant activity and their

most abundant phenolic compounds were detected. The maximum retention of these antioxidant compounds during microwave-assisted extraction (MAE) requires optimized process conditions. Therefore, two-level fractional factorial designs in preliminary tests were used for assessing the magnitude of microwave extraction factors' effects on the extraction of bioactive compounds from Russian olive samples and the experimental domains were determined. Subsequently, Response surface methodology (RSM) was conducted to determine the optimized MAE process variables including citric acid's molarity (X1: 1-5 M), extraction temperature (X2: 60–110 °C), ethanol concentration (X3: 0–100%) and solid to solvent ratio (X4: 5–15) for maximum recovery of total phenolic content, total flavonoid content and total antioxidant compounds in the extracts. Based on the results, the optimal extraction conditions were as follows: solid to solvent ratio of 7.5 (w/v), citric acid molarity of 2 M, ethanol concentration of 59.8 and 66.4%, and temperature of 97.4 and 97.5 °C for Russian olive leaves and flowers, respectively. In addition, the influence of extraction parameters on the responses was modeled using a second-order regression equation and a good agreement between the predicted values and the experimentally obtained values was indicated. Finally, among phenolic compounds which were previously identified through Quadrupole Time of Flight Mass Spectrometer (QTOF-MS) analysis, three abundant flavonoids of the extracts with therapeutic properties including isorhamnetin, luteolin and rutin contained in the optimized extract from MAE were compared with that of ultrasound-assisted extraction and conventional extraction using HPLC analysis. Results confirmed MAE as an efficient method for extraction of bioactive phytochemicals from Russian olive.

4.2 Introduction

Phenolic compounds such as flavonoids, which are considered a key source of natural antioxidants for humans, are highly unstable and can be remarkedly lost during different stages of food

processing. Therefore, supplementation of phenolic-rich compounds as a food ingredient is beneficial for retaining the required health beneficial amount of these compounds in the diet, which requires an efficient extraction process for their preparation as a food ingredient (Srivastava 2006; Rusconi and Conti 2010; Routray and Orsat 2012). The addition of herbal extracts in foods can enrich the chemical composition of food products by adding plant-derived metabolites such as polyphenols with health-promoting effects such as against oxidative stress and cardiovascular diseases (Ciulu et al. 2017). In North America, the *Elaeagnus angustifolia* tree, known as the Russian-olive or oleaster has been widely cultivated for horticultural and ornamental purposes. Extracts from leaves and flowers of Russian olive are proved by different studies to be rich in phytochemicals with antioxidant properties (Saboonchian et al. 2014; Bucur et al. 2008; Bendaikha et al. 2014; Okmen and Turkcan 2014). This along with our preliminary test results provide scientific validation for their potential for being used as an ingredient with effective and safe antioxidant properties in functional food products. Moreover, Russian olive is among the medicinal plants with various known therapeutic properties, which are believed to be vastly due to the antioxidant activity of this plant (Farzaei et al. 2015). The extraction of antioxidant compounds from plants depends on a variety of factors. These factors include the plant matrix and chemical properties of phenolic compounds such as the number of hydroxyl groups and aromatic rings, their molecular structure, concentration and polarity. In addition, complexes of phenolics with other sample elements such as proteins and carbohydrates prevent the complete extraction of some of the phenolic compounds (Khoddami et al. 2013). Therefore, obtaining phenolic-rich extracts from Russian olive leaves requires an efficient extraction process for their preparation as a food ingredient.

Different methods for phenolic compounds extraction exist including conventional methods such as solvent extraction and advanced methods including ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) (Orsat and Routray 2018). MAE is one of the most employed extraction methods since, when compared to conventional extraction, requires a lower amount of solvent and is less time-consuming (Vieira et al. 2017). Factors affecting the efficiency of MAE include the level and application time of the microwave power, the consequently achieved temperature during the process and dielectric properties of the applied solvent and the biomatrix (Orsat and Routray 2018). To obtain antioxidant-rich extracts from Russian olive leaves and flowers, maximum retention of these phytochemicals during extraction requires optimization of MAE parameter conditions. Despite the importance of the antioxidant activity of Russian olive leaves and flowers, no study has been dedicated to microwave-assisted extraction of the antioxidant-rich compounds in leaves and flowers of this plant and optimization of this process for the production of maximum yield of these compounds. Therefore, the objective of this study is to develop the microwave-assisted extraction of Russian olive leaves and flowers. We also aim to investigate the effect of different parameters of the microwave-assisted extraction process on total antioxidant activity, total phenolic content (TPC) and the total flavonoid content (TFC) of Russian olive flowers and leaves. Subsequently, response surface methodology (RSM) will be conducted to determine the optimized MAE process variables for maximum recovery of TPC, TFC and antioxidant capacity in the extracts. Finally, the extract from optimized MAE conditions will be compared with extracts prepared by ultrasound-assisted extraction and conventional solvent extraction using high-performance liquid chromatography (HPLC).

It is worth mentioning that to confirm the antioxidant activity of this plant as reported by other studies (Saboonchian et al. 2014; Bucur et al. 2008; Bendaikha et al. 2014; Okmen and Turkcan

2014), preliminary studies including conventional extraction of samples using 50, 60 and 70 % of ethanol and methanol were conducted and evaluated for the *in vitro* antioxidant activity of Russian olive leaves and flowers through 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing ability of plasma (FRAP) assays. We also measured the TFC, TPC of the samples. Results confirmed the significant antioxidant activity of Russian olive. Subsequently, extraction using 70 % ethanol and methanol was used to prepare the extract for screening the phenolic profile of leaves and flowers of Russian olive using Quadrupole Time of Flight Mass Spectrometer analysis (QTOF-MS), which later was used for HPLC analysis in this study.

4.3 Materials and Methods

4.3.1 Experimental design

The design of experiments in this study was performed using the software Design-Expert (version 10.0.7.0, Stat-Ease Inc., Minneapolis, USA). Experiments were developed based on a Central Composite Rotatable Design (CCRD) with four factors and including the central point with five replicates. The independent variables were selected as extraction temperature (60-110 °C), solid to solvent ratio (5-15 w/v), ethanol concentration (0-100) and citric acid concentration (1-5 M), each at five coded levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) for a total of sixty experiments conducted for optimization of the MAE process of Russian olive leaves (Table 4.1) and flowers (Table 4.2). The responses included extraction yield, antioxidant activity (FRAP and DPPH), total flavonoid content (TFC) and total phenolic content (TPC) of the extracts.

Table 4.1 *Matrix of the central composite design (CCD) and observed responses (Y_j) for different experimental conditions (mean values of three replicates) for MAE of Russian olive leaves.*

Run	Independent variables				Response variables				
	Acidity (x_1, M)	Temp. (x_2, ℃)	Ethanol Conc. (x_3, %)	Solid/solvent (x_3, ratio)	DPPH (Y_1, µmol Trolox Eq mL^{-1})	FRAP (Y_2, µmol FSHE/g bio)	TPC (Y_3, µg GAE/g bio)	TFC (Y_4, mg Quercetin Eq /g bio)	Extraction yield (%)
1	4	97.5	25	7.5	10.42	28.3	27.9	302.6	60.02
2	5	85	50	10	12.98	27.8	32	192.6	60.94
3	2	97.5	75	7.5	14.04	54.7	32.7	466.3	42.8
4	4	97.5	75	7.5	14.90	31	31.4	300.6	69.25
5	3	85	50	10	10.32	43.7	31.8	299.2	46.4
6	4	72.5	25	12.5	7.47	22	21.4	188.6	64.72
7	3	60	50	10	12.57	32.3	30.2	284.4	44.68
8	1	85	50	10	10.09	54.5	29.5	346.1	21
9	2	72.5	25	7.5	8.49	44.4	22.7	414.1	39.28
10	2	97.5	25	12.5	6.79	34.6	20.7	307.1	44.98
11	3	85	100	10	7.71	37.2	24.7	363.1	34.34
12	4	97.5	75	12.5	14.83	25.6	29.4	219.2	45.552
13	4	72.5	75	12.5	13.69	23.8	27	181.5	75.68
14	2	72.5	75	12.5	12.04	49.5	27.2	318.9	46.34
15	3	85	50	10	11.97	42.5	31.2	301.5	86.12
16	2	97.5	25	7.5	8.01	42.1	26.7	492.7	24.57
17	3	85	50	10	12.57	43	33.1	354.5	29.25
18	3	85	50	10	11.79	37.1	30.7	315.9	46
19	2	72.5	75	7.5	12.28	59.1	29.9	487.6	22.91
20	4	72.5	25	7.5	8.29	25.5	24	214	36.57
21	3	85	0	10	4.86	38.5	20.3	346.2	30.8
22	3	85	50	10	12.93	34.2	30.2	280.5	46.22
23	3	85	50	5	17.70	54.5	39.5	594.8	39.42
24	3	85	50	15	12.75	35.3	26	223.8	37.88
25	3	110	50	10	15.59	51.4	37.7	389.6	48.5
26	2	72.5	25	12.5	7.24	37.1	18.4	265.5	25.55
27	2	97.5	75	12.5	11.09	46.4	26.1	348.2	26.3
28	3	85	50	10	13.55	45.9	32.7	348.6	41.08
29	4	97.5	25	12.5	7.14	20.9	22.2	170.4	40
30	4	72.5	75	7.5	14.62	32.1	31	329.3	34.89

Table 4.2 *Matrix of the central composite design (CCD) and observed responses (Y_j) for different experimental conditions (mean values of three replicates) for MAE of Russian olive flowers.*

Run	Independent variables				Response variables				
	Acidity (x_1, M)	Temp. (x_2, ℃)	Ethanol Conc. (x_3, %)	Solid/solvent (x_3, ratio)	DPPH (Y_1, mg Trolox Eq/g bio)	FRAP (Y_2, mg FSHE/g bio)	TPC (Y_3, mg GAE/g bio)	TFC (Y_4, mg Quercetin Eq /g bio)	Extraction yield (%)
1	4	97.5	25	7.5	37.11	52.29	26.26	2244.94	70.50
2	5	85	50	10	49.09	54.14	45.80	1226.66	62.02
3	2	97.5	75	7.5	51.77	111.17	48.65	3014.57	50.10
4	4	97.5	75	7.5	60.75	69.37	47.68	2273.54	63.25
5	3	85	50	10	34.63	64.32	45.08	1879.52	47.72
6	4	72.5	25	12.5	26.86	37.68	26.09	470.82	69.36
7	3	60	50	10	55.07	52.08	38.76	516.02	50.68
8	1	85	50	10	39.65	104.92	43.26	2025.2	24.56
9	2	72.5	25	7.5	35.50	84.24	31.46	2118.44	42.34
10	2	97.5	25	12.5	23.98	68.62	25.93	1311.67	45.17
11	3	85	100	10	40.05	39.82	27.30	1958.73	47.38
12	4	97.5	75	12.5	50.65	55.50	45.79	885.58	50.20
13	4	72.5	75	12.5	51.69	51.98	38.33	666.79	73.01
14	2	72.5	75	12.5	43.97	91.02	40.58	1373.4	48.96
15	3	85	50	10	48.04	67.69	43.82	1789.31	95.92
16	2	97.5	25	7.5	29.00	95.00	39.17	3096.46	27.6
17	3	85	50	10	45.95	77.84	47.13	2103.04	30.61
18	3	85	50	10	47.27	73.89	44.28	1868.35	45.06
19	2	72.5	75	7.5	78.87	107.1	42.83	2093.76	29.21
20	4	72.5	25	7.5	28.26	53.98	31.02	1932.35	41.44
21	3	85	0	10	16.24	41.33	17.95	1921.08	40.94
22	3	85	50	10	42.77	55.51	42.93	1545.48	57.14
23	3	85	50	5	51.13	79.65	52.09	2234.41	48
24	3	85	50	15	45.88	49.61	40.56	808.859	55.12
25	3	110	50	10	55.00	72.96	54.82	1905.89	58.34
26	2	72.5	25	12.5	30.34	69.75	28.63	1425.54	28.51
27	2	97.5	75	12.5	36.93	102.03	44.70	1455.44	31.68
28	3	85	50	10	54.94	68.82	46.60	2035.57	47.56
29	4	97.5	25	12.5	33.46	42.45	32.04	642.53	44.97
30	4	72.5	75	7.5	66.48	63.57	43.32	2917.91	40.38

4.3.2 Sample preparation

Field-grown fruits, leaves and flowers of *E. angustifolia* were harvested from the Cramer Centre (a horticultural vendor in Montreal) and were transported to the research facility at McGill University. Fresh plant materials were air-dried to the moisture content of 6.2 and 7.4 % for leaves and flowers, respectively and stored at ambient room temperature until the initial sample preparation stage. To increase the enzymatic action and extraction yield of the bioactive compounds, samples were ground into smaller sizes in a blender (NutriBullet), for achieving higher surface area. Subsequently, the ground powder was passed through a standard 500 µm sieve and stored in airtight bags in a desiccator.

4.3.3 Extraction processes

4.3.3.1 Preliminary MAE experiments

Prior to the optimization process, two-level fractional factorial designs were used for assessing the magnitude of microwave-assisted extraction variables' effects on extraction yield, total phenolic content, total flavonoid content and antioxidant activity of the extracts from Russian olive leaves and flowers (data not shown). Preliminary screening of the MAE process was performed using 0 and 4 % citric acid, and 20 and 100 % ethanol concentration in the solvent, at solid to solvent ratios of 10 and 30 w/v. The extraction processes were performed for 5 and 15 minutes at an extraction temperature of 50 and 80 °C. Then, based on the preliminary results, experimental domains and their ranges were determined and used in the experimental design of the following optimization process in this study. These factors included the extraction temperature, solid to solvent ratio and the concentrations of ethanol and citric acid in the solvent.

4.3.3.2 Microwave extraction in this study

The microwave system was a focused-type, closed-vessel system (Star System 2, CEM Matthews, USA) operating at a nominal power level of 800 W and frequency of 2,450 MHz. The applied microwave power was intermittent with power on for 30 s/min. Dried powders from leaves and flowers were placed in a 250-ml quartz vessel. Samples were stirred into different concentrations of aqueous ethanol (0-100 %), at various solid to solvent ratios (5-15 mg/ml), and the vessel was placed into the microwave cavity for extraction. The other MAE extraction parameters studied were extraction temperature (60-110 °C) and citric acid concentration in the solvent (1-5 M) and the experiments were performed as presented in Tables 4.1 and 4.2. After each MAE treatment, the extract was filtered through a Whatman No. 1 filter paper using vacuum filtration and the supernatant was stored at 4 °C for further analysis.

4.3.3.3 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction (UAE) of the samples was carried out following a procedure by Dahmoune et al. (2015) with modifications using an ultrasonic bath (Ultrasonic Cleaner FS 30, Fisher Scientific) with a working frequency fixed at 20 kHz and the mixture of sample and solvent was exposed to acoustic waves for 15 minutes under a controlled temperature of 27°C. Subsequently, the supernatant was recovered and stored for further analysis.

4.3.3.4 Conventional extraction (CE)

Conventional solvent extraction of samples was conducted following the procedures by Dahmoune et al. (2015) with slight modifications. The mixture of solvent and sample was placed in a conical flask for 120 minutes at 60°C, with shaking at a speed of 110 strokes/minute using a thermostatic water bath. Subsequently, the supernatant was recovered for further analysis.

4.3.4 Chemical analysis

4.3.4.1 Determination of total phenolic content (TPC)

For measuring the total phenolic content of the samples, the Folin-Ciocalteu assay was used to detect the presence of total phenolics and quantify using gallic acid as standard using ISO 14502-1:2005(E) method with slight modifications. 1 ml aqueous solution of each sample and 4 mL of sodium carbonate (7.5% in deionized water) were added to 5 mL of the Folin-Ciocalteu reagent (diluted 10-fold in deionized water). After 60 minutes of incubation at room temperature, the absorbance is measured at 765 nm. Tests were carried out in triplicate, and the results were expressed as mg gallic acid equivalent (GAE) /g of sample.

4.3.4.2 Extraction yield

The yield of extracts was determined following Bampouli et al. (2014) with modifications. First, the ethanol portion of the solvent in the extract was evaporated using a nitrogen evaporator (N-EVAP 111, Organomation Associates, Berlin, MA, USA). Then remaining water extracts were frozen overnight at −80 °C and water was removed using a lab-scale vacuum freeze-dryer (7670520, Labconco Co., Kansas City, USA) for 48 h. Subsequently, the dried extract powders were collected, and the extraction yield was calculated as the ratio of the extract powder's weight to the initial dried weight of samples used for extraction and results were expressed as a percentage.

4.3.4.3 Determination of flavonoids contents

The total flavonoid content of the samples was measured with the aluminum chloride colorimetric method by Kamtekar et al. (2014) with slight modifications. 1 ml aqueous solution of plant extract or 1ml of standard quercetin solution (0.1-10 mM) were mixed with 4 ml of deionized water and 0.3 ml of 5 % sodium nitrite solution. After 5 minutes, 0.5 ml of 2 % aluminum chloride was added and in the next 6 minutes, 0.5 ml of 1 M sodium hydroxide was added. Finally, after 10 minutes,

when orange yellowish colour was developed, the absorbance was measured at 510 nm using a spectrophotometer. The tests were performed in triplicates and the results were expressed as mg of quercetin equivalents/g of the sample.

4.3.4.4 Determination of antioxidant activity

The antioxidant activity of the extracts was evaluated using two of the most widely used spectrophotometric assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity test and ferric reducing ability of plasma (FRAP) assay.

4.3.4.4.1 DPPH radical scavenging activity

The antioxidant activities of the samples are determined using the DPPH assay introduced by Brand-Williams et al. (1995) with slight modifications. Serial dilution of the standard using 2 mM Trolox was prepared in methanol. Also, 6 × 10^{-5} M of the DPPH• solution in methanol was prepared freshly. Then 3.9 ml of prepared DPPH solution was added to 100 Microliter of aqueous solutions of diluted samples extracts. Similarly, 3.9 ml of DPPH• solution in methanol was mixed with a serial dilution of the standard Trolox solution previously made. The mixtures are incubated for 30 minutes at room temperature and in darkness. DPPH solution was used as a control and the absorbance of standard solutions and samples were measured at 515 nm using a spectrophotometer. The experiment was carried out in triplicate and the radical scavenging activity of the samples is calculated and reported as mg Trolox equivalent per gram of sample.

4.3.4.4.2 FRAP antioxidant activity

For measuring the antioxidant activity of the samples using the Ferric reducing antioxidant power (FRAP) assay, a method introduced by Benzie and Strain (1996) with slight modifications was followed. Briefly, three millilitres of prepared FRAP reagent was mixed with 300 µL DI water and

100 µL of diluted sample. Consequently, after 30 min incubation at room temperature, the absorbance at 593 nm was measured using a spectrophotometer. Results were expressed as mg $FeSO_4$ Equivalent per gram of sample (Alam et al. 2013; Floegel et al. 2011).

4.3.5 Analytical techniques for separation of phenolic compounds

Among phenolic compounds identified in the preliminary QTOF-MS analysis, three of the most abundant compounds with anticarcinogenic activity, which were common between Russian olive leaves and flowers, were quantified and compared in the extracts from optimized MAE, CE and UAE. These compounds were isorhamnetin, rutin and luteolin and were analyzed using HPLC analysis following a method described by Wang et al. (2019) with modifications. Briefly, the extracts were centrifuged at 5000 rpm for 10 min and the supernatants of the samples were filtered using a syringe 0.45 µm filter and were analyzed using an HPLC system (model:1100 series, Agilent Technologies, USA). Before each run, the column was equilibrated for 5 min with the mobile phase. The flow rate was kept at 0.5 mL/min and the UV detection was set at 280 nm for phenolic compounds. For each sample, 10 µL injection volume was applied into the column with the temperature set at 30 °C. The samples were loaded to the Discovery C18 column (250×4.60 mm, 5 µm; Sigma, USA) connected to an ALS automatic sampler (G1313A), diode array detector (DAD, G131A) and a quaternary pump (G1311A) for identification of individual phenolic acids. Samples were eluted using the mobile phases consisting of a mixture of 0.17% acetic acid and water, v/v (A), and 100% acetonitrile (B). The solvent gradient in volume ratios was as follows: 0–3 min, 0–9% B; 3-8 min, 81% B; 8–13 min, 76% B; 13-15 min, 70% B and 15–25 min, 91% B (Wang et al. 2019). Samples were analyzed in triplicates and the quantitative determination of the target compounds in the samples was carried out using standards and their previously confirmed

calibration curves. Subsequently, obtained data were analyzed using an OpenLAB CDS ChemStation software (Agilent Technologies, Palo Alto, CA, USA).

4.3.6 Statistical analysis

Response surface methodology (RSM), which is a powerful optimization tool was used for the optimization of the microwave extraction process, and to determine the effects of experimental variables and their interactive effects on the response variables of the process. All responses were equally weighted and the analysis of variance Fisher test (ANOVA F-test) was used to analyze the process response variables and to identify the significant factors, and subsequently, the accuracy of the suggested model was determined. Finally, using P-value ($p \leq 0.05$), as well as the coefficient of variation (CV) and the coefficient of determination R^2 and R^2_{adj}, the quality of the fit of the polynomial model was expressed.

4.3.7 Verification of the models

When the microwave extraction process's optimal conditions were determined, the process was conducted, and the corresponding experimental values were compared with the model-predicted response values to verify the models' validity.

4.4 Results and Discussion

4.4.1 Results from preliminary studies

The results from the preliminary *in vitro* study of antioxidant activity, total phenolic content and total flavonoid content of the extracts confirmed the significant antioxidant activity of Russian olive leaves and flowers reported by other studies (Caliskan et al. 2010; L. Li et al. 2012; Mehrabani 2013; Okmen and Turkcan 2014). In addition, using high-resolution quadrupole time-of-flight mass spectrometry (QTOF-MS), we identified a series of phenolic compounds in Russian olive leaves and flowers among which, three of the common phenolic compounds between Russian

olive leaves and flowers namely, rutin, luteolin and isorhamnetin, were selected as the standards in the HPLC analysis of this study to compare the extracts obtained by the optimized MAE and UAE and conventional extraction methods of Russian olive extracts.

4.4.2 Microwave-assisted extraction processes

Tables 4.1 and 4.2 represent the results of the microwave-assisted extraction processes performed in this study. The effect of experimental factors on extraction process dependent variables was investigated and the statistical significance for each response and linear, quadratic and interaction coefficients of experimental factors are presented in Tables 4.3 and 4.4.

Table 4.3 *Regression equation coefficients for the MAE of Russian olive leaves response models.*

	Response							
Effects	**AA_{DPPH} (mg Trolox Eq/g)**		**AAFRAP (mg FeSO4 Eq/g)**		**TPC (GAEq/g)**		**TFC (mg Quercetin Eq/g)**	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Intercept								
β_0	+21.88		+6.362		+0.148		+6.75	
Linear								
β_1	+0.47	0.05	-0.486	<0.0001**	-4.2E-3	0.0018**	-0.21	<0.0001**
β_2	-0.26	0.27	+5.11E-3	0.307	+1.77E-4	<0.0001**	+4.44E-3	0.047*
$\beta 3$	+0.25	<0.0001**	+5.68E-3	0.029*	-2.89E-3	<0.0001**	+1.19E-3	0.084
$B4$	-1.63	0.022*	-0.085	0.0018**	+2.94E-3	<0.0001**	-0.08	<0.0001**
Quadratic								
β_{12}	+0.016	0.619			+2.68E-5	0.55		
β_{13}	+0.014	0.393			+4.45E-5	0.06		
β_{14}	+0.014	0.933			-4.52E-4	0.06		
β_{23}	+2.7E-4	0.840			+5.1E-6	0.01*		
β_{24}	-8.56E-3	0.526			+2.54E-5	0.17		
β_{34}	+2.36E-3	0.725			-2.77E-5	0.008*		
Interaction								
β_{11}	-0.34	0.295	+2659.65	<0.0001**	+4.18-4	0.37		
β_{22}	+1.88E-3	0.366	+860.42	0.6424	-5.88E-6	0.06		
β_{33}	-2.64E-3	<0.0001**	-62419.63	0.0003**	+2.24E-5	<0.0001**		
β_{44}	+0.09	0.085*			+1.68E-6	0.98		

Effects are statistically significant if 0.01≤P< 0.05 or P** < 0.01. The coefficients of the polynomial model include the constant term (β_0), linear effects (β_1, β_2, β_3, β_3), quadratic effects (β_{11}, β_{22}, β_{33}, β_{44}) and interaction effects (β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34}).*
(Abbreviations: AA_{FRAP}= Ferric reducing antioxidant power (FRAP) assay, AA_{DPPH}=1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, TPC= Total phenolic content, TFC: Total flavonoids content)

Table 4.4 *Regression equation coefficients for the MAE of Russian olive flowers response models.*

Effects	Response							
	AA_{DPPH} (mg TroloxEq/g)		AAFRAP (mg FeSO4 Eq/g)		TPC (mg GAEq/g)		TFC (mg Quercetin Eq/g)	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Intercept								
β_0	+202.73		+163.18		+15.93		+3332.09	
Linear								
β_1	-27.72	0.20	-37.98	<0.0001**	-3.77	0.4217	-227.16	0.006**
β_2	-3.33	0.26	-0.05	0.15	-0.14	<0.0001**	+15.68	0.016*
$\beta 3$	+2.38	<0.0001**	+0.99	0.01*	+1.31	<0.0001**	+2.52	0.41
$B4$	-6.50	0.0081*	-7.60	0.0039**	-0.76	<0.0001**	-238.52	<0.0001**
Quadratic								
β_{12}	+0.27	0.056	-0.06	0.77	+0.06	0.159		
β_{13}	+0.02	0.68	-0.09	0.36	-0.01	0.44		
β_{14}	+0.74	0.28	+0.36	0.73	+0.03	0.89		
β_{23}	-8.69E-3	0.12	+2.32E-3	0.78	+1.03E-4	0.95		
β_{24}	+0.04	0.41	-1.41E-3	0.98	-0.02	0.15		
β_{34}	-0.05	0.04	+0.01	0.70	+0.01	0.07		
Interaction								
β_{11}	-0.50	0.70	+4.61	0.0382*	-0.14	0.71		
β_{22}	+0.01	0.11	+2.33E-3	0.859	+2.69E-3	0.30		
β_{33}	-7.2E-3	0.0029**	-8.19E-3	0.02*	-0.01	<0.0001**		
β_{44}	+0.08	0.68	+0.14	0.66	+0.04	0.45		

Effects are statistically significant if 0.01≤P< 0.05 or P** < 0.01. The coefficients of the polynomial model include the constant term (β_0), linear effects (β_1, β_2, β_3, β_3), quadratic effects (β_{11}, β_{22}, β_{33}, β_{44}) and interaction effects (β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34}).*
(Abbreviations: AA_{FRAP}= Ferric reducing antioxidant power (FRAP) assay, AA_{DPPH}=1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, TPC= Total phenolic content, TFC: Total flavonoids content)

As demonstrated in Tables 4.3 and 4.4, the regression coefficients of the developed models demonstrated significant ($p < 0.05$) relationships between the MAE variables and corresponding responses of the produced extracts. Extraction temperature, acidity and ethanol concentration of the solvent and solid to solvent ratio, showed to have a significant effect on the extraction of antioxidant compounds from Russian olive leaves and flowers. Also, the combined effects of MAE

experimental variables on corresponding responses are illustrated in Figures 4.1-4.4, which will be discussed in great detail in the following sections.

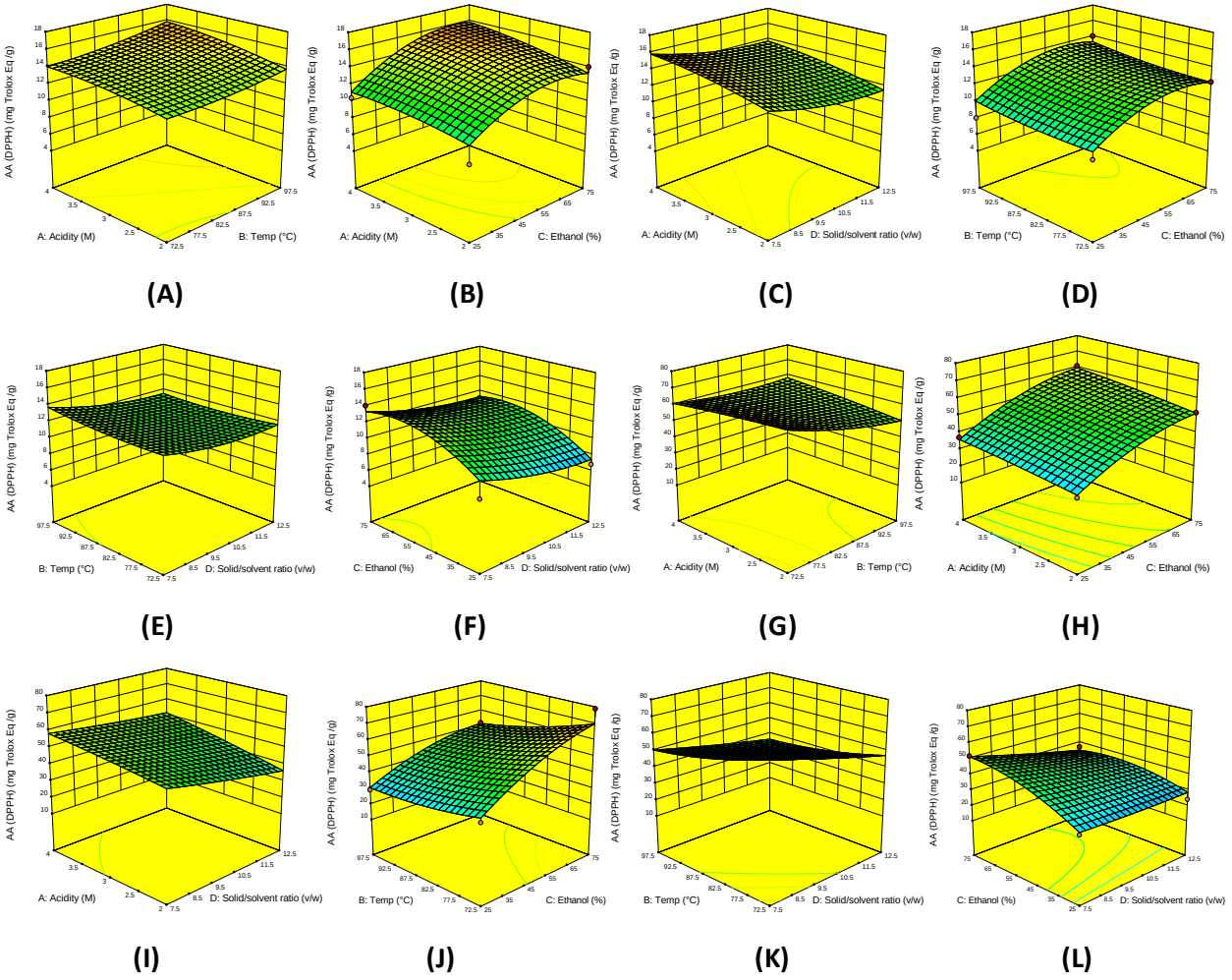
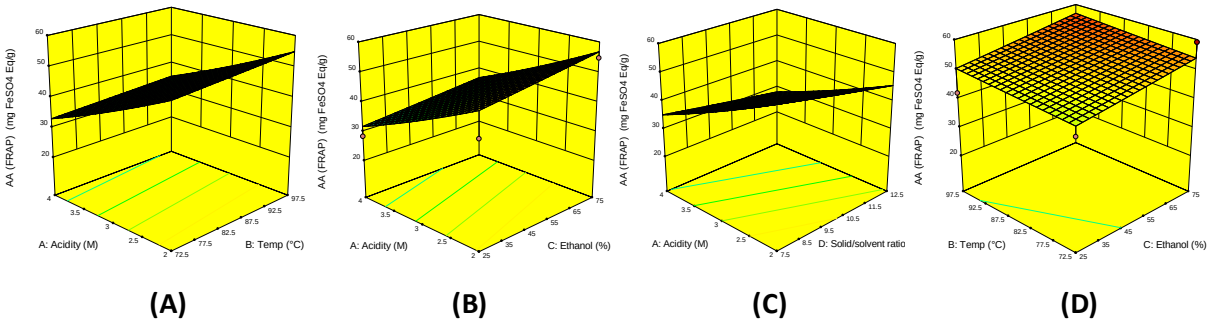


Figure 4.1 *3D plots showing the combined effect of citric acid molarity, ethanol concentration, solid to solvent ratio and extraction temperature on* AA_{DPPH} *radical scavenging activity of microwave-assisted extracts of Russian olive leaves (A-G) and flowers (G-L).*



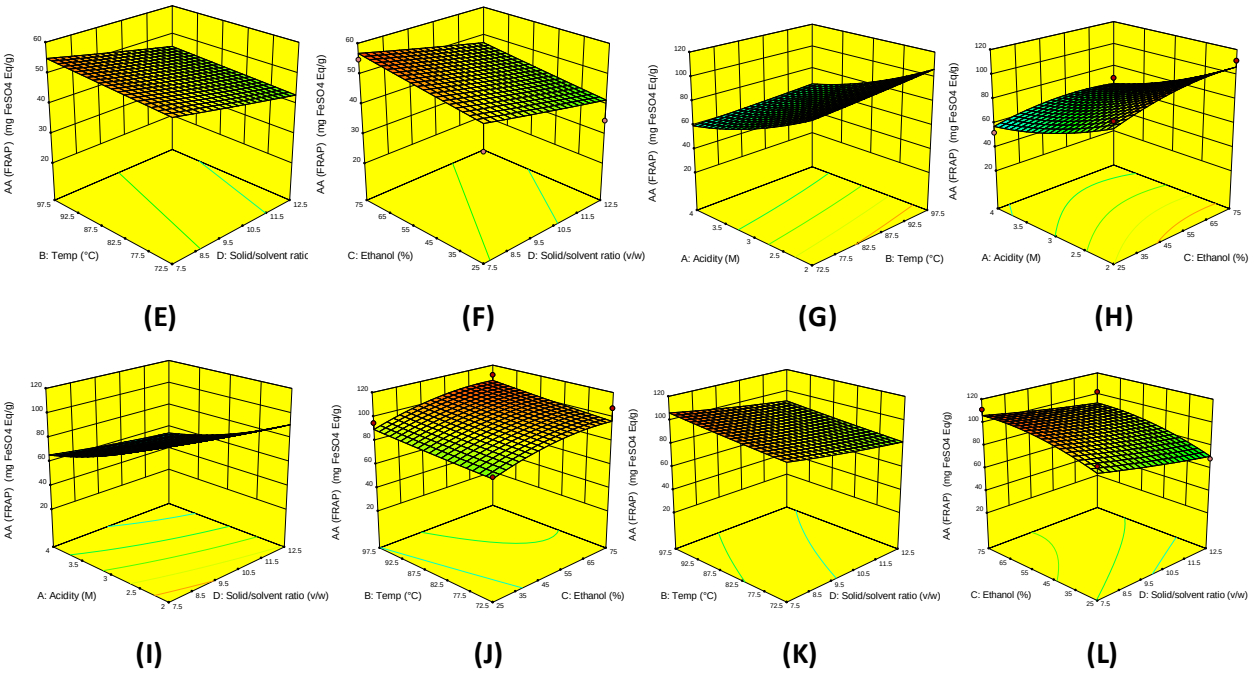
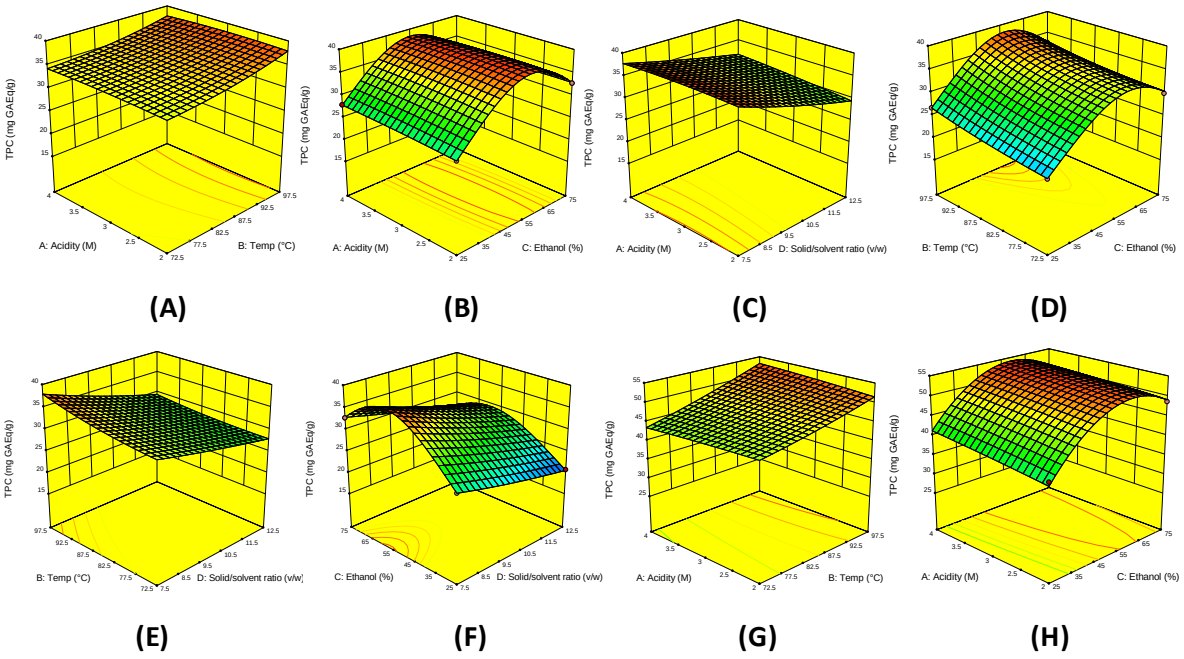
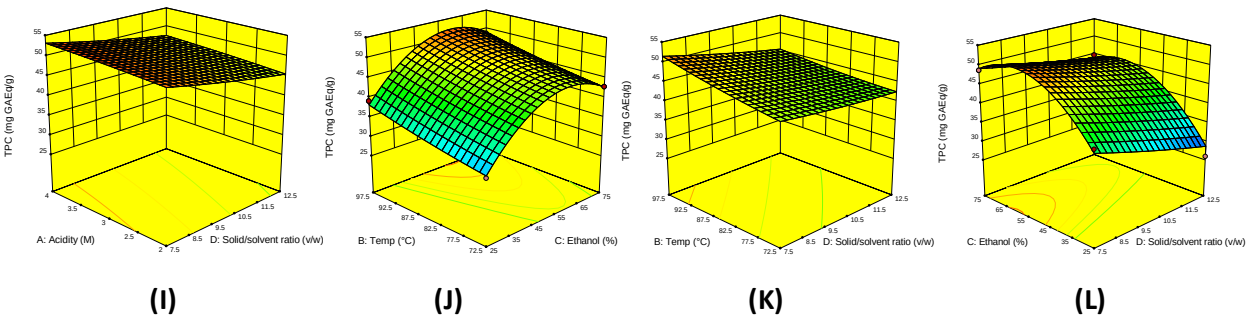


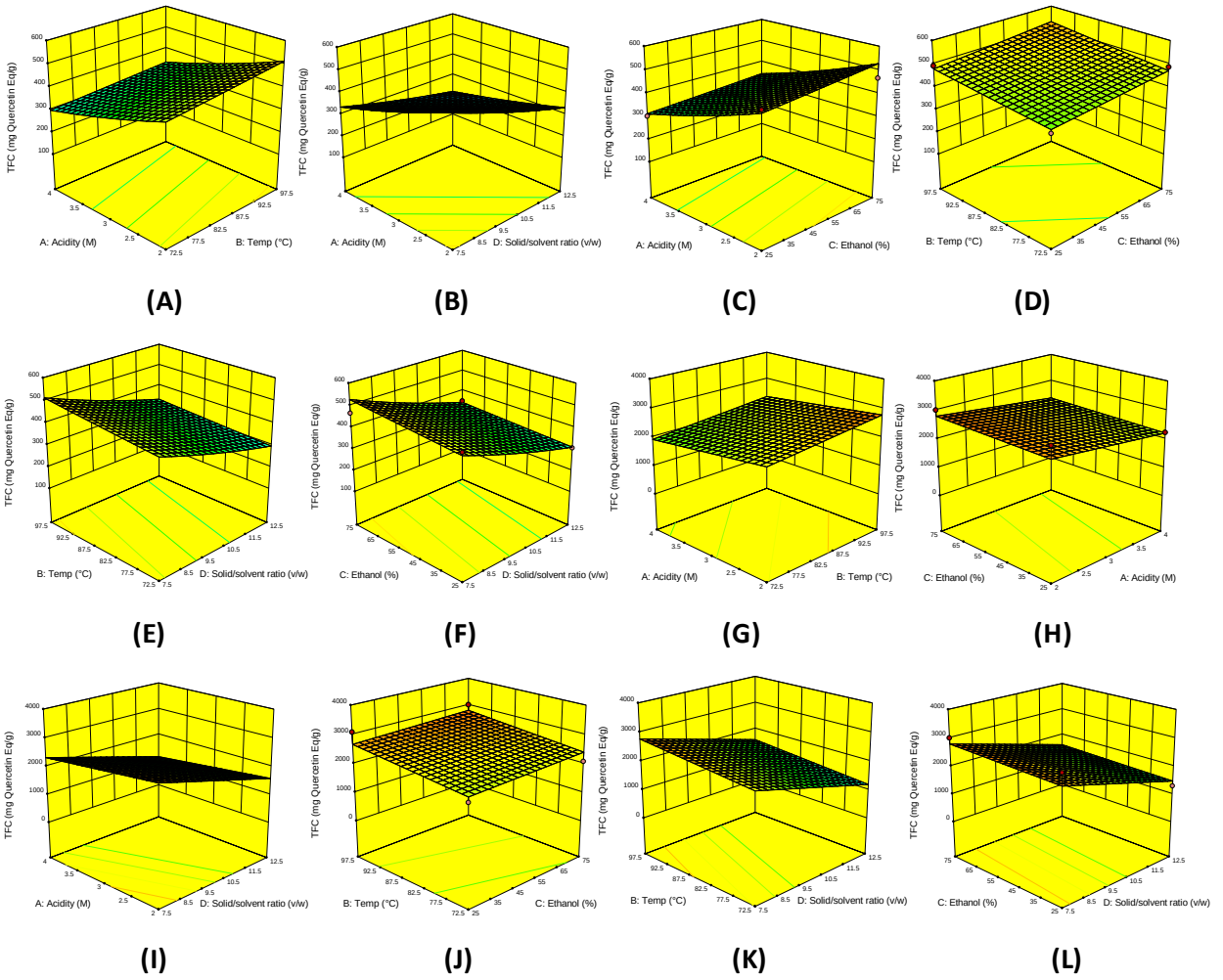
Figure 4.2 *3D plots showing the combined effect of citric acid molarity, ethanol concentration, solid to solvent ratio and extraction temperature on* AA_{FRAP} *of microwave-assisted extracts of Russian olive leaves (A-G) and flowers (G-L).*





(I) **(J)** **(K)** **(L)**

Figure 4.3 *3D plots showing the combined effect of citric acid molarity, ethanol concentration, solid to solvent ratio and extraction temperature on Total phenolic contents (TPC) of microwave-assisted extracts of Russian olive leaves (A-G) and flowers (G-L).*



(A) **(B)** **(C)** **(D)**

(E) **(F)** **(G)** **(H)**

(I) **(J)** **(K)** **(L)**

Figure 4.4 *3D plots showing the combined effect of citric acid molarity, ethanol concentration, solid to solvent ratio and extraction temperature on Total flavonoids contents (TFC) of microwave-assisted extracts of Russian olive leaves (A-G) and flowers (G-L).*

4.4.2.1 Effect of extraction factors on experimental responses

4.4.2.1.1 Effect of Solvent (ethanol concentration and acidity)

Due to differences in the properties of phenolics in different plants, the choice of optimal extraction solvent vastly depends on the type of plant sample and target molecule. Phenolics in different plants have different properties. Therefore, optimum recovery of the phenolic compounds requires the solvent to be selected based on the plant and affected yield of phenolics extraction. As an example, while water can extract a high yield of phenolics from sorghum leaves, in the case of *Vitis vinifera*, pure methanol can extract the highest level of phenolics while in wheat bran, 80% aqueous ethanol is required (Khoddami et al. 2013). Extraction of antioxidant compounds is usually through organic solvents, among which the most common are ethanol and methanol. Based on preliminary results on the efficiency of ethanol in the extraction of phenolic compounds and due to the lower toxicity of ethanol as recommended by the Food and Drug Administration (FDA), ethanol was selected in this study. In addition, since a single solvent might not be efficient enough for the maximum extraction of antioxidant compounds, a combination of aqueous-organic solvents (different concentrations of aqueous ethanol) was selected for maximum recovery of these compounds in the produced extracts from Russian olive leaves and flowers. Therefore, this study designed the experiments to compare the application of water as the solvent to water with different concentrations of ethanol and citric acid and to evaluate the effect of the combination of different concentrations of ethanol and acids in water on the total yield of phenolic, flavonoids and total antioxidant components extracted from Russian olive. Overall, the concentration of ethanol in water as the extraction solvent was one of the important factors affecting extraction efficiency. Antioxidant activity (FRAP and DPPH) and TPC of Russian olive extracts of leaves and flowers were significantly enhanced by increasing the concentration of ethanol in the solvent ($p<0.05$) but the effect was less significant for total flavonoid content (Tables 4.3 and 4.4). Conversely, the

quadratic term of ethanol concentration was highly significant and had a negative parameter estimate, which implies a point of maxima at 59.8 and 66.6% for leaves and flowers, respectively. In other words, the yield of compounds increased with increasing ethanol concentration up to a certain level after which it started decreasing with further increase in the concentration of ethanol in the solvent (Figures 4.1-4.4). The reason is that ethanol and water are both great solvents for efficient extraction of antioxidant compounds and for maximizing the efficiency of the solvent in extracting these compounds, the ratio of their mixture is important. Therefore, based on the results, 66.6 and 59.8 % of aqueous ethanol solution were the best combinations of these two solvents for the maximized recovery of antioxidant compounds including phenolics and flavonoids in Russian olive leaves and flowers, respectively.

Acids, in particular weak acids (to avoid degradation of antioxidant compounds), are widely applied for successful extraction of antioxidant compounds, among which hydrochloric acid, citric acid and acetic acid are commonly used (Routray and Orsat 2014). Routray (2014) has reported greater efficiency of ethanol with HCl combinations compared to citric acid or acetic acid solutions for microwave-assisted extraction of phenolics, at all concentration levels of acids. However, considering overall observations including dielectric properties and value of dissipation factor, they also reported the combination of citric acid solution with aqueous ethanol as an efficient combination of solvents for microwave extraction. Similarly, Nicoue et al. (2007), who applied different combinations of acids with ethanol as the extraction solvent, reported less but sufficient efficiency of ethanol with citric acid compared with ethanol with phosphoric acid for extraction of phenolic compounds from blueberries. The application of organic acids, which are part of different food commodities, such as acetic acid and citric acid in extraction is considered less toxic. Subsequently, due to the better efficiency of citric acid compared to acetic acid (reported by

Routray (2014)), microwave-assisted extraction of antioxidant compounds was conducted using citric acid. Due to the interaction of solvent components, different patterns are observed in the figures. For example, according to Figure 4.2B, at all concentrations of ethanol, FRAP antioxidant activity of the Russian olive leaves extract increased with increasing acidity. Where in Figure 4.3B for TPC yield of Russian olive leaves extract, it is noticed that when using a low concentration of ethanol in water with a higher citric acid concentration, efficiency of extraction was increased, whereas, in extraction with a higher concentration of ethanol in water, TPC yield decreased when using a higher concentration of citric acid. In other words, alcohol and water interact differently with increasing concentration of citric acid, and when the proportion of ethanol to water in the solvent is low, increasing citric acid concentration has a different effect compared to when more ethanol is present in the solvent. Similarly, Halee et al. (2021) reported that the proportion of ethanol and water in the solvent affects the influence of increasing acid concentration on the extraction yield of antioxidant compounds. Adding 2 M citric acid in the solvent, showed to be the optimum concentration for maximum extraction yield of antioxidant, phenolic and flavonoid content of both leaves and flowers extracts of Russian olive (Table 4.5). Results showed increasing the concentration of acetic acid to more than 2 M significantly reduces the TFC of the extracts from Russian olive leaves and flowers, which can be due to the free form of flavonoid compounds being destroyed by a higher concentration of the acid (Figure 4.4). Overall, the results showed that the 59.8 % ethanolic extract with 2M citric acid gives the highest yield of antioxidant activity (FRAP and DPPH), TPC and TFC for Russian olive leaves, whereas for Russian olive flowers extract, the highest values were obtained using 66.6 % aqueous ethanol with 2M citric acid.

4.4.2.1.2 Effect of extraction temperature

According to the information reported in Tables 4.3 and 4.4, increasing extraction temperature significantly improved the recovery yield of TPC and TFC in all extracts. Increasing temperature can enhance the diffusivity of the extraction solvent into plant matrix for better solubility of phenolic and flavonoid compounds and can decrease the solvent viscosity for accelerating dissolution of these compounds (Alara et al. 2018). Similar results are reported by Dahmoune et al. (2015) on the effect of temperature on enhancing microwave-assisted extraction of polyphenol compounds. Also, increasing extraction temperature resulted in better extraction yield of total antioxidant compounds in all extracts; however, in contrast to the significant effect of temperature on TPC and TFC, the effect of temperature on total antioxidant yield of the extracts was not significant, which can be attributed to a balance between the release of some antioxidant compounds with degradation of some other thermolabile antioxidant compounds leading to insignificant enhancing effect of temperature on total antioxidant activity of the extracts (Radojković et al. 2018). The interaction effects of extraction temperature with other experimental variables are shown in Figures 4.1-4.4, among which the interaction of temperature and ethanol concentration in the solvent had a significant influence on the yield of total phenolic contents of Russian olive leaves extract as illustrated in Figure 4.3D. A significant increase in the recovery of TPC in leaves extract to 37.92 mg GAE/g of the extract was observed at 97.5 °C by increasing the ethanol concentration to 54.67 %, followed by a decline with a further increase of ethanol concentration in the solvent. Also, Figure 4.3-E illustrates that at a lower solid to solvent ratio, the level of TPC in leaves and flowers extracts was enhanced more significantly with increasing extraction temperature and reached a maximum of 51.47 and 37.92 mg GAEq/g in extracts of flowers and leaves, respectively. Whereas in the case of high solid to solvent ratio, increasing temperature showed a less significant effect on yield of extraction, which can be as a result of

saturation of the solvent at high solid to solvent ratio resulting in the less noteworthy effect of temperature on increasing the extraction efficiency. A similar trend was observed for DPPH antioxidant activity of the leaves extract (Figure 4.1E).

4.4.2.1.3 Effect of the ratio of solid to solvent

The ratio of the solvent to the sample showed to be the most important factor affecting microwave extraction of Russian olive leaves and flowers. The decreasing solid to solvent ratio was found to significantly enhance antioxidant activity (FRAP and DPPH), total phenolic and flavonoid content in all extracts by providing a better contact surface for extraction of bioactive phytochemicals from the plant matrix. As can be seen in Tables 4.3 and 4.4, the solid to solvent ratio showed significant interaction with other experimental variables such as with ethanol concentration of the solvent. For example, as it can be observed from the response surface plot of DPPH antioxidant activity of Russian olive flowers with respect to solid to solvent ratio and ethanol concentration, at higher ethanol concentration the yield increased with decreasing solid to solvent ratio, while for the lower level of ethanol concentration the yield remained mostly consistent with decreasing solid to solvent ratio, which can be attributed to saturation of ethanol at its low concentration level (Figure 4.4L). Based on the results, the highest yield of the extraction process was obtained at a 7.5 mg/mL ratio of Russian olive leaves and flowers to solvent. However, at an industrial scale, a balance between minimizing the high costs of solvent and its waste and maximizing extraction yield needs to be obtained for the cost-effectiveness of the extraction process (Alara et al. 2018; Dai and Mumper 2010).

4.4.2.2 Optimal MAE processing conditions

Based on the previously discussed effects of experimental factors, the microwave-assisted extraction process for Russian olive leaves and flowers was optimized using the RSM response

optimizer. Numeric and graphic optimizations were performed while desired goals were defined by high values of antioxidant activity (DPPH and FRAP), total phenolic content (TPC) and total flavonoid content (TFC). The software-generated most desirable solution of the MAE process of Russian olive leaves and flowers and the predicted responses are presented in Table 4.5. The optimum values of 0.82 and 0.79 were selected for the desirability function of MAE of Russian olive leaves and flowers, respectively.

Table 4.5 *The most desirable solution of MAE of Russian olive leaves and flowers and the predicted values of responses at the optimum experimental condition.*

Solution / Sample	Acidity (M)	Temp (°C)	Ethanol Conc. (%)	Solid/solvent (Ratio)	DPPH (mg Trolox Eq/g)	FRAP (mg FeSO4 Eq/g)	TPC (mg GAE q/g)	TFC (mg Quercetin Eq/g)	Yield (%)
Leaves	2	97.4	59.8	7.5	13.70	54.85	37.98	512.91	33.71
Flowers	2	97.5	66.6	7.5	50.48	106.31	51.47	2786.17	39.74

The optimal experimental conditions obtained in this study is within the range of optimal MAE conditions for maximum yield of antioxidant compounds from different materials such as leaves from basil, batal and walnut, reported by Şen et al. (2019), Xie et al. (2015) and Vieira et al. (2017), respectively.

4.4.3 Model fitting

The software Design-Expert (version 10.0.7.0, Stat-Ease Inc., Minneapolis, USA) was employed for regression analysis of the data, and model building. Results showed that the models developed for corresponding responses including antioxidant activity (FRAP and DPPH), total phenolic content and total flavonoids were significant ($p < 0.05$) (Tables 4.6-4.7). Moreover, according to these tables, non-significant ($p > 0.05$) lack of fit was observed for all the developed models, which indicates there is a satisfactory level of accuracy of the proposed models for representing the relationship between the dependent and independent variables and the prediction of the corresponding variable responses. The coefficients of determination (R^2 values) of the developed

models were ranging from 0.76 to 0.98 which implies that the experimental data successfully fit the equation. Also, adj- R^2 ranging from 0.72 to 0.96 and low coefficient of variation (CV) values, indicating a low deviation from mean values, are desirable in the model and consequently represent acceptable reliability of the models.

Table 4.6 *Analysis of variance (ANOVA) for the response of Russian olive leaves MAE experimental variables.*

Response	Source						*F*-value	*P-value Prob>f*	*P vale Lack of fit*	R^2	*Adj* R^2
	residual			model							
	df	SS	MS	*df*	SS	MS					
AA_{DPPH} (mg Trolox Eq/g)	15	40.96	2.73	14	240.36	17.17	6.29	<0.0005	0.13	0.86	0.72
AA_{FRAP} (mg FeSO4 Eq/g)	25	2.26	0.09	4	7.37	1.84	20.36	<0.0001	0.26	0.77	0.73
TPC (mg GAEq/g)	13	6.48E-5	4.98E-6	14	3.85E-3	2.75E-4	55.23	<0.0001	0.44	0.98	0.96
TFC (mg Quercetin Eq/g)	25	0.43	0.017	4	2.34	0.59	34.29	<0.0001	0.183	0.85	0.82

F-values imply the models are significant. These values indicate a significant ($p < 0.05$) relationship between independent and response variables and can be used to predict the corresponding responses of the Russian olive leaves extract.

Table 4.7 *Analysis of variance (ANOVA) for the response of Russian olive flowers MAE experimental variables.*

Response	Source						*F*-value	*P-value Prob>f*	*P vale Lack of fit*	R^2	*Adj* R^2
	residual			model							
	df	SS	MS	*df*	SS	MS					
AA_{DPPH} (mg Trolox Eq/g)	15	677.69	45.18	14	4489.22	320.66	7.1	0.0003	0.53	0.87	0.75
AA_{FRAP} (mg FeSO4 Eq/g)	15	1679.10	113.14	14	10937.58	781.26	6.91	0.0003	0.18	0.87	0.74
TPC (mg GAEq/g)	13	53.62	4.12	14	1618.33	115.6	28.02	<0.0001	0.24	0.97	0.93
TFC (mg Quercetin Eq/g)	25	3.45E+6	1.38E+5	4	1.079E+7	2.69E+6	19.53	<0.0001	0.058	0.76	0.72

F-values imply the models are significant. These values indicate a significant ($p < 0.05$) relationship between independent and response variables and can be used to predict the corresponding responses of the Russian olive flower extract.

4.4.4 Verification of the models

The validity of the developed models was determined by conducting the extraction process under obtained optimum processing conditions by the models, at the extraction temperature of 97.4 and 97.5°C and ethanol concentration of 59.8 and 66.6 % for MAE of Russian olive leaves and flowers,

respectively. For both samples solid to solvent ratio of 7.5 (w/v) and citric acid molarity of 2 M was applied. The corresponding experimental values including antioxidant activity, TPC and TFC of the extracts were determined and compared with model-predicted response values and showed a good correlation between the predicted and experimental values, which indicated the suitability of the model in predicting quality attributes of Russian olive extract produced by the optimum microwave extraction process.

4.4.5 HPLC analysis (Comparison of MAE with CE and UAE techniques)

Figure 4.5 provides a comparison of MAE with UAE and CE for extraction of some phenolic compounds of Russian olive including rutin, isorhamnetin and luteolin which are known to have different therapeutic properties including anti-cancer activity (Ganeshpurkar and Saluja 2017; Gong et al. 2020; Seelinger et al. 2008). MAE showed to have superiority over UAE in extracting rutin and luteolin, and when comparing MAE and CE, they showed closely related efficiency in extracting these two Russian olive phenolic compounds. On the other hand, isorhamnetin was only detected in the conventially extracted samples, which shows the superiority of CE over MAE and UAE for extraction of isorhamnetin. However, conventional extraction requires considerably more solvents compared to MAE and also it is more time-consuming. The reason is explained by the greater rate of release of phenolic compounds into the solvent with the greater destruction of the cellular structure by microwaves with higher temperature levels possible in a shorter time (Routray 2014). Thus, the application of MAE as a modern extraction technique with advantages over conventional extraction methods such as lower solvent consumption and extraction time is beneficial in applying bioactive phytochemicals such as rutin and luteolin with therapeutic properties from Russian olive; however, isorhamnetin was only detected in conventionally extracted samples.

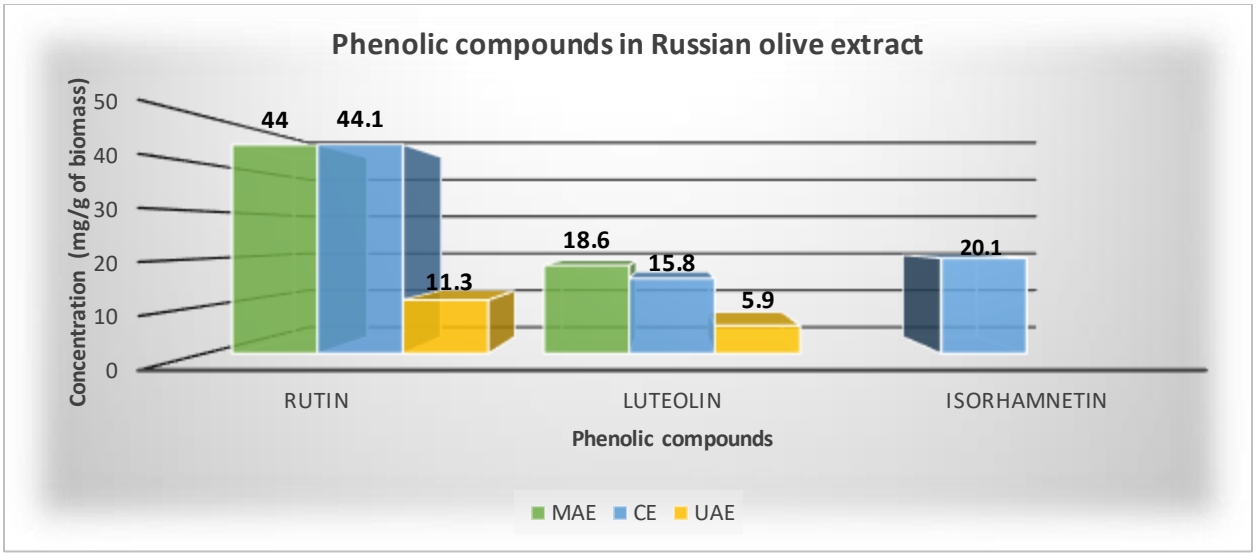


Figure 4.5 *HPLC analysis for comparison of Rutin, Luteolin and Isorhamnetin (mg/g of biomass) in Russian olive extracts obtained by MAE, CE and UAE processes.*

4.5 Conclusion

In this study, the leaves and flowers of the *Elaeagnus angustifolia* tree were shown to be rich in phytochemicals with high antioxidant properties. Microwave extraction, which is one of the most advanced extraction methods, showed promising results for the extraction of active antioxidant compounds in leaves and flowers of Russian olive. The response surface methodology was successfully employed for optimization of microwave-assisted extraction intending to recover maximum yield of compounds with antioxidant properties and an optimized process for maximum extraction of compounds with antioxidant activity from Russian olive was developed. Ethanol concentration in the solvent and the solid to solvent ratio were the most significant factors affecting the MAE of Russian olive leaves and flowers. The overall results confirmed the MAE method as an efficient and time-saving method for the extraction of valuable compounds from natural sources such as antioxidant compounds from Russian olive. The optimized MAE extraction process developed in this study can be useful in the development of industrial extraction processes for the preparation of antioxidant-rich extracts from Russian olive leaves and flowers. The produced

extracts with a maximized yield of antioxidant compounds can have various applications in the food industry for the development of functional foods. Also, considering the potential therapeutic properties of this plant, which can be attributed to its antioxidant compounds, optimized MAE of antioxidant compounds from this plant can have functional food applications towards a healthy society.

4.6 Acknowledgement

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CONNECTING TEXT

The preceding chapter demonstrated that the leaves and flowers of the *Elaeagnus angustifolia* tree are rich in phytochemicals with high antioxidant properties, and an optimized MAE process was developed for the extraction of these compounds. Chapter V of this study reports the use of Russian olive fruit for developing a new non-dairy kefir product called Russian olive water kefir. The fermentation process is developed using a face-centred Central Composite Design (CCD) with three factors including substrate concentration (% of Russian olive), fermentation duration (Time) and temperature (Temp). One of the important properties of Russian olive and water kefir is their antioxidant activity, therefore we aim to optimize the fermentation process with respect to the antioxidant activity of the product, and to develop a beverage with high antioxidant activity. Additionally, due to the importance of the nutritional health benefits of water kefir, which is associated with its water-kefir microorganisms, the viability of these microorganisms is evaluated and optimized during the fermentation process. Finally, The effects of the fermentation factors on the quality of developed RWK product are modeled, and the validity of the model is verified.

CHAPTER V

PROCESS OPTIMIZATION FOR DEVELOPMENT OF A NOVEL WATER KEFIR DRINK WITH HIGH ANTIOXIDANT ACTIVITY AND POTENTIAL PROBIOTIC PROPERTIES FROM RUSSIAN OLIVE FRUIT (ELAEAGNUS ANGUSTIFOLIA)

5.1 Abstract

Kefir is a dairy-based probiotic beverage with high antioxidant activity, among other health benefits. To extend kefir's beneficial health effects to non-dairy consumers, studies on kefir fermentation using alternative matrices (referred to as water kefir) are needed. As such, the purpose of this study was to formulate a novel water kefir beverage using Russian olive, as a non-dairy product with high antioxidant activity and potential probiotic properties. To this end, the Russian olive kefir water (RWK) fermentation process was optimized to maximize the total phenolic content, antioxidant activity, and microbial viability of this product. The experimental design was set using a face centered central composite design with response surface methodology (RSM). The optimized independent variables included: the substrate concentration (20-30 % of Russian olive juice), fermentation time (24-48 h), and incubation temperature (20-32 °C). The optimal fermentation conditions were observed to be 31.2ºC incubation temperature, 24 hours incubation time, and 30 % Russian olive juice concentration. Under these conditions, the values for FRAP antioxidant activity, DPPH radical scavenging, and TPC in RWK were 0.22 (µmol FSHE/mL), 0.096 (µmol Trolox Eq/mL) and 98.32 (µg GAE/mL), and the microbial viability (of AAB, LAB, and yeasts) was 7.20, 7.06, and 7.17 log10 CFU/mL, respectively.

Keywords: Russian olive water kefir, Fermentation, Antioxidant activity, Probiotic

5.2 Introduction

The use of lactic acid bacteria as the primary agent for the production of fermented dairy products has a long history worldwide (Bitaraf et al. 2012; F. Zare et al. 2013; Fonteles et al. 2013; Nguyen et al. 2014). Alternatively, kefir grains, consisting of a symbiotic culture of lactic acid bacteria (LAB), yeast species, and acetic acid bacteria (AAB), can be used as starter cultures for the fermentation of dairy matrices, resulting in a viscous pourable liquid product called kefir. In comparison with traditional fermentation processes, where single-species are used as a starter culture, kefir grains can be adapted to a variety of substrates due to their wide microbial diversity (Fiorda et al. 2017). The main alternative non-dairy substrate for kefir fermentation is sugar, which renders a beverage called water kefir (Marsh et al. 2013). Production of the water kefir is induced by mucilaginous and translucent water kefir grains, which have a polysaccharide matrix backbone (Pidoux 1989; Neve and Heller 2002). In fact, they contain a water-soluble matrix of dextran, a glucose polymer, which can retain the water during the process of fermentation and is firmly embedding the containing microbiota, whereas, in milk kefir, a glucogalactan, which is synthesized mainly by *Lactobacillus kefiranofaciens* holds the starter culture community of kefir (Gulitz et al. 2011; Martínez-Torres et al. 2017). In contrast with milk kefir grains, which employ milk as the substrate for fermentation, water kefir grains use sucrose as the substrate. However, in both products, the microbial communities include yeast, lactic acid bacteria, and acetic acid bacteria, each having different compositions and relative abundance (Martínez-Torres et al. 2017).

Traditionally water kefir is produced by fermentation of 8% sucrose solution containing dried fruits, some lemon slices, and the water kefir grains kept for 1-2 days at room temperature. As a

result of such a fermentation process, a low acid carbonated drink is produced, yellowish, and slightly alcoholic (Gulitz et al. 2011). Kefir and water kefir contain a variety of functional substances providing health benefits such as antioxidant, anti-hyperglycemic, anti-hyperlipidemic, anti-inflammatory, anti-ulcerogenic, and antimicrobial activities (Rodrigues et al. 2005, 2016; Alsayadi et al. 2013; Alsayadi et al. 2014). Water kefir grains contain LAB (including *Lactobacillus paracasei*, *Lactobacillus parabuchneri, Lactobacillus kefiri, Lactobacillus Casei,* and *Lactococcus. lactis*), AAB (including *Acetobacter lovaniensis*), and yeasts (including *Saccharomyces cerevisiae*), and various studies have shown the potential probiotic features of some of these water kefir grains associated microorganisms for application in the development of functional foods. In some water kefir microorganisms, these probiotic features include the capacity for antagonistic activity against potential pathogenic strains, adherence to epithelial cells, resistance to simulated gastric juice and bile salts, surviving transit through the gastrointestinal tract, and the potential for inducing activation of the gut mucosal immune system (Magalhães et al. 2010; Schneedorf 2012; Laureys and Vuyst 2014b; Diosma et al. 2014; Zanirati et al. 2015; Romero-Luna et al. 2020).

The ability of water kefir fermentation to improve the phenolic content profile of a product is mainly attributed to the action of microbial enzymes, which are produced over fermentation, cause degradation of the cell wall structure and subsequent release of bond phenolics. In addition, microbial metabolism of phenolic compounds through a variety of bioconversion pathways, such as glycosylation, deglycosylation, ring cleavage, methylation, glucuronidation, and sulfate conjugation, releases a broad range of new metabolisms (Huynh et al. 2014). For example, lactic acid bacteria in the water kefir grains can contribute to the depolymerization of high molecular weight phenolic compounds (Hur et al. 2014). Hole et al. (2012) reported an increased release of

phenolic acids and flavanols and a significant increase of ferulic acid and p-coumaric acid during fermentation of barely with some LAB strains. Studies have also indicated the scavenging activity of LAB against reactive oxygen species, and water kefir grains contain some of these species (Fiorda et al. 2016b). In addition to LAB strains, different yeasts, such as *Saccharomyces cerevisiae,* available in water kefir grains, are known to improve the wheat bran's free phenolic profile (Moore et al. 2007). On the other hand, various studies reported decreased total phenolic content and antioxidant activity during water kefir fermentation of fruits and vegetables (Corona et al. 2016; Randazzo et al. 2016). Therefore, the final water kefir product's total phenolic content and antioxidant activities are a balance between degradation and synthesis. Moreover, fermentation is desirable for improving the developed product's antioxidant profile because the bio-availability and bio-accessibility of a variety of compounds, including antioxidant compounds such as polyphenols and vitamins, are improved by the activity of a series of different enzymes. In fact, modification of the molecular nature of phenolic compounds during the fermentation process releases new derived compounds with the potential of biological activities such as changing the level of gut immunoglobin and populations of the microbiota. Improved bioavailability of polyphenols during fermentation improves their potential for in situ radical scavenging and enhances natural antioxidant body defences (Septembre-Malaterre et al. 2018). In addition to changes in bioactive compounds, degradation of anti-nutritional factors, increase of nutrient density and secondary metabolites, short-chain fatty acids, and vitamins generated by the water kefir fermentation process result in improving health-related properties of the product.

To date, research has mainly focused on milk substrates to produce kefir as an excellent source of probiotics (Athanasiadis et al. 2004; Varga et al. 2006; Ghasemlou et al. 2012; Melo and Silva 2014; M'hir et al. 2019). To provide kefir's beneficial health effects to non-dairy consumers, a

diversification in alternative non-dairy substrates used for kefir production is necessary. Adaptation of kefir grain with a variety of other non-dairy sources, including fruits and vegetables, has been tested for production of water kefir products as new functional beverages (Puerari et al. 2012; Baú et al. 2013; Cui et al. 2013; Corona et al. 2016; Fiorda et al. 2016b; Fiorda 2016; Randazzo et al. 2016; Rodrigues et al. 2016; Koh et al. 2017; Du and Myracle 2018; Łopusiewicz et al. 2019); however, currently, there is no reported study on the development of non-dairy kefir based on Russian olive fruit (*Elaeagnus angustifolia*). Extracts from fruits of Russian olive are rich in phytochemicals with high antioxidant properties (Wang et al. 2013; Incilay 2014; Farzaei et al. 2015). Investigation of the considerable antioxidant activity of Russian olive by other authors and our preliminary tests confirms the potential of this plant to be used as a valuable food ingredient, including in fermentation processes for producing beneficial fermented products. However, despite its excellent nutritional value and health benefits, Russian olive has not been widely used in the food sector (Farzaei et al. 2015). Moreover, there are numerous bioactivities associated with the consumption of kefir beverages, among which Russian olive shares many, including antioxidant, anti-inflammatory, anti-ulcerogenic, and antimicrobial activities (Rodrigues et al. 2005, 2016; Alsayadi et al. 2013; Alsayadi et al. 2014; Farzaei et al. 2015). Therefore, in this study, water kefir production using Russian olive develops a unique product, in which particular health benefits of water kefir can be enhanced.

Moreover, a standardized industrial process for water kefir production is not presently established, and this beverage is mostly produced at home or on a small scale. The production of water kefir is based on a symbiotic relationship among the kefir microbial community. Different factors can affect their metabolic activities and, in turn, the quality of the produced kefir. These factors include the type of substrate and its ratio to the water kefir grains, incubation time, and temperature

(Randazzo et al. 2016). Given the importance of antioxidant and probiotic activities among water kefir properties, the aim of this study is to develop and optimize the fermentation process with respect to both these activities in the beverage. Accordingly, the effects of fermentation factors such as time, temperature, and substrate concentration on total phenolic content (TPC), antioxidant activity, and viability of microorganisms of RWK were investigated. In addition, characteristics of the optimized water kefir beverage, including total soluble solids Brix (°Br), pH, viscosity, and ethanol content, were assessed. In our study, the use of Russian olive juice as the main substrate in the formulation of a water kefir beverage is proposed.

5.3 Materials and Methods

5.3.1 Experimental design

The experimental design, regression analysis of the data, and model building were performed using the software Design-Expert (version 10.0.7.0, Stat-Ease Inc., Minneapolis, USA). The experiments were developed with three factors using a face-centered Central Composite Design (FCCD), including the central point (five replicates). Factors included concentration of Russian olive (20, 25, or 30 %), fermentation duration (24, 36 or 48 h), and temperature (20, 26, or 32°C), each at three coded levels −1, 0, 1. Twenty experiments in duplicates were conducted for optimization of the RWK fermentation process (Table 5.1).

Table 5.1 *Matrix of the central composite design (CCD) and observed responses (Yj) for different experimental conditions (mean values of three replicates).*

Run	Independent variables			Response variables					
	Time (x_1, h)	Temperature (x_2, ℃)	Russian olive juice (x_3, % v/v)	DPPH (Y_1, µmol Trolox Eq mL^{-1})	FRAP (Y_2, µmol FSHE mL^{-1})	TPC (Y_3, µg GAE mL^{-1})	AAB viability (Y_4, Log_{10} CFU mL^{-1})	Yeast viability (Y_5, Log_{10} CFU mL^{-1})	LAB viability (Y_6, Log_{10} CFU mL^{-1})
1	24.00	20.00	20.00	0.090	0.124	68.803	6.070	5.977	6.875
2	24.00	32.00	20.00	0.097	0.152	56.205	7.197	7.050	7.105
3	24.00	20.00	30.00	0.129	0.175	113.51	6.740	6.633	6.903
4	24.00	32.00	30.00	0.124	0.197	100.765	7.249	7.075	7.130
5	24.00	26.00	25.00	0.111	0.148	89.931	7.431	7.027	7.161
6	36.00	20.00	25.00	0.068	0.154	68.411	6.583	6.201	7.017
7	36.00	26.00	20.00	0.064	0.142	67.215	7.124	6.844	7.208
8	36.00	26.00	25.00	0.073	0.185	86.5	7.163	6.580	7.269
9	36.00	26.00	25.00	0.102	0.168	83.411	7.111	6.538	7.054
10	36.00	26.00	30.00	0.084	0.168	99.147	7.293	6.646	7.278
11	36.00	26.00	25.00	0.073	0.130	59.637	7.547	7.082	7.153
12	36.00	26.00	25.00	0.030	0.113	60.215	7.392	6.830	7.166
13	36.00	26.00	25.00	0.083	0.129	55.470	7.369	6.960	7.236
14	36.00	32.00	25.00	0.083	0.160	74.539	7.288	7.019	7.140
15	36.00	26.00	25.00	0.080	0.115	56.254	7.353	6.752	7.208
16	48.00	26.00	25.00	0.069	0.157	69.637	7.361	6.216	7.021
17	48.00	32.00	20.00	0.017	0.110	46.107	7.432	6.720	7.108
18	48.00	32.00	30.00	0.040	0.225	85.176	7.217	6.907	7.153
19	48.00	20.00	20.00	0.045	0.081	48.411	7.021	6.120	6.645
20	48.00	20.00	30.00	0.051	0.163	88.362	7.047	6.447	7.243

5.3.2 Preparation of water kefir grains and fermentative medium

Field-grown fruits of Russian olive were harvested from a local farm in Iran and Water kefir grains which contain LAB (including *Lactobacillus paracasei*, *Lactobacillus parabuchneri*, *Lactobacillus kefiri*, *Lactobacillus Casei*, and *Lactococcus. lactis*), AAB (including *Acetobacter lovaniensis*) and yeasts (including *Saccharomyces cerevisiae*) were provided by the Happy Kombucha Company, Eastbourne, England. In order to eliminate the influences associated with the previous cultivation process of the supplier, grains (50g/L) were activated in 4 liters of sterilized sucrose solution (10%, w/v) and incubated at room temperature for 24 hours (Magalhães et al. 2010). Incubation was repeated three times before the grains were considered ready to be used. Sucrose solution was prepared by dissolving sucrose in deionized water and was sterilized through filtration using membrane filters with pore sizes of 0.22µm. To prepare Russian olive juice for the experiments, pesticide-free Russian olive fruits were air-dried. Seeds were separated, and exocarp plus endocarp, with a moisture content of 11.8 %, were milled into powder in a blender. Fruit powder was extracted in deionized water by mixing 15.4 g Russian olive with 100 mL water. Following stirring for 30 minutes, the juice was separated through centrifugation for 10 minutes at 10,000 x g. Prepared Russian olive fruit juice was pasteurized at 75 °C for 5 min before its use in the fermentation process.

5.3.3 Preparation of RWK beverage and fermentation conditions

Experiments were performed (2 biological replicates per condition) in 1.0 L Biostat Qplus bioreactors (Sartorius Stedim Biotech, Germany). The working volume in each fermenter vessel was 0.4 L containing 20 grams of water kefir grains (50 g/L), pasteurized Russian olive juice (20, 25, or 30 % v/v, depending on experimental run), and 32 grams of sucrose (dissolved in a volume of deionized water equal to the remaining volume of each bioreactor to give a final concentration

of 8% w/v of sucrose in a 0.4 L bioreactor). The prepared sucrose solutions were filter-sterilized before adding to each bioreactor. During the experiments, the temperature was maintained at 20, 26, or 32°C and fermentation continued for 24, 36, or 48 hours (depending on each condition) while pH was monitored using pH sensors (Model EasyFerm Plus K8 160, Hamilton). Each bioreactor was equipped with an impeller, and stirring was constant at 100 rpm to assure consistency between different runs. At the end of each experiment, samples were taken out from the water kefir liquor through a sampling tube using a syringe. Samples were analyzed in triplicates, and the results were expressed as mean values.

5.3.4 Evaluation of the total phenolic content through the Folin–Ciocalteu assay

For measuring the total phenolic content of the samples, the Folin-Ciocalteu assay was used using gallic acid as the standard following a method by Pientaweeratch et al. (2016) with slight modifications. Briefly, 20 µL of each test sample was mixed with 80 µL of sodium carbonate (7.5% in deionized water) and 100 µL of Folin-Ciocalteu reagent (diluted 10-fold in deionized water) in a 96-well plate. After 60 minutes of incubation at room temperature, the absorbance was measured at 765 nm using a microplate reader, and the results were expressed as µg gallic acid equivalent (GAE) /mL of sample.

5.3.5 Determination of antioxidant activity

The total antioxidant capacity of the samples was measured using 2 spectrophotometric assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and ferric reducing antioxidant power (FRAP) assay (Brand-Williams et al. 1995; Benzie and Strain 1996).

5.3.5.1 Estimation of DPPH radical-scavenging capacity

The antioxidant activities of the samples were determined using a modified DPPH assay introduced by Brand-Williams et al. (1995). Briefly, 250 µL of prepared DPPH solution (0.2 mM) was added to 25 µL of water kefir samples in a 96-well microplate. The mixtures were incubated for 30 minutes, and then absorbance was determined at 517 nm using a microplate reader. Finally, the results were expressed as µmol Trolox equivalent /mL of sample.

5.3.5.2 Determination of ferric reducing antioxidant power (FRAP)

For measuring the antioxidant activity of the samples using the FRAP assay, a modified method introduced by Benzie and Strain (1996) was followed. Briefly, 250 µL of the freshly made FRAP reagent was mixed with 25 µL of water and 8.5 µL of the samples in a 96-well microplate. After 30 minutes of incubation, the absorbance was read at 593 nm using a microplate reader. The results were expressed as µmol $FeSO_4.7H_2O$ equivalent (FSHE)/mL of the sample.

5.3.6 Determination of lactic acid bacteria, yeast, and acetic acid bacteria cell viability

Samples were prepared according to the Association of Official Analytical Chemists' guidelines (AOAC 1990). Isolation of LAB, AAB, and yeasts in the samples was carried out by plating different dilutions on appropriate media following methods by ISO (1998), Gulitz (2013), Atalar and Dervisoglu (2015) and Hsieh et al. (2012) with slight modifications. Viable bacteria or yeasts in the samples were quantified using the drop plate method. Following sequential dilution stages, a serial dilution was prepared using a sterile saline solution (0.85% sodium chloride solution, pH 7.2–7.4). Then, ten µL of each dilution was transferred to the appropriate agar plate for LAB, AAB, and yeasts. After the growth of colonies at optimum conditions, they were counted, and the number of bacteria/yeast in the original samples was calculated as colony-forming units per mL (CFU/mL). LAB count was performed on de Man, Rogosa and Sharpe (MRS) agar (pH 5.7), in

which cycloheximide (150 µg/mL) was added to inhibit the growth of yeasts. The MRS agar plates were incubated for 72 hours at 30 °C. AAB was enumerated on GM agar (pH 6.0) containing cycloheximide (150 µg/mL) to inhibit the growth of yeasts followed by incubation at 30 °C for 72 hours. For yeast isolates, YPG agar (pH 6.5) was used, in which chloramphenicol (100 mg/L) was added to inhibit bacterial growth and bromphenol blue (0.01 g/L) was mixed for morphological differentiation following 3 days of incubation at room temperature.

5.3.7 Characterization of optimized RWK beverage

Samples of the optimized RWK beverage were subjected to several physicochemical characterizations following the standard methodologies described by AOAC (2000). The pH values were determined electrometrically using a pH probe (Mettler Toledo, Switzerland). Soluble solids content (SSC) was evaluated using a portable refractometer and reported as degree Brix (°Br). Rheological properties of the sample were measured according to Nindo et al. (2005) with modifications and using a controlled stress rheometer (Discovery Hydrid Rheometer HR-3, TA Instruments, Delaware, USA). The instrument was equipped with a concentric cylinder geometry with a 30.33 mm cup, and a gap of 1 mm was employed. Before measurements were taken, five minutes was allowed for sample equilibration, and sample viscosity was determined by linearly increasing the shear rate from 1 to 100 1/s at 25 C. The ethanol content of water Kefir samples was measured following Atalar and Dervisoglu (2015) method. Briefly, samples were filtered on a 0.45 mm membrane filter and injected into a gas chromatograph equipped with a flame-ionization detector. In addition, the TPC and antioxidant activity of the optimized product were measured using previously discussed assays.

5.3.8 Statistical analysis

Response surface methodology (RSM) was used to determine the effects of three experimental variables and their interactive relationship on the response variables. All responses were equally weighted and using analysis of variance Fisher test value (ANOVA F-test), response variables were analyzed to identify the significant factors and accuracy of the suggested model. Subsequently, P-value ($p \leq 0.05$), as well as the coefficient of determination R^2 and R^2_{adj}, were used to express the quality of the fit of the polynomial model.

5.3.9 Verification of the models

When the Russian olive water kefir fermentation process's optimal conditions were determined, the fermentation process was conducted under these conditions to verify the models' validity by comparing the corresponding experimental values with model-predicted response values.

5.4 Results and discussion

5.4.1 Effects of the experimental variables on total phenolic content (TPC) and antioxidant activities (FRAP and DPPH) of RWK

Figure 5.1 illustrates the variation of TPC and antioxidant activity with Russian olive juice concentration, time, and temperature. The data obtained indicates that with the increase in Russian olive juice concentration, TPC and DPPH radical scavenging activity significantly ($p < 0.01$) increased, reaching the highest level at the concentration of 30 % Russian olive juice. On the other hand, the values for TPC and DPPH of RWK decreased by increasing fermentation time and temperature (Table 5.2). It was observed that at a time of 24 h and a temperature of 20°C, TPC and DPPH radical scavenging activity reach the highest level. At a higher temperature than 20°C and after 24 hours of fermentation, lower DPPH radical scavenging activity was observed. One possible explanation might be that at higher temperatures, close to the optimum temperature for

microbial metabolic activities, the structure of phenolic compounds is influenced by the action of microbial enzymes converting them into other molecules, which in turn affects the antioxidant activity of the beverage (Ekbatan et al. 2016). Also, the stability of some of the natural phenols and antioxidant compounds is pH-dependent; therefore, the content and structure of the phenolic compounds are influenced by changes in pH throughout the fermentation, which ultimately affects the total phenolic content and antioxidant activity of the product (Hur et al. 2014). However, there could be a certain level of improved antioxidant activities attributed to microbial-derived phenolic metabolites (Ekbatan et al. 2016). At first, water kefir fermentation can increase the total phenolic content and antioxidant activity by inducing the Russian olive cell wall's structural breakdown through hydrolysis by microbial enzymes, releasing various bioactive compounds inducing their synthesis. Moreover, Huynh et al. (2014) illustrated a summary of various metabolic pathways of phenolic compounds by microbial fermentation, among which there are particular metabolic pathways for the phenolic compounds and microorganisms, which are shared with Russian olive and water kefir grains, respectively. Therefore, in addition to microorganisms' action on releasing bound phenolic compounds from the plant cell walls, they can also convert phenolic compounds into more simplified metabolites for improving their bioactivities (Heim et al. 2002; Huynh et al. 2014). Subsequently, enhancement of the phenolic profile of Russian olive and its antioxidant properties over water kefir fermentation is anticipated.

Due to synergism between the polyphenolic compounds and other components in the fermentation, which contributes to the total antioxidative activity of the RWK, it cannot be predicted based on TPC alone. However, there is a correlation between TPC and antioxidant activity, particularly with the DPPH radical scavenging observed in this study. The conclusion is that an increase in TPC with higher Russian olive juice concentration and reduced fermentation time can increase the

antioxidant activity (FRAP and DPPH radical scavenging activity) of RWK. Overall, our results showed that total phenolic content and antioxidant activity of RWK are decreased throughout the fermentation process (compared to the starting point of the fermentation process (time zero) with the values of 132.4 µg GAE/mL, 0.35 µmol FSHE/mL, and 0.18 µmol Trolox Eq/mL for TPC, FRAP antioxidant activity and DPPH radical scavenging activity, respectively). It can be explained by fermentative changes to the bioactive compounds, including phenolic compounds, which are converted to other molecules with varied biological activities. However, they remain significantly higher than the control water kefir sample produced using sucrose solution (with the values of 6.3 µg GAE/mL, 0.014 µmol FSHE/mL, and 0.021µmol Trolox Eq/mL for TPC, FRAP antioxidant activity, and DPPH radical scavenging activity, respectively). In a similar study by Randazzo et al. (2016) on water kefir fermentation of different fruit juice, all fruit juices showed a decreased TPC (up to 53 % decrease) and reduced DPPH antioxidant activity (up to 19%) after water kefir fermentation. Corona et al. (2016) also reported an up to 49 % decrease in the total phenolic content of juice after water kefir fermentation. When sucrose is used as the only carbon source for water kefir production, Fiorda et al. (2016b) and Alsayadi et al. (2013) indicated improvement in the total phenolic compound and antioxidant activity after fermentation. Similar observations of increased antioxidant activity were made in our control sample, in which sucrose was used as the only carbon source in the media for the water kefir fermentation process (increased level of 1.92 µg GAE/mL, 0.009 µmol FSHE/mL, and 0.014 µmol Trolox Eq/mL for TPC, FRAP antioxidant activity and DPPH radical scavenging activity, respectively). In this study, when Russian olive and sucrose were combined as the source of carbon for fermentation, the total antioxidant activity of the RWK samples decreased after fermentation. However, despite the decrease in antioxidant activity, the results were significantly higher than for the water kefir samples fermented using

sucrose alone. This is in accordance with our hypothesis; by replacing the standard sucrose solution or even part of it with Russian olive with high antioxidant activity, the antioxidant properties of the produced water kefir can be considerably increased compared to water kefir in which only sucrose is used as the substrate.

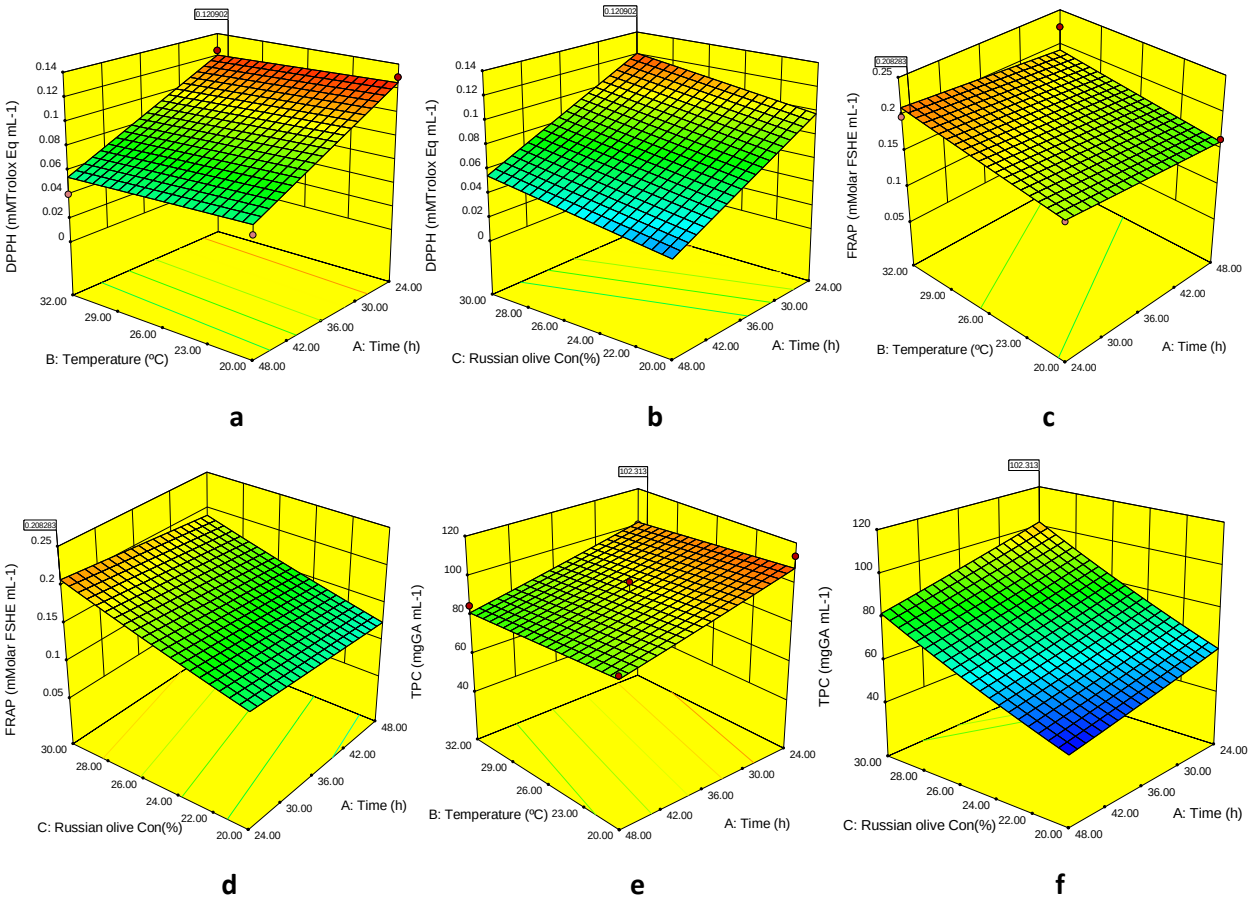


Figure 5.1 *3D plots showing the combined effect of time, temperature and Russian olive juice concentration on (A-B) AA_{DPPH} radical, (C-D) AA_{FRAP} and (E-F) TPC.*

5.4.2 Effects of the experimental variables on LAB, AAB, and yeast cell viability of water kefir microorganisms in RWK

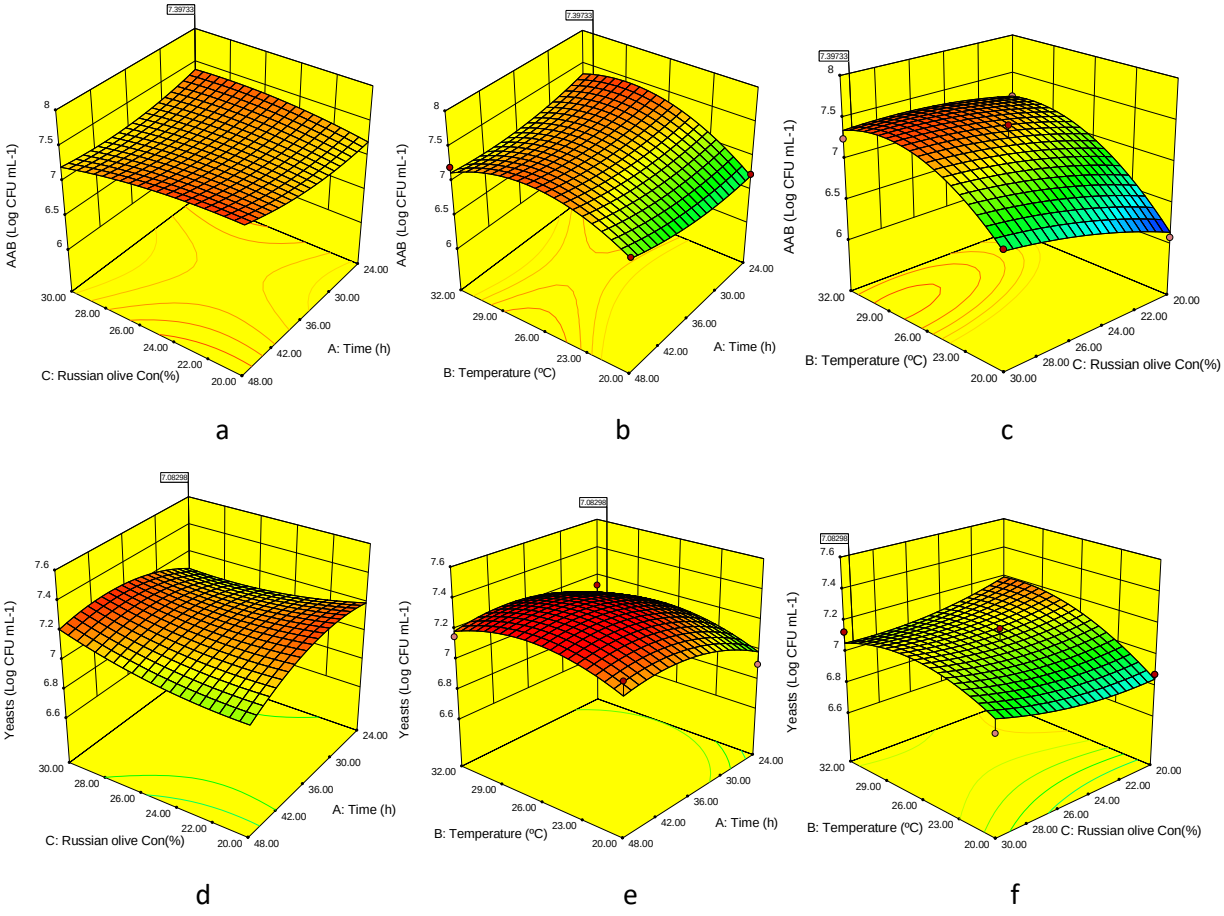
Production of water kefir is based on a symbiotic relationship among the kefir microbes (including yeasts, LAB and AAB). Different factors can affect their metabolic activities, among which the concentration of substrate, incubation time, and temperature were evaluated in this study. Despite some unique features, the results regarding the effect of independent experimental variables on

AAB, LAB, and yeasts of RWK beverage showed a similar trend, including the positive effect of temperature and Russian olive juice concentration on cell viability of RWK microorganisms (Figure 5.2). From the ANOVA of the response surface quadratic model, there were significant effects ($p < 0.05$) of the fermentation variables on the microbial proliferation of RWK microorganisms, producing first-order significant ($p < 0.01$) linear effect of temperature on AAB, LAB, and yeasts and significant interactive effects (Time × Temp) and (Time × Russian olive Con) on AAB and yeast respectively (Table 5.2). The number of viable cells of AAB and LAB was shown to be significantly ($p < 0.05$) affected by the time of fermentation; however, it showed to have no significant effect on the viability of yeast cells. (Figure 5.2B, E, and G). The highest number of cells for AAB and yeasts was shown to be at 47 and 36 hours of fermentation, whereas Figure 5.2 showed the highest number of cells for LAB at 27 hours of fermentation. Longer fermentation time for optimum growth and viability of AAB and yeasts compared to the LAB can be due to AAB and yeasts' symbiotic interaction during water kefir fermentation. The presence of ethanol, produced by the yeast cells, facilitates the growth of acetic acid bacteria. Acetic acid bacteria transform glucose to gluconic acid and fructose into acetic acid and, thereby, the increased production of acetic acid stimulates the yeast cells to convert sugar and produce ethanol via the glycolysis metabolic pathway (Ayed et al. 2017). Moreover, increased fermentation time was shown to positively affect AAB's proliferation but negatively affected the growth of the LAB and yeast cells. This can be due to the increased production of ethanol with increased fermentation time, which consequently facilitates the growth of acetic acid bacteria. During the kefir fermentation, other microbial interactions can exist; however, the mechanisms of symbiotic interaction of microorganisms of kefir grains are still not well known (Fiorda et al. 2017). The optimal proliferation of AAB, LAB, and yeasts was observed at 28, 31, and 26 °C, respectively.

Figure 5.2 (B, E, and G) presents a gradual linear increase in cell growth of AAB, LAB, and yeasts when the fermentation temperature increased to close to their optimum temperature for growth. The increase in LAB and AAB was more significant ($p < 0.05$) than for yeasts, as indicated by the highest coefficients in Table 5.2. This linear effect is confirmed as the concentration of Russian olive juice increased. Mishra and Mishra (2015) also reported free sugar and temperature as important factors for guaranteeing a sufficient number of viable probiotic cells in fermented products. An increase in AAB, LAB, and yeast cells upon the increasing concentration of Russian olive juice in RWK could be attributed to the increased availability of nutrients, which may sustain water kefir microorganisms' metabolic activities at the accumulation of a higher concentration of organic acids. Consequently, the accessibility to essential nutrients, including carbon and nitrogen sources for the growth of bacteria and yeast, can maintain a high level of microbial proliferation.

Overall, the fermentation of water kefir is traditionally performed at room temperature. Commonly used incubation time and temperatures are recommended as 25°C /24 h by Magalhães et al. (2010) and Marsh et al. (2013) and 21 °C /24 h by Alsayadi et al. (2013). However, for the growth of mesophilic bacteria and yeasts present in the water kefir grains, 32 °C is reported as the optimum temperature by Koh et al. (2017). The results of our study indicate 24 hours of fermentation at 31.2°C as the optimized conditions for the maximum level of cell growth. This aligns with the most relevant study by Koh et al. (2017), who reported 24 h at 32 °C as the optimal conditions for the fermentation of pumpkin water kefir with good overall acceptability and higher microbial proliferation. According to the Canadian Food Inspection Agency (2019) and Codex Alimentarius Commission (2018), in order to exert a beneficial health impact, the recommended number of viable cells in fermented food products is 1.0×10^9 CFU of one or more of the eligible

probiotic microorganism(s) per serving size of a product. On the other hand, according to Bertazzoni et al. (2013), the volume of the consumed probiotic drink is often 100-200 mL. According to Table 5.4, the number of viable cells of AAB, LAB, and yeasts in the optimized RWK is 7.20, 7.06, and 7.17 $\log_{10}$ CFU/mL, respectively. Therefore, a minimum number of 10^9 live probiotics per 100-200 mL serving size of RWK is expected to be reached and shows that the optimized RWK can potentially deliver health benefits to the target host as a potential probiotic drink, which can be confirmed through future in vivo studies.



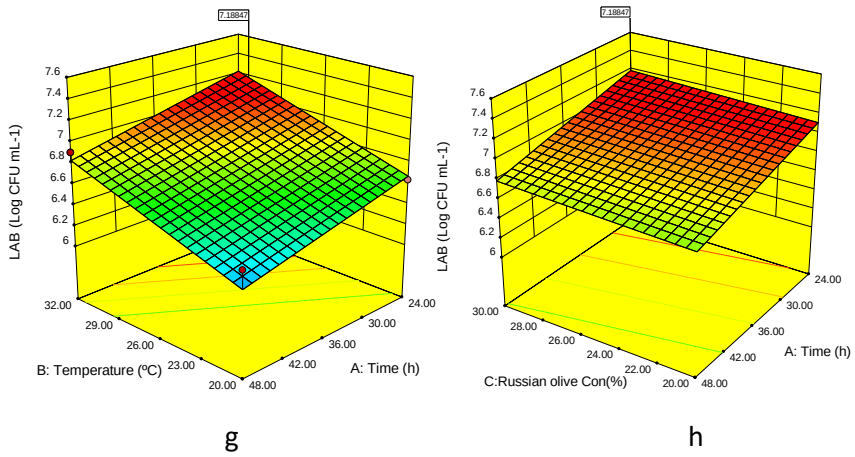


Figure 5.2 *3D plots showing the combined effect of time, temperature and Russian olive juice concentration on the cell viability of Acetic acid bacteria (A–C), yeasts (D–F) and Lactic acid bacteria (G–H).*

5.4.3 Model fitting

Using a second-order polynomial equation, the independent and response variables were fitted to the experimental data. Table 5.2 presents the linear and quadratic equations, as well as the statistical significance for each response. As shown in Table 5.3, an insignificant P-value ($p > 0.05$) for lack of fit for all investigated variables indicates satisfactory accuracy of the six proposed mathematical models for estimation of the corresponding variable responses. The high coefficients of determination (R^2 values) of the linear and quadratic polynomial models indicate that the experimental data fit the equation effectively. In addition, to validate the model's accuracy, adj- $R^{2,}$ and coefficient of variation (CV) were estimated. The low CV values (0.67-15.35%) indicate high precision of the experiments, and adequate precision values greater than 4 (9.17-16.79) are desirable and imply reliability of the models with good signal to noise ratios.

Table 5.2 *Regression equation coefficients for the response of RWK fermented product response models.*

Effects	Response											
	AAB viability (Log_{10} CFU mL^{-1})		**Yeast viability (Log_{10} CFU mL^{-1})**		**LAB viability (Log_{10} CFU mL^{-1})**		**AA_{DPPH} (µmol Trolox Eq mL^{-1})**		**AA_{FRAP} (µmol FSHE mL^{-1})**		**TPC (µg GAE mL^{-1})**	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Intercept												
β_0	7.30		1.972		6.712		0.078		0.387		6.880	
Linear												
β_1	0.13	0.016*	-0.0002	0.962	-0.196	0.007**	-0.032	<0.0001**	-0.009	0.299	-0.380	0.015*
β_2	0.29	0.0001**	0.013	0.008**	0.277	0.0006**	-0.002	0.593	0.018	0.056	-0.100	0.484
$\beta 3$	0.07	0.178	0.010	0.025*	0.038	0.562	0.011	0.009**	0.041	0.0004**	0.837	<0.0001**
Interaction												
β_{12}	-0.13	0.035*	-0.001	0.787								
β_{13}	-0.11	0.063	0.010	0.046*								
$\beta 23$	-0.11	0.075	-0.010	0.057								
Quadratic												
β_{11}	0.11	0.260	-0.014	0.102								
$\beta 22$	-0.35	0.003**	-0.016	0.072								
$B33$	-0.07	0.426	0.006	0.402								

Effects are statistically significant 0.01≤P< 0.05, P** < 0.01. The coefficients of the polynomial model include the constant term (β_0), linear effects (β_1, β_2, β_3), quadratic effects (β_{11}, β_{22}, β_{33}) and interaction effects (β_{12}, β_{13} and β_{23}).*
(RWK: Russian olive water kefir, CFU: Colony-forming unit, AAB: Acetic acid bacteria, LAB: Lactic acid bacteria, AA_{DPPH}: Antioxidant activity value using 1,1-diphenyl-2-picrylhydrazyl method, AA_{FRAP}: Antioxidant activity value using ferric reducing antioxidant power test, FSHE: Ferrous sulfate heptahydrate equivalent, TPC: Total phenolic content, GAE: Gallic acid equivalent)

Table 5.3 *Analysis of variance (ANOVA) for the response of Russian olive water kefir fermented products experimental variables.*

Response	Source						*F*-value	*P-value Prob>f*	*P vale Lack of fit*	R^2	*Adj* R^2
	residual			model							
	df	SS	MS	*df*	SS	MS					
AA_{DPPH} (µmol Trolox Eq mL^{-1})	15	0.002	0.0001	3	0.012	0.004	27.98	<0.0001	0.518	0.84	0.81
AA_{FRAP} (µmol FSHE mL^{-1})	15	0.013	0.0008	3	0.022	0.0072	8.66	0.0014	0.908	0.64	0.56
TPC (µgGA mL^{-1})	16	3.17	0.20	3	8.56	2.85	14.42	<0.0001	0.949	0.73	0.68
AAB viability (Log_{10} CFU mL^{-1})	10	0.24	0.024	9	2.01	0.22	9.49	0.0008	0.574	0.90	0.80
Yeast viability(Log_{10} CFU mL^{-1})	10	0.0017	0.00017	9	0.0074	0.0008	4.77	0.0114	0.214	0.81	0.64
LAB viability (Log_{10} CFU mL^{-1})	10	0.31	0.031	3	1.16	0.39	10.8	0.0005	0.711	0.68	0.62

F-values imply the models are significant. These values indicate a significant (p < 0.05) relationship between independent and response variables *and can be used to predict the corresponding responses of the RWK beverage*

5.4.4 Process optimization

Using the RSM response optimizer, the overall optimum region was determined to be at the temperature of 31.2ºC, fermentation time of 24 hours, and the 30 % concentration of Russian olive. According to the results of the optimization process, predicted response values under the optimum conditions for FRAP, DPPH radical scavenging, and TPC were 0.199 (µmol FSHE/mL), 0.121 (µmol Trolox Eq/mL), and 101.939 (µg GAE/mL) respectively. Also, the predicted values for microbial viability (of AAB, LAB, and yeasts) were 7.39, 7.18, and 7.08 $\log_{10}$ CFU/mL, respectively. Regression coefficients of the developed linear and quadratic models exhibited significant ($p < 0.05$) relationships between the dependent variables and corresponding responses of the final RWK product. The optimum value of 0.718 was selected for the desirability function.

5.4.5 Verification of the models

The models' validity was accomplished by conducting the water kefir fermentation process under optimal conditions determined by the models, at the temperature of 31.2ºC, fermentation time of 24 hours, and using the 30 % concentration of Russian olive. The corresponding experimental values were compared with model-predicted response values (Table 4). The RMSE value, which is the square root of the residuals variance, indicates the absolute fit of the model to the data and illustrates how close the observed values for the optimized fermentation condition are to the model's predicted values. The low RSME values prove that the experimental model's predicted values are in good agreement with the experimental results. RSME values (ranging from 0.02-3.62) imply that the proposed model could accurately predict the responses in real experimental conditions. Finally, the physicochemical properties of the produced RWK, including pH, ethanol content, soluble solid content, and viscosity, were measured and reported as 4.1, 0.65 (%), 9.7 (°Br), and 1.52 (cP), respectively.

Table 5.4 *Predicted and experimental values of the responses at optimum conditions.*

Predicated	Experimental	Response variable	Root Mean Square Error
101.939	98.32	TPC (µg GAE/mL)	3.62
0.121	0.096	DPPH (µmol Trolox Eq/mL)	0.03
0.199	0.22	FRAP (µmol FSHE/mL)	0.02
7.39	7.20	AAB viability (Log_{10} CFU/mL)	0.19
7.18	7.06	LAB viability (Log_{10} CFU/mL)	0.12
7.08	7.17	Yeast viability (Log_{10} CFU/mL)	0.09

5.5 Conclusion

By using Russian olive in water kefir fermentation, a novel non-dairy (water kefir) beverage with enhanced bioactivities is developed. In addition, this is the first study where the fermentation process of RWK is optimized using advanced bioreactors and response surface methodology. The developed predictive models for all responses of interest of the water kefir yielded predictable and reproducible results, and the verification of the models showed a close agreement between the experimental values and the predicted values. RSM predicted that a set level of 30 % Russian olive juice concentration over 24 hours of fermentation at the temperature of 31.2ºC would provide the optimum conditions for preparing water kefir beverages with maximal TPC, antioxidant activity, and microbial viability of water kefir microorganisms. Under the optimum fermentation conditions, the values for FRAP, DPPH radical scavenging, and TPC in RWK were 0.22 (µmol FSHE/mL), 0.096 (µmol Trolox Eq/mL) and 98.32 (µg GAE/mL), and the microbial viability (of AAB, LAB, and yeasts) were 7.20, 7.06, and 7.17 log10 CFU/mL, respectively. The optimized RWK process in this study can facilitate its production at a larger scale for satisfying a wider range of non-dairy consumers with limited availability of kefir-like products.

5.6 Acknowledgments

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5.7 Conflicts of interest

The authors declare no conflict of interest.

CONNECTING TEXT

Considering water kefir as a probiotic product beneficial to health, consumer's interest in the consumption of this product is expected to increase (Atalar and Dervisoglu 2015). Therefore, drying water kefir into its powder form improves commercialization of this product by minimizing challenges that affect its stability, such as post-acidity, gas production and released microbial metabolites (K. Chen et al. 2011). Also, it is important to microencapsulate water kefir microorganisms in Russian olive water kefir to improve their viability against possible severe conditions in the food matrices throughout storage. Therefore, Chapter VI of this study investigates spray drying for microencapsulation of water kefir microorganisms and antioxidant compounds in Russian olive water kefir. Also, this chapter aims to evaluate the effects of spray drying conditions on the bioactive properties of Russian olive water kefir and optimize the process conditions for producing a high-quality Russian olive water kefir powder. In addition, we aim to evaluate the functional properties of the spray-dried powder and compare it with freeze drying, which is known as the best drying method for preservation of bioactive properties of powders (Atalar and Dervisoglu 2015).

CHAPTER VI

ENCAPSULATION OF RUSSIAN OLIVE WATER KEFIR AS AN INNOVATIVE FUNCTIONAL DRINK WITH HIGH ANTIOXIDANT ACTIVITY

6.1 Abstract

Processing of Russian olive water kefir (RWK), as a fermented functional drink made with Russian olive juice and water kefir grains with high antioxidant activity, into a powder is crucial for improving its stability for the commercialization of this product. For the first time, this study aimed to encapsulate water kefir microorganisms and bioactive compounds in RWK using carrier materials to develop a synbiotic functional powder using spray drying as an encapsulation method. The goal was to maximize antioxidant activity, product yield, and survival rates of water kefir microorganisms in the Russian olive water kefir powder. The optimal spray drying conditions were observed to be at an inlet air temperature of 120ºC, 35 % feed flow rate, and 7% concentration of drying aid. The effects of spray drying conditions on the quality of microcapsules were assessed and modeled, and the validity of the model was verified. Also, the spray-dried powder's physicochemical properties were assessed and showed promising microbial and physicochemical characteristics compared with the freeze-dried powder.

Keywords: Russian olive, Water kefir, Encapsulation, Spray drying, Optimization, Fermentation

6.2 Introduction

Water kefir is a non-dairy fermented drink based on a sugar solution, which is low acid, straw-colored, and slightly alcoholic. Production of the water kefir is induced by mucilaginous and

translucent water kefir grains, consisting of a symbiotic culture of lactic acid bacteria (LAB), yeast species, and acetic acid bacteria (AAB) (A. Gulitz et al. 2011; Pidoux 1989). Due to the associated nutritional-health benefits, high antioxidant properties of water kefir, and its specific sensory properties, non-dairy consumers' interest in this product's consumption is expected to increase (Atalar and Dervisoglu 2015). On the other hand, according to existing studies, Russian olive shares many bioactivities with water kefir, including antioxidant, anti-inflammatory, anti-ulcerogenic, and antimicrobial activities (Alsayadi et al. 2013; Alsayadi et al. 2014a; Rodrigues et al. 2005; Farzaei et al. 2015). Therefore, by using Russian olive in water kefir fermentation, a novel non-dairy kefir (water kefir) beverage with enhanced bioactivities, called Russian olive water kefir (RWK), is developed by Darvishzadeh et al. (2021a). Water kefir fermentation can improve the antioxidant activity of the product through hydrolysis by microbial enzymes which induces the Russian olive cell wall's structural breakdown, by releasing numerous bioactive compounds and inducing their synthesis. In addition to microorganisms' action on releasing microbial-derived phenolic metabolites, they can also improve bioactivities of phenolic compounds by converting them into more simplified metabolites and subsequently, enhance the phenolic profile of Russian olive and its antioxidant properties over water kefir fermentation (Darvishzadeh et al. 2021a). RWK could be considered as an attractive product meeting modern consumer demands, while similar to other fermented products, the short shelf life of water kefir is a common limitation in the commercial production of this product. Microbial metabolites affect water kefir sensory properties such as taste and flavour during the storage. In addition, probiotic-containing food products require previous encapsulation by different methods such as spray drying and freeze drying to guarantee probiotics' survival and provide a powder for easier storage and handling (Barbosa and Teixeira 2017). The purpose of this study is to encapsulate water kefir

bioactive compounds by spray drying and using carrier materials with prebiotic properties, thus improving the stability of this product and its commercialization potential through reduced storage, packaging, transportation, and refrigeration associated costs. In addition, considering spray drying as a thermal process, it is essential to maintain the viability of the beneficial water kefir microorganisms and antioxidant properties of water kefir. Therefore, this study aims to use an optimization method to produce a high-quality Russian olive water kefir powder with maximized product yield, microbial viability, and antioxidant activity, and finally to compare the spray drying and freeze drying for producing RWK powder.

6.3 Materials and Methods

6.3.1 Sample preparation

To prepare Russian olive juice for the experiments, air-dried and pesticide-free Russian olive fruits were milled into powder which was extracted in deionized water. Then the juice was separated through centrifugation and was pasteurized at 75 °C for 5 min before its use in the experiments. Then, following Darvishzadeh et al. (2021a), Russian olive water kefir was produced. Briefly, 5% water kefir grains were added to 8% sterilized sucrose solution and 30 % pasteurized Russian olive juice and were allowed to ferment for 24 hours at 31.2 °C in a bioreactor (Sartorius Stedim Biotech, Germany). Water kefir grains which contain LAB (including *Lactobacillus paracasei*, *Lactobacillus parabuchneri*, *Lactobacillus kefiri*, *Lactobacillus Casei*, and *Lactococcus. lactis*), AAB (including *Acetobacter lovaniensis*), and yeasts (including *Saccharomyces cerevisiae*) provided by the Happy Kombucha company, Eastbourne, England (Magalhaes et al. 2010). Water kefir grains were removed from the produced RWK drink at the end of fermentation. RWK samples were mixed with an appropriate concentration of drying aid (maltodextrin13-17 DE (MD)/gum Arabic (GA) 50:50 mix), and the homogenized mixtures were prepared for the drying

experiments. The number of viable cells of AAB, LAB, and yeasts in RWK drink was 7.46, 7.44, and 7.17 $\log_{10}$ CFU/mL, respectively.

6.3.2 Drying experiments

6.3.2.1 Spray drying and freeze drying

Spray drying processes were performed based on the generated experimental design presented in Table 6.1 and using a lab-scale dryer (Buchi™ B-290 mini spray dryer), while the aspiration and airflow rate were set constant at 100% and 414 L/h, respectively. For freeze drying, RWK samples were frozen at -40 °C and lyophilized for 72 h using a lab-scale vacuum freeze-dryer (7670520, Labconco Co., USA). The dried RWK powders were collected in glass jars, impermeable to oxygen and moisture for further analysis.

6.3.3 Determination of antioxidant activity

Samples were rehydrated in 50 % methanol solution (1:40 ratio) and centrifuged at 5000 rpm for 10 min. The supernatant was separated for measuring the total antioxidant capacity of the samples, using two spectrophotometric assays, including ferric reducing antioxidant power (FRAP) assay following Benzie and Strain (1996), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay introduced by Brand-Williams et al. (1995).

6.3.4 Determination of water kefir microorganism' cell viability

Samples of RWK powders were rehydrated to the initial solids content of the RWK drink. Following sequential dilution stages and using sterile saline solution, a dilution series of the rehydrated samples were prepared and following the drop plate method aliquots of 0.1 ml of the suspension were plated on the appropriate agar plate for isolation of water kefir microorganisms. Isolation of LAB and AAB cells was performed on de Man, Rogosa and Sharpe (MRS) agar (pH 5.7) and GM agar (pH 6.0), respectively. Both plates, which contained cycloheximide (150 µg/mL)

to inhibit yeasts' growth, were incubated for 72 hours at 30 °C. Isolation of the yeast cells was performed on YPG agar (pH 6.5), in which chloramphenicol (100 mg/L) was added to inhibit bacterial growth and bromphenol blue (0.01 g/l) was mixed for morphological differentiation. The YPG agar plates were incubated for 72 hours at room temperature. After the growth of colonies at optimum conditions, the number of viable bacteria/yeast cells was enumerated and calculated as colony-forming units per gram of RWK powder (CFU/g) (A. J. Gulitz 2013; Atalar and Dervisoglu 2015).

6.3.5 Characterization of optimized RWK powder

6.3.5.1 Particle size, rehydration time, and water solubility index

The microcapsules' particle size was measured using a vapour pressure scanning electron microscope (VPSEM, Hitachi® S-3400N, Japan), operating at 10 Pa and 5 kV, and using two magnifications of 100x and 1000x under vacuum pressure. To determine the dried samples' rehydration time, the required time for complete rehydration of the powder was recorded following a procedure by Pereira et al. (Pereira et al. 2014). The powders' water solubility index was calculated as the ratio of dried supernatant weight to the initial powder weight, as described by Singh et al. (Singh et al. 2019) with modifications.

6.3.5.2 Product yield, moisture content, and water activity

To calculate the powder yield, the total solids collected after drying were divided by the soluble solids in the samples before drying and was reported as a percentage (%) (Pereira et al. 2014). The samples ' moisture content and water activity were determined following the standard methodologies and using a Decagon Aqualab water activity meter, respectively (International 2005).

6.3.5.3 Soluble solids content, titratable acidity, and pH

Soluble solids content (SSC) of the samples was measured using a portable refractometer and reported as degree Brix (°Br). The samples were rehydrated in water to the initial concentration of RWK dry matter, and pH values were measured using a pH probe (Mettler Toledo, Switzerland). Titratable acidity of the samples was determined (as citric acid) by titration with 0.1 N NaOH.

6.3.6 Experimental design and statistical analysis

The software Design-Expert (version 10.0.7.0, Stat-Ease Inc., Minneapolis, USA) was used for designing the experiments, model building, and regression analysis of the obtained data. Experiments were developed based on a Central Composite Rotatable Design (CCRD), and the independent variables were selected as inlet air temperature (120-170 °C), feed flow rate (15-35%), and drying aid concentration (5-15%), each at each at five coded levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) (Table 6.1). The responses included product yield, antioxidant activity, and water kefir microorganisms' survival rates in the water kefir powder. Response surface methodology (RSM) was used to evaluate three experimental factors' effects and their interactive effects on the spray drying process's dependent variables. For analyzing the process response variables and identifying the significant factors, the analysis of variance Fisher test (ANOVA F-test) was used, and the suggested model's accuracy was determined. Subsequently, to express the quality of the polynomial models' fit and their accuracy, P-values, coefficient of variation (CV), and the coefficient of determination R^2 and adj- R^2 were determined (Table 6.2).

Table 6.1 *Matrix of the central composite design (CCD) and observed responses (Yj) for different experimental conditions (mean values) of spray drying of RWK.*

Run	Independent variables			Response variables							
	Temp.	Feed flow rate	Drying aid Con.	AA_{FRAP}	AA_{DPPH}	LAB viability	AAB viability	Yeast viability	Recovery	Moisture	Outlet temp.
	(°C)	(%)	(% w/v)	(µmol AAEq/g)	(µmol TroloxEq/g)	(log CFU/g)	(log CFU/g)	(log CFU/g)	(%)	(%)	(Mean value, °C)
1	145	25	18.4	12.80	8.30	6.13	6.68	4.27	54.36	2.739	94
2	170	15	15	10.92	9.15	6.17	6.21	4.61	58.64	2.45	117
3	120	35	15	10.58	6.62	6.31	6.58	6.92	48	5.03	74
4	120	15	15	11.10	6.72	6.07	6.11	4.61	52.48	3.68	74
5	170	35	15	11.79	6.52	5.61	5.57	4.39	54.96	3.88	117
6	145	25	10	9.22	5.64	5.77	6.19	4.60	60.8	3.33	94
7	187	25	10	8.70	6.03	4.82	4.82	3.82	60.1	2.69	130
8	145	25	10	8.25	5.83	6.16	6.11	4.77	58.6	3.92	94
9	145	42	10	8.53	5.83	6.30	6.53	5.23	46.4	3.54	94
10	145	25	10	9.53	5.87	6.02	6.38	4.86	56.6	3.33	94
11	145	25	10	9.19	5.21	5.87	6.09	4.30	55.7	3.8	94
12	145	25	10	8.63	5.52	5.60	6.20	4.12	56.6	3.71	94
13	145	25	10	8.98	5.72	5.90	6.14	4.42	54.1	3.56	94
14	145	8.2	10	8.67	5.72	5	5	3.82	59.6	2.56	94
15	103	25	10	8.56	6.07	7.04	7.09	7.86	51.5	4.87	59
16	170	15	5	6.84	3.05	4.87	5.47	4.60	45.46	2.98	117
17	120	15	5	6.76	4.95	5.77	6.23	5.72	53.2	4.23	74
18	170	35	5	6.79	5.24	4.87	5.77	4.17	40.4	3.88	117
19	120	35	5	6.06	4.86	6.96	7.08	7.75	39.6	6.03	74
20	145	25	1.6	5.59	4.00	4.88	5.28	5.35	27.24	4.58	94

Note: (Abbreviations: AA FRAP= Ferric reducing antioxidant power (FRAP) assay, AA DPPH=1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, LAB= Lactic acid bacteria, AAB= Acetic acid bacteria)

6.4 Results and Discussion

6.4.1 Model fitting

Results in Table 6.2 showed that the models developed for corresponding responses including antioxidant activity (FRAP and DPPH), product yield, and survival rates of water kefir microorganisms (LAB, AAB and yeasts) were significant ($p<0.05$). According to Table 6.2, which shows an insignificant *p*-value ($p>0.05$) for lack of fit for all responses, there is a satisfactory level

of accuracy of the proposed models for the prediction of the corresponding variable responses. The high coefficients of determination (R^2 values) of the developed models indicate that the experimental data successfully fit the equation. Also, adj- R^2 and low coefficient of variation (CV) values (4.04-25.15%) indicate the experiments' high precision and validate the model's accuracy.

Table 6.2 *Analysis of variance (ANOVA) for the responses of spray drying variables.*

Response	Source						*F*-value	*P-value Prob>f*	*P value Lack of fit*	R^2	*Adj* R^2
	residual			model							
	df	SS	MS	*df*	SS	MS					
AA_{FRAP} (µmol AAEq/g)	16	2.48	0.15	3	66.59	22.20	143.42	<0.0001	0.768	0.96	0.95
AA_{DPPH} (µmol Trolox Eq/g)	12	2.16	0.18	6	19.15	3.19	17.73	<0.0001	0.058	0.90	0.85
LAB Viability (CFU/g)	13	5.30	0.41	6	37.90	6.32	15.48	<0.0001	0.146	0.88	0.82
AAB Viability (CFU/g)	8	3.4E+12	4.3E+11	9	2.2E+14	2.4E+13	57.07	<0.0001	0.061	0.98	0.97
Yeasts Viability (CFU/g)	10	5.05	0.51	9	137.20	15.24	30.18	<0.0001	0.374	0.96	0.93
Product Yield (%)	10	90.70	9.07	9	1264.22	140.4	15.49	<0.0001	0.711	0.93	0.87

Note: (Abbreviations: AA FRAP= Ferric reducing antioxidant power (FRAP) assay, AA DPPH=1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, LAB= Lactic acid bacteria, AAB= Acetic acid bacteria)

The statistical significance for each response and the linear and quadratic equations are presented in Table 6.3, which will be discussed in the following sections.

Table 6.3 *Regression equation coefficients for RWK spray-dried powder response models.*

Effects	**Response**											
	LAB viability (CFU/g)		**AAB viability (CFU/g)**		**Yeasts viability (CFU/g)**		**AA_{FRAP} (µmol AAEq/g)**		**AA_{DPPH} (µmol Trolox Eq/g)**		**Product Yield (%)**	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
Intercept												
β_0	+15.54		+2.6E+7		+52.56		+3.71		+7.95		+47.79	
Linear												
β_1	-0.04	<0.0001**	-6.5E+5	<0.0001**	-0.56	<0.0001**	+6E-3	0.17	-0.04	0.42	-0.01	0.09
β_2	+0.46	0.0105*	+1.9E+6	0.0067**	+0.8	0.0003**	-4.6E3	0.66	-0.06	0.32	-0.54	0.0013**
$\beta 3$	-0.45	0.0027**	+1.5E+6	0.0002**	-1.21	0.0116*	+0.44	<0.0001**	+0.15	<0.0001**	+2.27	<0.0001**
Quadratic												
β_{12}	-2.2E-3	0.0252*	-10625.68	<0.0001**	-5.7E-3	0.0002**			+9.6E-4	0.1912	+4.6E-003	0.2989
β_{13}	+5.5E-3	0.0091**	+2373.62	0.3339	+5E-3	0.0312*			+1.6E-3	0.2549	+0.02	0.0404*
β_{23}	-8.6E-3	0.0773	-42128.44	<0.0001**	+2.7E-3	0.5991			-6.6E-3	0.0826	+0.02	0.2456
Interaction												
β_{11}			+2659.65	<0.0001**	+1.9E-3	<0.0001**					-8.3E-4	0.5276
β_{22}			+860.42	0.6424	+1.3E-3	0.4821					-0.01	0.0863
β_{33}			-62419.63	0.0003**	+0.014	0.0785					-0.23	<0.0001**

Note: Effects are statistically significant 0.01<P< 0.05, P** < 0.01. The coefficients of the polynomial model include the constant term (β0), linear effects (β1, β2, β3), quadratic effects (β11, β22, β33), and interaction effects (β12, β13 and β23). F-values imply the models are significant. These values indicate a significant (p < 0.05) relationship between independent and response variables and can be used to predict the corresponding responses of the RWK beverage. (X1 Inlet air temperature; X2 Feed temperature; X3 Feed flow rate).*
(Abbreviations: AA FRAP= Ferric reducing antioxidant power (FRAP) assay, AA DPPH=1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, LAB= lactic acid bacteria, AAB= Acetic acid bacteria)

6.4.2 Product yield

In order to produce RWK powder as an economically feasible product at an industrial scale, it is necessary to optimize the spray drying process for achieving a high product yield and minimized production costs. Similar to other studies on spray drying of beverages containing high concentrations of low molecular weight sugars, the walls of the spray drying chamber were layered with RWK solids causing a significant loss in product recovery, which was as a result of the stickiness of the solutions, and use of drying aids allowed product recovery ranging from 27.2 to 60.8 %. According to Table 6.3, temperature showed no significant effect in powder recovery, whereas both drying aid concentration and feed flow rate affected the recovery of the produced powder significantly. As it is illustrated in Figure 6.1B, the interaction between inlet air temperature and drying aid concentration (AC) produced distinct curvatures showing the optimum conditions for maximum recovery of dried powder over 60.8 % with a 10 % drying aid concentration, where the other parameters were constant at a 25 % feed flow rate and 145ºC. Figure 6.1 illustrates that increasing drying aid concentration in RWK significantly improves the product yield. The reason is that, like other fruit juices, Russian olive juice contains a high concentration of low-molecular-weight sugars and hence has a low glass transition temperature (T_g). This makes RWK, which contains sucrose and Russian olive juice, very sticky during spray drying and consequently makes its drying difficult in its pure state. Therefore, the application of drying aids such as maltodextrins or gum Arabic with high molecular weights, and hence high T_g values, can reduce thermal plasticity and stickiness of the solution by increasing the glass transition

temperature of the RWK, thus improving the recovery of the dried powder (Barbosa and Teixeira 2017). However, at the excessive concentration of drying aid (increasing from 10 % to 15 %), the powder recovery dropped from 60.8 % to 52 % (Figure 6.1A-B). Figure 6.1B-C illustrates that increasing inlet air temperature increases product yield, which is explained by the fact that at the higher temperature, greater efficiency of heat and mass transfer is expected (Fazaeli et al. 2012); however, increasing the inlet air temperature above the glass transition of the solution, when only a low concentration of drying aid is used, resulted in melting of the powder and adherence of the powder to the walls to occur, leading to reduced recovery yield. As illustrated in Figure 6.1, a faster rate of feed flow affects the moisture content of the powder by limiting the time required for complete drying of RWK droplets and hence reduces recovery of the powder due to deposition of the powder on the dryer walls (Barbosa and Teixeira 2017).

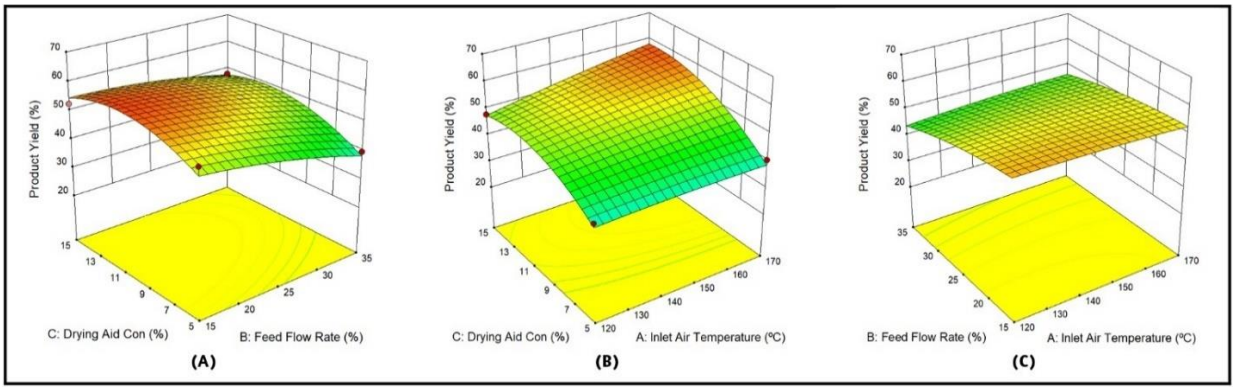


Figure 6.1 *3D plots showing the combined effect of inlet air temperature, feed flow rate, and drying aid concentration on recovery % of RWK spray-dried powder.*

6.4.3 Dry matter content

The dry matter content of dried powder is an important factor affecting the produced powder's quality, including its shelf life (Atalar and Dervisoglu 2015). According to other studies, for the powder's storage stability, the optimal moisture content of spray-dried powders is between 4 and 7% (Teijeiro et al. 2018). Moisture contents of RWK powders were between 2.4 and 6 % (Table

6.1). As inlet air temperature increased from 120 to 170 °C, a higher rate of heat transfer and a greater driving force for moisture evaporation reduced the moisture content of the produced powder from 4.43 to 3.03%. Similarly, increasing concentration of maltodextrin/gum Arabic showed a positive effect on the powder's dry matter content, resulting from the feed's increased total solids content. In contrast, the feed rate showed a significantly negative effect on the evaporation of moisture content from the powder, which results from shorter contact time between product and drying air and less efficient heat transfer (Figure 6.2) (Atalar and Dervisoglu 2015).

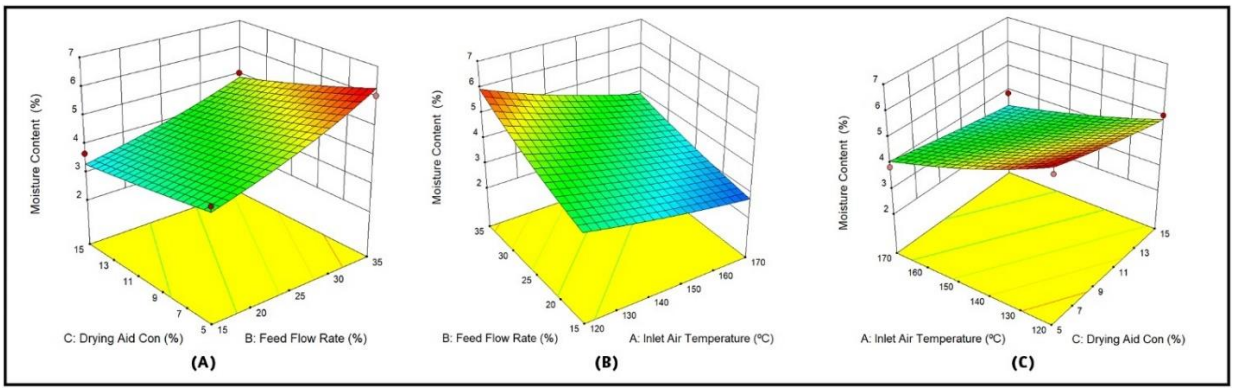


Figure 6.2 *3D plots showing the combined effect of inlet air temperature, feed flow rate, and drying aid concentration on dry matter content of RWK spray-dried powder.*

6.4.4 Survival of water kefir microorganisms

Table 6.3 presents the significant effects ($p < 0.05$) of temperature, feed flow rate, and drying aid concentration, and Figure 6.3 illustrates the combined effect of selected parameters on survival of LAB, AAB, and yeast cells in RWK powder during the spray drying process. Our results indicate that the survival of all water kefir microorganisms decreased with increasing temperature from 120 to 170°C and sharply increased when reducing exposure to heat by using a higher feed flow rate. In fact, a shorter drying time (higher feed rate) and lower level of heat exposure (lower temperature) are associated with the increased risk of cell inactivation resulting from the damages to the wall, membrane, and DNA of the cells at high-temperature exposure (Barbosa and Teixeira

2017; Atalar and Dervisoglu 2015). Our results showed that at elevated inlet air temperature and decreased feed flow rate, which provides higher heat exposure to microorganisms, increasing concentration of the thermo-protectants from 5 to 10% significantly improved survival of AAB cells during the dehydration process (Figure 6.3D-E). Similarly, the positive effect of drying aid concentration on the survival rate of LAB cells was observed at a higher temperature and all range of feed flow rates (Figure 6.3A-B). The thermo-protective effect of drying aids can be due to the protective effect of saccharides, which is correlated to the number of equatorial OH groups in the molecular structure of sugars (Teijeiro et al. 2018). However, as it is illustrated in Fig 6.3.D-E, increasing the concentration of drying aid to more than 10 % resulted in a negative impact on the survival of AAB cells, because very high solids content creates larger particles, which results in longer drying time, more prolonged exposure of microorganisms to high temperature, and consequently, decreasing their survival. At the optimized concentration of drying aid, the maximum viability of microorganisms was shown to be 7.04, 7.09, and 7.86 log CFU/g for LAB, AAB, and yeast cells, respectively. The ability of the yeast cells to survive the dehydration process varied considerably compared to the other two investigated RWK microorganisms and showed to have more susceptibility during the dehydration process (Figure 6.3G-I), which can be associated with membrane condensation caused by increased cell surface to volume ratio (s/v) of the yeast cells, which in turn results in restructuration of the cells during the spray drying process (Teijeiro et al. 2018). In a similar study by Golowczyc et al. (2010), yeasts (*Saccharomyces lypolytica*) were found to be the most damaged microorganism in kefir during the dehydration process.

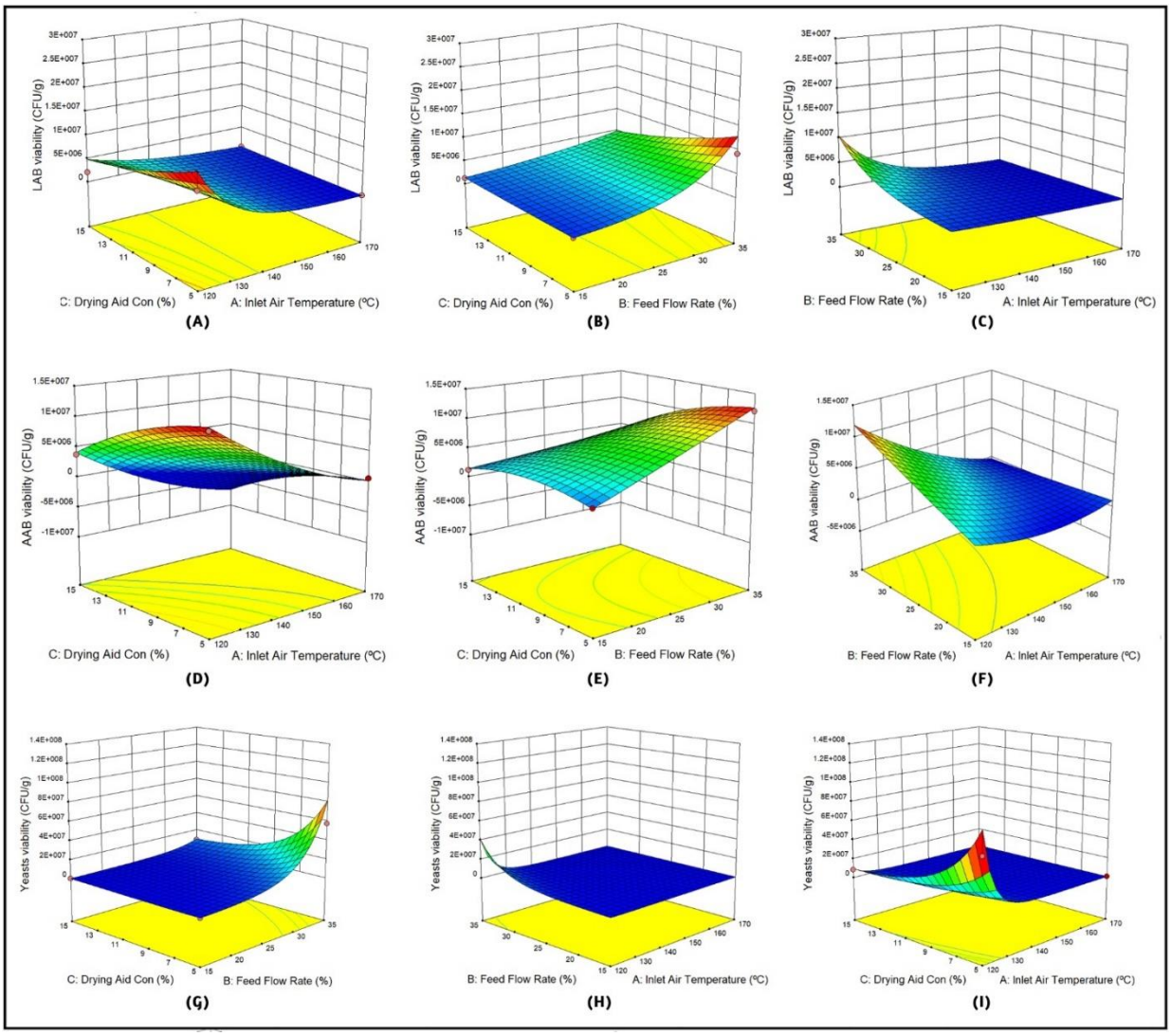


Figure 6.3 *3D plots showing the combined effect of inlet air temperature, feed flow rate, and drying aid concentration on the survival rate of Acetic acid bacteria (AAB) (a-c), Lactic acid bacteria (LAB) (d-f), and yeast cells (g-i) in Russian olive water kefir (RWK) spray-dried powder.*

6.4.5 Antioxidant properties

Retention of the antioxidant property of RWK powder (FRAP and DPPH) during spray drying at different experimental conditions is shown in Table 6.1. The values of FRAP (µmol AAEq/g) and DPPH scavenging activity (µmol TroloxEq/g) varied between 5.5 to 12.8 and 3.05 to 9.15, respectively. Inlet air temperature and feed flow rate collectively showed no significant effect ($p < 0.05$) on the antioxidant properties of water kefir powders (Table 6.3). However, Figure 6.4 shows

the significantly higher retention of antioxidant components with increased levels of thermo-protectants reaching the values of 9.15 (μmol AAEq/g) and 12.6 (μmol TroloxEq/g) for DPPH and FRAP, respectively, at the maximum concentration of 15 percent of carrier agents (Figure 6.4E-F; Table 6.1). However, even though increasing the concentration of drying aid has a positive effect on the antioxidant properties of the powder, high solids content creates larger particles, which may result in longer drying time with associated prolonged exposure of antioxidant compounds to high temperature and consequently decreasing the retention of the antioxidant activity in the produced powder (Barbosa and Teixeira 2017). Similarly, a variety of studies showed the successful application of maltodextrin and gum Arabic for preserving the antioxidant properties in powders and reported around 10 percent as the optimum concentration for producing powder with acceptable overall quality (Igual et al. 2014; Singh et al. 2019). Overall, results of the present study suggest an acceptable model with a non-significant lack of fit (Table 6.2) for the improved retention of antioxidant content in RWK during spray drying and predict the highest level of antioxidant compounds (DPPH and FRAP values) at spray condition of 120 ºC, 15 % feed flow rate and using 15 % drying aid.

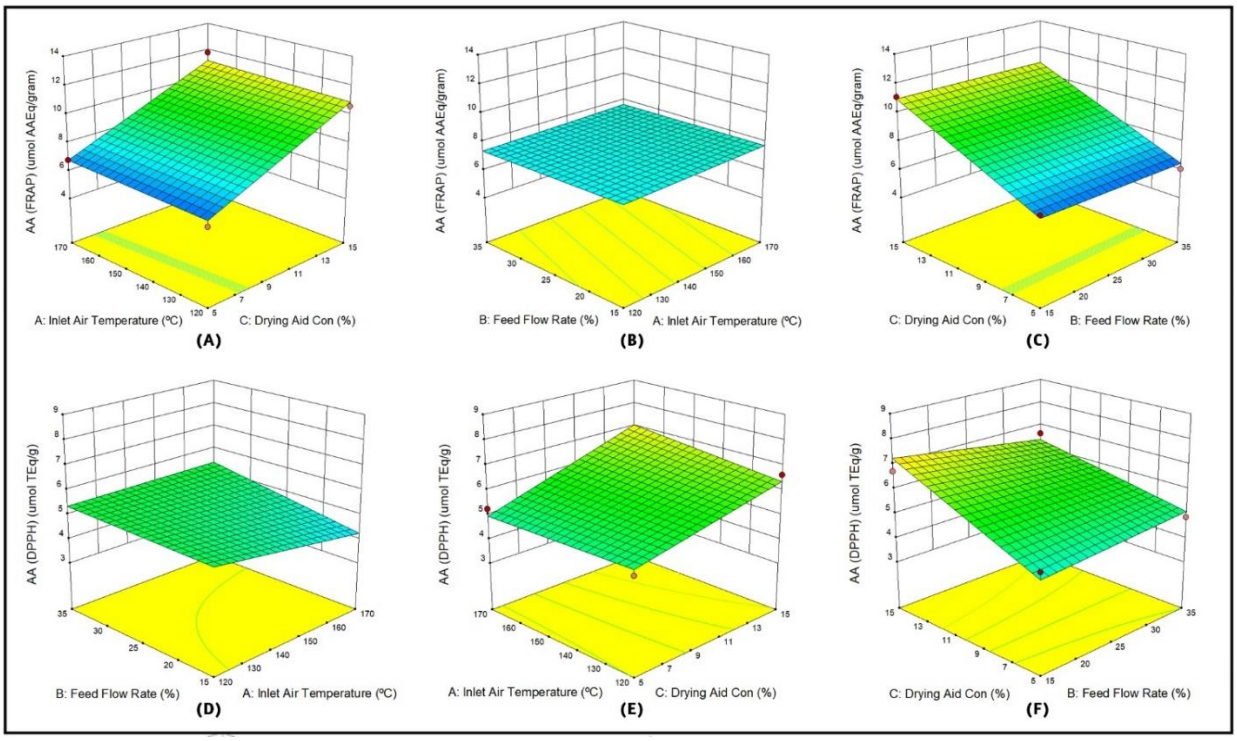


Figure 6.4 *3D plots showing the combined effect of inlet air temperature, feed flow rate, and drying aid concentration on Ferric reducing antioxidant power AA (FRAP) (a-c) and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity AA (DPPH) (d-f) of Russian olive water kefir (RWK) spray-dried powder.*

6.4.6 Optimization

The process development for spray-dried RWK powder using maltodextrin/gum Arabic as thermo-protectants was optimized using the RSM response optimizer. Numeric and graphic optimizations were performed while desired goals were defined by high values of survival rate of water kefir microorganisms, antioxidant activity, product yield, and dry matter. The software-generated most desirable solution of the spray drying process was shown as the combination of the temperature of 120 ºC, the feed flow rate of 35, and the 7.03 % concentration of drying aid, resulting in a 44% recovery of RWK powder and a moisture content of 5.9 %. At the overall optimum region, the predicted response values for FRAP, DPPH radical scavenging are 7.37 (µmol AAEq/g) and 5.35 (µmol TroloxEq/g), respectively. Also, the predicted values for the viable number of water kefir

microorganisms (LAB, AAB, and yeasts) in the final optimized RWK powder were 7.02, 7.09, and 7.62 log CFU/g, respectively.

6.4.7 Verification of the models

In order to check the validity of the developed model, the Russian olive water kefir spray-dried powder was produced using the obtained optimum processing conditions, and the experimental values of the developed powder were determined and compared with the predicted values of the model (Table 6.4). The low Root Mean Square Errors values (RMSE) indicate that the experimental values (mean of three trials) are in good agreement with the predicted values indicating the suitability of the model in predicting quality attributes of Russian olive water kefir powder.

Table 6.4 *Predicted and experimental values of the responses at optimum conditions.*

Predicated	Experimental	Response variable	Root Mean Square Error
5.35	5.63	AA_{DPPH} (µmol TroloxEq/g)	0.28
7.37	7.97	AA_{FRAP} (µmol AAEq/g)	0.6
7.02	7.27	LAB viability (log_{10} CFU/g)	0.25
7.09	7.19	AAB viability (log_{10} CFU/g)	0.1
7.62	7.01	Yeast viability (log_{10} CFU/g)	0.61

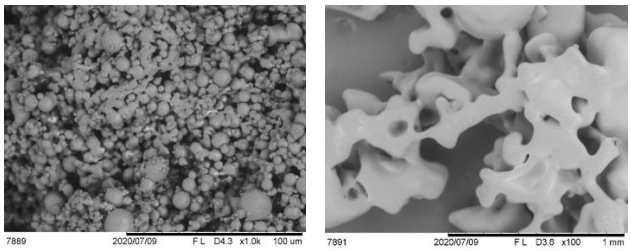
Note: (Abbreviations: AA FRAP= Ferric reducing antioxidant power (FRAP) assay, AA DPPH=1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, LAB= Lactic acid bacteria, AAB= Acetic acid bacteria)

6.4.8 Characterization of the optimized spray-dried RWK

The physicochemical and microbiological properties of the spray-dried RWK powder (at the optimized condition) were assessed, and the results were compared with the samples produced using Freeze drying (lyophilization), which is considered as the best drying method for producing a high-quality product considering bacterial viability and sensory properties (Atalar and Dervisoglu 2015). The results are presented in Table 6.5 and Figure 6.5.

Table 6.5 *Physiochemical and microbiological characterization of spray-dried RWK powder at optimized condition compared to freeze-dried RWK powder.*

	AA_{FRAP} (µmol AAEq/g)	AA_{DPPH} (µmol TroloxEq/g)	LAB viability Viability (log CFU/g)	AAB viability Viability (log CFU/g)	Yeast viability Viability (log CFU/g)		
Spray-dried RWK powder	7.97	5.63	7.27	7.19	7.01		
Lyophilized RWK powder	8.09	6.9	7.48	7.40	6.95		
	Water Solubility Index (%)	**Water activity** (a_W)	**Particle size** (µ)	**Rehydration time** (s)	**Recovery** (%)	**Titratable acidity** (%)	**pH**
Spray-dried RWK powder	91	0.272	73	33	47	0.793	4.57
Lyophilized RWK powder	89	0.368	200	80	100	0.793	4.53



(A) (B)

Figure 6.5 *Scanning electron microscopic (SEM) images of Russian olive water kefir (RWK) microcapsules produced by spray drying (magnification 1000×, bar length 100 µm) (a) and by freeze drying (magnification 100×, bar length 1 mm) (b) with maltodextrin and gum Arabic as wall materials.*

Microencapsulated samples at optimized spray drying conditions showed good viability of water kefir microorganisms and antioxidant activity, which is closely related but lower than those measured in the freeze-dried samples and could be attributed to the higher temperatures achieved during spray drying. The freeze-dried powder had superior quality considering antioxidant activity and survival of water kefir bacteria; however, the spray-dried product showed better characteristics for a stable and desirable product, including lower water activity, moisture content, and rehydration time, and might be a more economical method for producing Russian olive water kefir powder.

6.5 Conclusions

Our results show that spray drying is an efficient encapsulating method for guaranteeing the survival of water kefir microorganisms and also for preserving the antioxidant activity of RWK. Based on the results of this study, for the development of RWK powder as a functional product containing probiotics, maximizing the viability of the water kefir microorganism (LAB, AAB, and yeasts) was achieved to be 7.27, 7.19, and 7.01 log CFU/g, respectively at the most desirable solution of the spray drying process, which is the combination of the temperature of 120 ºC, feed flow rate of 35, and the 7.03 % concentration of drying aid. At this optimized process conditions, $AA_{(FRAP)}$ and $AA_{(DPPH)}$ are 7.37 (µmol AAEq/g) and 5.35 (µmol TroloxEq/g) respectively. Based on the results, yeasts were shown to be the most susceptible microorganism of water kefir during the dehydration process, and application of gum Arabic/maltodextrin showed significant importance for survival rates of water kefir microorganisms, preserving antioxidant compounds, satisfying product yield and moisture content of RWK during spray drying. In addition, due to the prebiotic properties of these carriers, their application in the beverage formulation and, therefore, in Russian olive water kefir may contribute to the production of a synbiotic functional product. The physicochemical and microbial analysis of the produced RWK powder in our study showed that spray drying is a promising method compared to freeze drying for encapsulation of Russian olive water kefir. Therefore, our findings support the application of spray drying as a potential dehydration process for producing dehydrated products derived from water kefir. Due to the associated health benefits of water kefir and the increasing trend in the consumption of water kefir related products, producing its powder through the optimized spray drying process in this study can facilitate storage, transportation, and commercialization of this product. Also, microencapsulation of RWK is expected to improve the viability of water kefir microorganisms

against possible severe conditions in the food matrix to provide them a higher level of resistance to storage conditions and finally to passage through the gastrointestinal tract following consumption. In future studies, it is important to evaluate the sensory properties of the spray-dried RWK as well as its quality retention during the storage period.

6.6 Acknowledgments

The authors would like to thank the Canadian Natural Sciences and Engineering Research Council (NSERC) for their funding of this project.

CONNECTING TEXT

In Chapter VI, spray drying process conditions were optimized and a high-quality Russian olive water kefir powder was developed. However, considering RWK as a functional food that can potentially provide nutritional health benefits to the consumer, the functional properties of RWK powder throughout its shelf-life and prior to its consumption must be preserved. Therefore, Chapter VII focuses on monitoring the quality of encapsulated RWK during a subsequent three months of storage where retention of RWK bioactive compounds and beneficial water kefir microorganisms is ensured. This chapter assessed the effects of storage conditions on the quality of spray-dried RWK powder. In addition, the *in vitro* gastrointestinal resistance of water kefir microorganisms present in RWK powder was investigated. At the end, all results are compared between RWK powder encapsulated by spray drying and freeze drying.

CHAPTER VII

STORAGE STABILITY AND IN VITRO DIGESTION OF MICROENCAPSULATED RUSSIAN OLIVE WATER KEFIR USING SPRAY DRYING

7.1 Abstract

This study investigated the storage stability of Russian olive water kefir (RWK) powder, obtained by an optimized spray drying (SD) based microencapsulation technique to determine the maximum shelf life of a high-quality RWK powder as a new functional product. Also, the effect of the storage conditions, including time and temperature on the kinetics of changes in total phenolic content, antioxidant activity and microbial viability of the spray-dried RWK powder was evaluated under two different storage temperatures (4 and 25°C) and at different time intervals (0, 15, 30, 45, 60 and 90 days) to determine the best storage conditions. In addition, the *in vitro* gastrointestinal resistance of the water kefir microorganisms present in spray-dried RWK was evaluated. Overall, degradation of antioxidant compounds and total phenolic content in encapsulated RWK samples showed first-order kinetics during 90 days of storage. Temperature showed no significant effect on the stability of antioxidant compounds in RWK powder; however, refrigeration resulted in significantly higher survival of the water kefir microorganisms. Retention of bioactive compounds and high survival of water kefir microorganisms in spray-dried RWK during 90 days of cold storage and also in transit through simulated gastrointestinal conditions confirmed spray drying as a successful technique for encapsulation of RWK. Moreover, the storage stability of spray-dried RWK was compared with samples produced using freeze drying (FD), known as one of the best

encapsulation methods, and the results showed that in cold storage SD samples have prolonged storage stability and high microbial survival closely related to FD.

7.2 Introduction

Due to the associated nutritional-health benefits of kefir, as a fermented dairy product, and its specific sensory properties, there is an increasing trend in the consumption of this product. In addition, the increasing number of vegetarians and vegans, in particular, are showing an increasing interest in non-dairy probiotic sources (Corona et al., 2016). To provide kefir's beneficial health effects for vegans, consumers with lactose intolerance, or with an allergy to milk-derived products, water kefir is produced as an alternative product with similar properties. Water kefir is a non-dairy and sugar-based fermented drink, which is produced using water kefir grains, containing a symbiotic culture of lactic acid bacteria (LAB), yeast species, and acetic acid bacteria (AAB) (Pidoux, 1989; Gulitz et al., 2011). Kefir and water kefir provide several health benefits such as antioxidant, anti-hyperglycemic, anti-hyperlipidemic, anti-inflammatory, anti-ulcerogenic, and antimicrobial activities. On the other hand, studies have reported many bioactivities of Russian olive fruit, among which, antioxidant, anti-inflammatory, anti-ulcerogenic, and antimicrobial activities which are shared with water kefir (Rodrigues et al., 2005; Alsayadi et al., 2013; Alsayadi et al., 2014; Farzaei et al., 2015; Rodrigues et al., 2016). Therefore, by using Russian olive in water kefir fermentation, Darvishzadeh et al. (2021a) developed a novel non-dairy (water kefir) beverage with enhanced bioactivities, called Russian olive water kefir (RWK). RWK could be considered as an attractive product to create diversification in water kefir beverages; however, similar to other fermented products, ongoing microbial metabolites can affect water kefir sensory properties and limit the shelf life of RWK (Nale et al., 2017). In addition, RWK requires encapsulation by different methods such as drying to guarantee the survival of probiotics and stability of bioactive

compounds with antioxidant activity during storage. Therefore, microencapsulation of Russian olive water kefir into powder format not only can extend the shelf life of this product through the preservation of flavour, phenolic content and survival of probiotics but also, can facilitate commercialization of this product through reduction of packaging and storage-related costs. The application of different drying methodologies for encapsulation and preservation of the microorganisms and bioactive compounds has a long history, among which, spray drying and freeze drying are more common processes (Reddy, 2007). To preserve the quality of RWK during storage and improving the stability and commercialization potential of this product, Darvishzadeh et al. (2021b) produced RWK powder using a spray drying process with gum Arabic and maltodextrin as wall materials and optimized the encapsulation process parameters for maximizing the viability of RWK's microorganisms and bioactive compounds. They proved spray drying as a promising and economical dehydration process for producing a high-quality RWK powder similar to freeze drying, which is an expensive encapsulation method. They used gum Arabic/maltodextrin, which due to the prebiotic properties of these carriers, their application for encapsulation of Russian olive water kefir can contribute to the production of a synbiotic functional RWK powder product.

However, many studies have reported a decreased level of bioactive compounds and survival of probiotics in functional products during drying and the subsequent storage period (Barbosa & Teixeira, 2017). Considering water kefir as a functional product with high antioxidant capacity and probiotic properties, it is essential to maintain its functional properties throughout the shelf-life of the powder and prior to its consumption. Currently, there are no studies on the storage stability of encapsulated RWK powder, in particular using spray drying. The purpose of this study is to investigate the stability of water kefir microorganisms (LAB, AAB and yeasts) and the

retention of radical-scavenging activity and total phenolics in RWK powder produced using spray drying at different common storage temperatures (4 and 25 °C) over 90 days of storage. Storage temperature and time are among the factors greatly affecting the quality of the produced powder and we aim to monitor the degradation kinetics of bioactive compounds and determine the best storage conditions and the maximum shelf life of a high-quality Russian olive water kefir powder as a new functional product.

Moreover, probiotics can potentially provide various health benefits only when they are consumed in sufficient quantity. Survival of probiotics is affected by various factors before spray drying (strain characteristics, growth medium, growth phase, and sub-lethal stress exposure), during spray drying (drying medium and drying parameters), and finally, after spray drying such as packaging, storage conditions, and rehydration (Barbosa & Teixeira, 2017). Darvishzadeh et al. (2021b) proved that the optimized spray drying process using wall materials for the production of Russian olive water kefir powder provides adequate numbers of viable probiotic bacteria; however, probiotic properties should be maintained throughout the shelf-life of the powder until consumption. Therefore, we aim to evaluate the survival of water kefir microorganisms, including lactic acid bacteria, acetic acid bacteria, and yeasts, in dehydrated RWK samples, obtained through optimized spray drying conditions as developed by Darvishzadeh et al. (2021b), which are stored at different common storage temperatures (4 and 25 °C) over 90 days of storage. In addition, since the survival of these bacteria in the human gastrointestinal system is important for imparting their health benefits to the host, we aim to evaluate the efficiency of microencapsulation in protecting the live probiotic cells in their transit through simulated gastrointestinal conditions. Based on the previous study by Darvishzadeh et al. (2021b), it is hypothesized that the optimized spray drying process using wall materials for the production of RWK powder can maintain the adequate

numbers of viable probiotic bacteria and stability of bioactive compounds to a comparable level to that of the freeze-dried powder during the storage period. Therefore, we aim to compare the functional properties of the spray-dried RWK during storage with the samples produced using freeze drying, which is considered the best drying method for producing a high-quality product considering bacterial viability and retention of bioactive compounds (Atalar & Dervisoglu, 2015).

7.3 Materials and Methods

7.3.1 Sample preparation and drying processes

Russian olive water kefir was produced using the optimum product formula and following the optimum fermentation conditions developed by Darvishzadeh et al. (2021a). Briefly, using 50 g/L water kefir grains, 30 % Russian olive juice and 8 % sucrose solution RWK was produced under the optimal fermentation conditions of 31.2ºC incubation temperature, 24 hours incubation time and constant stirring (100 rpm) in 1.0-L Biostat Qplus bioreactors (Sartorius Stedim Biotech, Germany). Subsequently, samples of RWK were encapsulated following the spray drying condition optimized for producing a product with maximized microbial viability and antioxidant activity, previously developed by Darvishzadeh et al. (2021b). Briefly, samples were mixed with a 7 % drying aid (maltodextrin13-17 DE/gum Arabic 50:50 mix). Prepared homogenized samples were spray-dried at the temperature of 120ºC and feed flow rate of 35%, while setting the aspiration and airflow rate constant at 100% and 414 L/h, respectively. For the freeze drying process, RWK samples were frozen overnight at -80°C and then freeze-dried for 72 h in a lab-scale vacuum freeze-dryer (7670520, Labconco Co., Kansas City, USA).

7.3.2 In vitro characterization of the survival of encapsulated RWK microorganisms in a simulated gastric solution

7.3.2.1 Gastric digestion

Preliminary information regarding the probiotic properties of the water kefir microorganism can be obtained through *in vitro* tests recommended by FAO/WHO (2002) including tolerance to gastrointestinal conditions (acid, pepsin, bile salts and pancreatin resistance). This test verifies whether probiotics can survive and grow sufficiently after passage through the gastric and intestinal phases to perform beneficial actions (FAO/WHO, 2002). To simulate the gastrointestinal digestion process of encapsulated RWK, a standardized *in vitro* digestion method developed by Minekus et al. (2014), including gastric and small intestinal digestion, was employed with modifications as follows: RWK samples were exposed to gastric phase in which SGF (Simulated Gastric Fluid) stock electrolyte solution was added to RWK at the ratio of 50: 50 (v/v) (including KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H2O)_6$ and $(NH4)_2CO_3$). Subsequently, porcine pepsin and $CaCl_2$ were added to achieve a concentration of 2000 U/mL and 0.075 mM, respectively in the final digestion mixture followed by adding the required amount of water. Then, pH was adjusted to 3.0 using 1 M HCl and digestion was carried out for 2 hours at 37°C and 150 rpm. Samples were taken at time 0 and after 1 hour and 2 hours of exposure to the gastric phase.

7.3.2.2 Small intestinal digestion

To simulate the intestinal phase, the gastric chyme from the previous gastric phase was mixed with the SIF (Simulated Intestinal Fluid) electrolyte stock solution (including KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H2O)_6$) for intestinal digestion. A final ratio of gastric chyme to SIF of 50: 50 (v/v) in the mixture was targeted after additions of enzymes including pancreatin from porcine pancreas (0.1%), bile salts (0.3 %) and water. 1 M NaOH was used to neutralize the mixture to pH 7.0 and

intestinal digestion was carried out for 2 hours. Samples were taken at time 0, and after 1 hour and 2 hours of exposure to the intestinal phase (at 37°C and 150 rpm) for analysis (Minekus et al., 2014; Fadda et al., 2017). Subsequently, microbial counts were determined and survival of dehydrated water kefir microorganisms in simulated gastric solution was estimated. LAB, AAB, and yeast cells in the digested samples were isolated on the appropriate media following methods by ISO (1998), Hsieh et al. (2012), Gulitz (2013) and Atalar and Dervisoglu (2015) with slight modifications. The viable number of LAB, AAB and yeasts were enumerated, and results were expressed as colony-forming units per 100 mL portion of consumed RWK (CFU/100mL).

7.3.3 Storage stability tests

RWK Russian olive water kefir microcapsules obtained from spray drying and freeze drying were collected in glass jars, impermeable to oxygen and moisture and were stored at 4 and 25 °C (representing cold storage and room storage, respectively). Samples were evaluated for storage stability and were periodically analyzed with respect to antioxidant activity, total phenolic content and microbial viability (LAB, AAB and yeasts) during three months of storage. Samples were analyzed in triplicates, at time intervals of the 1st, 15th, 30th, 45th, 60th and 90th days of storage, and the results were expressed as mean values. In addition, using a first-order kinetic model, the total phenolic content and antioxidant compounds loss in RWK powders during storage were evaluated and the reaction rate constants (k) were calculated for all samples using the Equations below:

$$-ln\left(\frac{C_t}{C_0}\right) = kt \quad (1)$$

$$t_{\frac{1}{2}} = \frac{ln\,2}{k} \quad (2)$$

In these equations, C_0 represents the initial concentration of TPC and antioxidant compounds of the samples and C_t is their concentration at storage time t, and half-life $t_{1/2}$ corresponds to the time

at which the TPC of the samples is reduced by 50% compared to the first day of storage (zero time) (Ferrari et al., 2012). In addition, the retention of total phenolic and antioxidant compounds was evaluated by calculating their ratio after the storage period to the beginning of storage.

7.3.3.1 Determination of antioxidant activity

Dried samples were rehydrated in methanol/H_2O (50:50, v/v) to the initial Brix of the RWK drink. The mixture was centrifuged at 5000 rpm for 10 min and the supernatants were used to determine the total antioxidant activity of the samples. In order to measure the antioxidant capacity of the samples, 2 widely known spectrophotometric assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and ferric reducing antioxidant power (FRAP) assay were used as follows.

7.3.3.1.1 Estimation of DPPH radical-scavenging capacity

To measure the antioxidant activities of the samples, a modified 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, introduced by Brand-Williams et al. (1995), was followed. Briefly, a serial dilution of the standard solution (0.01-0.4 mM Trolox) was prepared in methanol. Then, 3.9 mL of freshly prepared DPPH solution (0.06 mM) was added to 100 µL of rehydrated water kefir samples or standards and the mixtures were incubated for 30 minutes at room temperature and in darkness. The absorbance was read at 517 nm using a spectrophotometer, and the percentage of DPPH radical scavenging activity of the standards and each sample was calculated, while the antioxidant activity of each sample was expressed as mM Trolox equivalent/g of the samples.

7.3.3.1.2 Determination of antioxidant activity using FRAP assay

For measuring the ferric reducing antioxidant power (FRAP) of the samples, a method introduced by Benzie and Strain (1996) was followed with slight modifications. To prepare the working FRAP reagent, 300 mM acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM hydrochloric acid and 20 mM ferric chloride were mixed to the volume ratio of 10:1:1 respectively. Then, 250

μL of the freshly prepared FRAP reagent was mixed with 25 μL of water and 8.5 μL of the samples or standards (0.1-3.2 mM $FeSO_4.7H2O$) in a microplate and incubated for 30 minutes at room temperature and in darkness. Using a microplate reader, the absorbance of mixtures was read at 593 nm, and the results were expressed as μmol $FeSO_4.7H2O$ equivalent/g of the samples.

7.3.3.2 Microbial analysis/ Determination of lactic acid bacteria (LAB), yeast, and acetic acid bacteria (AAB) cell viability

Encapsulated RWK samples (spray-dried, freeze-dried or digested samples) were rehydrated in 0.85% sodium chloride solution to the initial solids content of RWK. The suspensions were kept at 25°C for 1 hour to release the cells. Then, following methods by ISO (1998), Hsieh et al. (2012) Gulitz (2013) and Atalar and Dervisoglu (2015) with slight modifications and using sequential dilution, different dilutions of the samples were plated on appropriate media for isolation and quantification of water kefir microorganisms. LAB and AAB cells were isolated on de Man, Rogosa and Sharpe (MRS) agar (pH 5.7) and Germination Medium (GM) agar (pH 6.0), respectively, in which cycloheximide (150 μg/mL) was added to inhibit the growth of yeasts and the plates were incubated for 72 hours at 30 °C. Yeast cells were isolated on YPG (yeast-peptone-glycerine) agar (pH 6.5), in which chloramphenicol (100 mg/L) and bromphenol blue (0.01 g/L) were added to inhibit bacterial growth and for morphological differentiation. Yeast cells were incubated for 3 days at room temperature. Subsequently, the viable number of LAB, AAB and yeast cells in the samples were enumerated, and results were expressed as colony-forming units per gram of encapsulated consumed RWK (CFU/g).

7.4 Results and Discussion

7.4.1 Storage stability of bioactive compounds in encapsulated RWK

The effects of storage time, temperature and encapsulation method on the stability of total phenolic content (TPC) and antioxidant capacity (FRAP and DPPH) of the encapsulated RWK powders during 90 days of storage at 4 and 25 °C are illustrated in Figures 7.1 and 7.2.

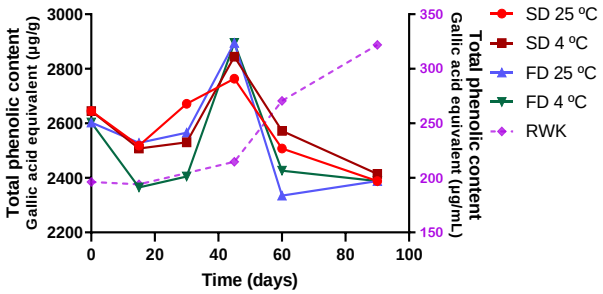


Figure 7.1 *Stability of total phenolic contents (TPC) in Russian olive water drink (RWK), and in RWK powder encapsulated using spray drying (SD) and freeze drying (FD), during 90 days of storage at room temperature (25 °C) and in the fridge (4 °C).*

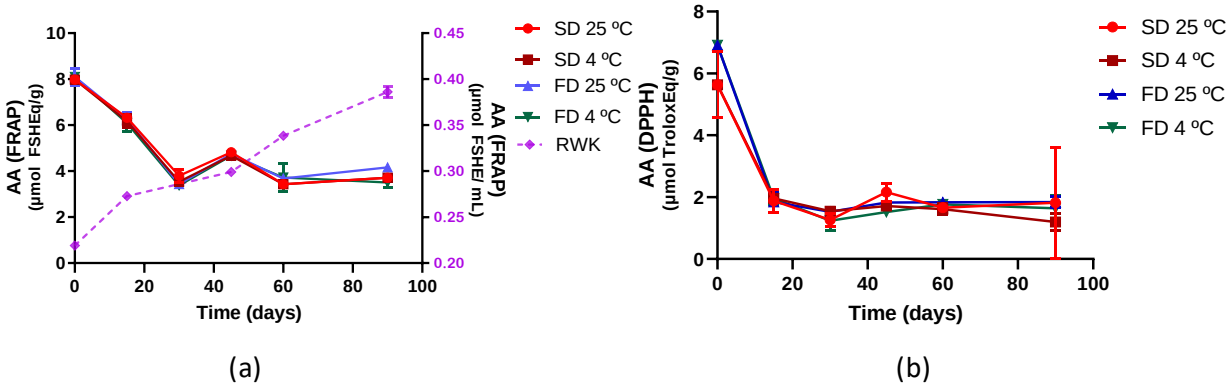


Figure 7.2 *Stability of antioxidant active compounds in Russian olive water drink (RWK), and in RWK powder encapsulated using spray drying (SD) and freeze drying (FD), a) Using FRAP assay and b) Using DPPH radical scavenging activity assay during 90 days of storage at room temperature (25 °C) and in the fridge (4 °C).*

Storage stability of phenolic compounds and the retention of radical-scavenging activity, which are responsible for certain health-promoting functions in humans, in encapsulated RWK powders are important (Zhang et al., 2020). As can be seen in Figure 7.1, comparing phenolic content of all samples, despite showing different values at different times of storage, they followed a similar trend and there was no significant difference in the overall loss in total phenolic content of the

samples. All samples showed to have an overall decreasing trend with some fluctuation at different stages of storage, which can be attributed to the release and biosynthesis of phenolic compounds or bioconversion of phenolic compounds to other bioactive compounds during the storage period. However, between days 30 and 45, a significant release of phenolic compounds was observed for all samples (Figure 7.1). Comparison of Figure 7.1 and Figure 7.2 suggests that the increased level of the phenolic content of the samples in this period, which can be attributed to the synthesis or release of phenolic compounds due to decomposition of phenolic polymers, can be responsible for the increased level of antioxidant capacity (DPPH and FRAP) in all samples between days 30 and 45. Similarly, an increasing trend in the total phenolics content of the samples was observed between days 15 to 30; however, the antioxidant level of the samples during this period did not show an increasing trend. The reason can be due to the fact that although a higher TPC level increases the antioxidant capacity, if it is as a result of bioconversion of other compounds with antioxidant activity to phenolics content, then the total antioxidant compounds present in the powder can be expected to show a decreasing trend as shown between days 15 to 30 (Figure 7.2).

The results showed that the storage time has a significantly important effect on the TPC and antioxidant activity of the powders ($P < 0.05$). The changes in antioxidant activity and TPC value of RW samples were fast at the beginning of storage, within the first two weeks, but rapidly decreased for the rest of the storage period for all samples. The changes in the antioxidant activity value and total phenolic content in spray-dried RWK powders were not significantly affected by storage temperatures ($P<0.05$) and the degradation behaviours of total phenolic compound and antioxidant activity compounds were found to be similar for both storage temperatures of 4 and 25°C. At the end of storage (12 weeks) at 25°C, SD RWK powder retained about 46.5% of initial FRAP antioxidant capacity and 90.2% of initial total phenolics, whereas for the refrigerated spray-

dried powder these numbers were 46.5% and 91.2%, respectively. Overall, after 3 months of storage at 4 and 25 °C, antioxidant activity (FRAP and DDPH) in all samples decreased (Figure 7.2). Similar to reports by Yang et al. (2010), the level of decrease in antioxidant activity of samples was greater when assayed with DPPH compared to FRAP; however, despite a slight difference in the stability of antioxidant capacity between the two assays DPPH and FRAP, which can be related to the difference in reaction mechanism in these assays, the degradation of antioxidant capacity of RWK powders assayed with these two assays demonstrated similar decreasing trend during 3 months of storage (Figure 7.2). The induced formation of compounds with antioxidant properties, and as a result increased overall antioxidant activity of products, with increasing storage time has been reported before (Pitalua et al., 2010). Tables 7.1 and 7.2 present the kinetics of the phenolics and antioxidant compounds degradation in RWK microcapsules produced by spray drying and freeze drying.

Table 7.1 *Kinetic parameters of total phenolics degradation in the RWK powder produced by spray-drying (SD) and freeze-drying (FD) using maltodextrin and gum Arabic as wall materials.*

Sample	$K*10^{\wedge 2}$/day (days:0-15)	$K*10^{\wedge 2}$/day (days:15-30)	$K*10^{\wedge 2}$/day (days:30-45)	$K*10^{\wedge 2}$/day (days:45-60)	$K*10^{\wedge 2}$/day (days:60-90)	Storage temperature (°C)
SD	0.64	0.32	0.32	-0.22	-0.39	25
SD	0.67	0.42	0.35	-0.06	-0.77	4
FD	1.43	0.19	-0.09	-0.15	-0.80	25
FD	1.17	0.63	0.10	-0.11	-1.22	4

Table 7.2 *Kinetic parameters of antioxidant compounds degradation in the RWK powder produced by spray-drying (SD) and freeze-drying (FD) using maltodextrin and gum Arabic as wall materials.*

Sample	$K*10^{\wedge 2}$/day (days:0-15)	$K*10^{\wedge 2}$/day (days:15-30)	$K*10^{\wedge 2}$/day (days:30-45)	$K*10^{\wedge 2}$/day (days:45-60)	$K*10^{\wedge 2}$/day (days:60-90)	Storage temperature (°C)
SD	3.401342	2.278394	1.555934	-0.5266	-1.6036	25
SD	3.713419	2.059436	1.713281	-0.5266	-1.85407	4

FD	3.993516	1.720898	1.701512	-0.82213	-2.15417	25
FD	3.928027	1.902872	1.516185	0.398303	-2.16028	4

Figure 7.3 shows that total phenolics and antioxidant compounds degradation in all encapsulated RWK samples while the degradation exhibited first-order kinetics throughout the storage at 4 and 25 °C with reaction rate coefficients fluctuating between -12×10^{-3} and 14×10^{-3} $days^{-1}$ and between -21×10^{-3} and 39×10^{-3} $days^{-1}$, respectively. According to this figure, it can be stated that the first-order reaction kinetic rate did not significantly change with increasing storage temperature; however, it increased with storage time which caused higher losses. The degradation rate of antioxidant capacity in all samples was rapid within the first month of storage and between days 45-60. However, a rapid increase was observed between days 30-45, which can be attributed to the increased total phenolic contents within this period. Similarly, the degradation of phenolic compounds experienced a sharp reduction within the first weeks and a jump between 15-45 days of storage, which can justify the similar changes in the antioxidant level of the sample within the corresponding period. Similarly, other studies reported first-order degradation rates of polyphenol contents under different storage conditions (Gradinaru et al., 2003; Saénz et al., 2009).

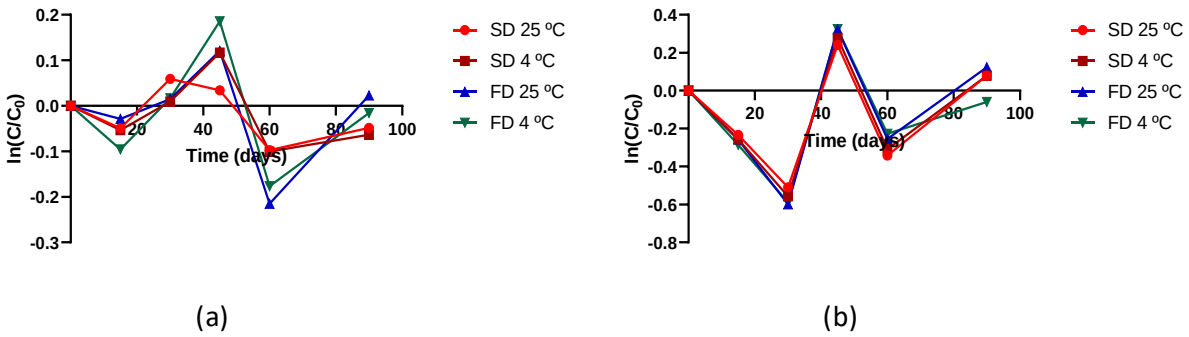


Figure7.3 *Degradation kinetics of phenolic content (a) and antioxidant compounds (b) in RWK encapsulated powders produced by spray drying and freeze drying during 90 days of storage at 4 C and 25 °C.*

7.4.2 Viability of microencapsulated RWK microorganisms during storage

Storage stability of water kefir microorganisms in RWK powder encapsulated using spray drying was evaluated during 90 days at 25 and 4ºC and the results were compared with freeze-dried RWK powder and RWK beverage (Figure 7.4).

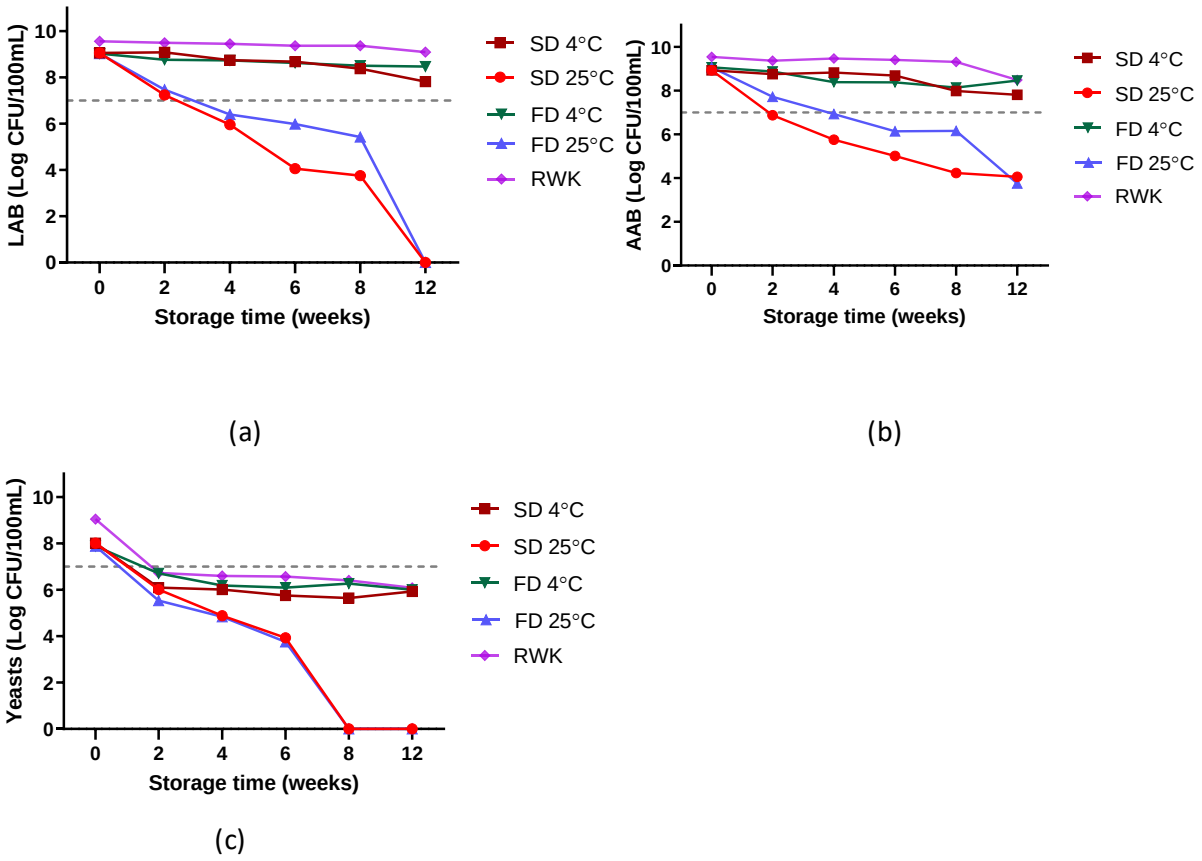


Figure 7.4 *Storage stability of Russian olive water kefir microorganisms including a) Lactic acid bacteria (LAB), b) Acetic acid bacteria (AAB) and c) Yeasts in RWK drink, and in RWK powder encapsulated using spray drying (SD) and freeze drying (FD), during 90 days of storage at room temperature (25 °C) and in the fridge (4 °C). The gray line indicates 10^7 CFU, which is the minimum required number of probiotics per portion of a consumed product for conferring health benefits to the consumer, defined by the standards.*

The refrigerated encapsulated powders (spray-dried and freeze-dried) showed to have significantly higher survivability of all water kefir microorganisms compared to the encapsulated RWK powders stored at room temperature. Results also showed that except for yeast cells, which dropped to less than 107 log CFU/100mL within the first two weeks of storage at both storage temperatures, if encapsulated RWK powders (both SD and FD) are

stored at the fridge temperature, they can retain the sufficient number of LAB and AAB (107 CFU/100 mL) for at least 3 months of storage. According to the Canadian Food Inspection Agency (2019) and Codex Alimentarius Commission (2018), for a fermented food product to confer a beneficial health impact to the host, 1.0 x 109 CFU of one or more of the eligible probiotic microorganism (s) per serving size of a product is required and according to Bertazzoni et al. (2013), the volume of the serving size of a probiotic drink is often 100-200 mL. Therefore, after a minimum of 90 days of storage, refrigerated RWK samples are expected to meet the standards for imparting the health benefits of probiotics to the consumer. Similarly, cold storage has been reported as a suitable preservation condition for dried cultures (Wang et al., 2004; Simpson et al., 2005; Teijeiro et al., 2018). The produced RWK drink stored at 4ºC, was showed to retain a high level of viability of its water kefir microorganisms at the end of 90 days for LAB and AAB, respectively; however, undesirable changes in the sensory properties of the RWK drink was observed, which can be due to the ongoing metabolic activity of water kefir microorganisms affecting taste and flavour of the product during extended storage. At room temperature, the RWK samples encapsulated using the optimized spray drying conditions showed good viability and storage stability of the water kefir microorganisms while they were slightly lower than those measured in the freeze-dried samples, which can be attributed to the higher temperature reached during spray drying. When encapsulated samples were stored at 4 C, spray-dried samples showed closely related survivability for LAB and AAB, particularly within the first two months of storage, while the yeast cells showed similar storage stability in both encapsulation methods regardless of storage temperature.

7.4.3 The resistance of RWK microorganisms in the simulated gastrointestinal transit

Table 7.3, Figure 7.5A-C and D-E illustrate the viable number of water kefir microorganisms in each stage of exposure to the gastrointestinal transit (SGF and SIF, respectively).

Table 7.3 *Viability of water kefir microorganism in RWK with or without encapsulation in gum Arabic/Maltodextrin using spray drying (SD) and freeze-drying (FD) during exposure to simulated gastric fluid (SGF, pH 3.0) and simulated intestinal fluid (SIF, pH 7.0) for 120 minutes.*

	Viable number of cells (log CFU/100mL)																	
	LAB						AAB						Yeasts					
Time (Hour)	Post SGF			Post SIF			Post SGF			Post SIF			Post SGF			Post SIF		
	RWK	*SD*	*FD*	*RWK*	*SD*	*FD*	*RWK*	*SD*	*FD*	*RWK*	*SD*	*FD*	*RWK*	*SD*	*FD*	*RWK*	*SD*	*FD*
0	*9.56*	*9.05*	*9.02*	*7.3*	*6.63*	*6.62*	*9.54*	*8.9*	*9.06*	*7.73*	*6.4*	*6.9*	*9.04*	*8*	*7.88*	*8.74*	*7.95*	*7.81*
1	*9.11*	*8.09*	*8.2*	*7.2*	*6.39*	*6.34*	*9.36*	*8.11*	*8.27*	*7.2*	*6.16*	*6.57*	*8.83*	*7.97*	*7.83*	*8.78*	*7.93*	*7.84*
2	*7.3*	*6.63*	*6.62*	*7.16*	*6.24*	*6.2*	*7.73*	*6.4*	*6.9*	*7.09*	*6*	*6.29*	*8.74*	*7.95*	*7.81*	*8.79*	*7.97*	*7.87*

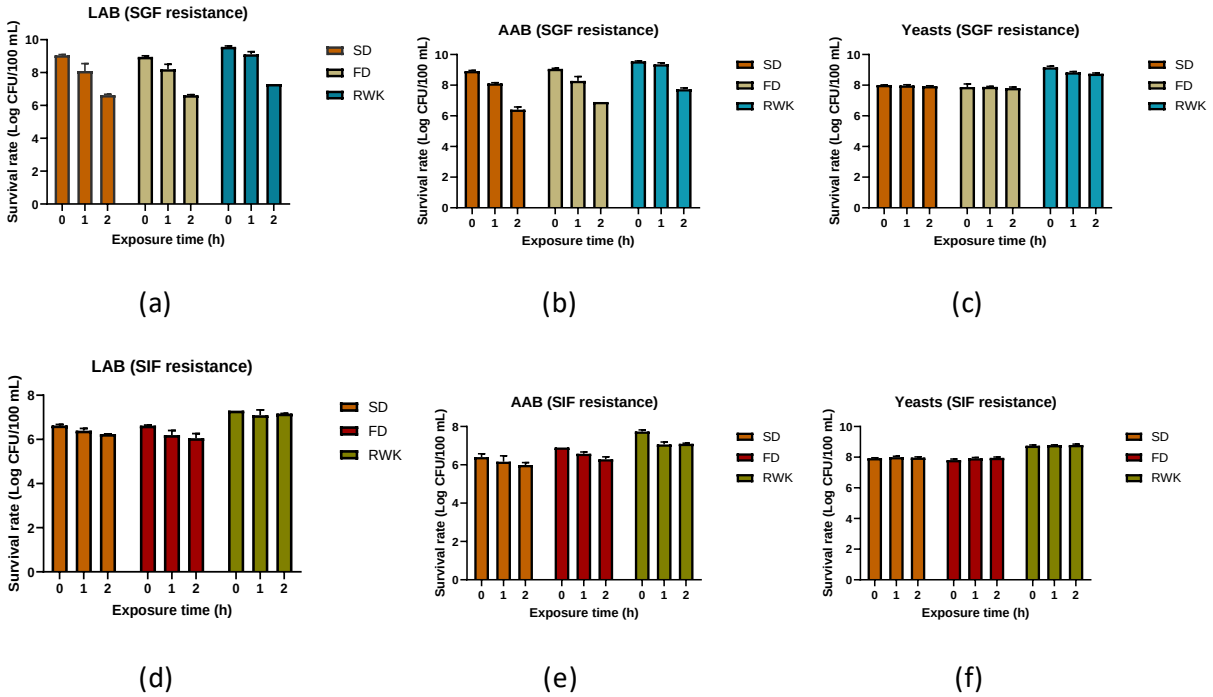
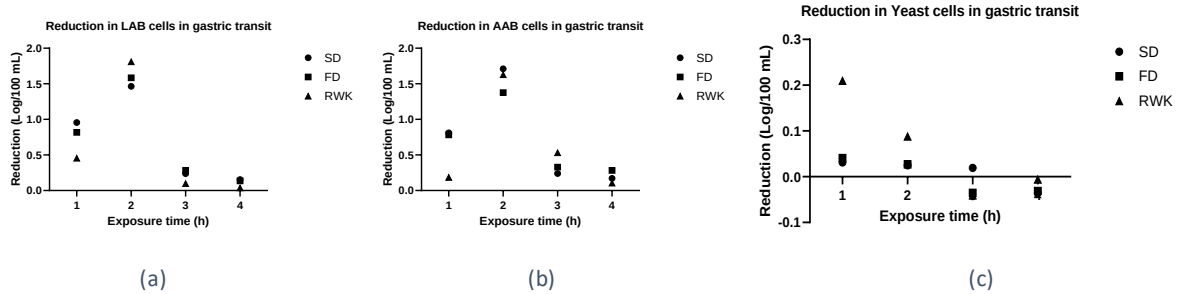


Figure 7.5 *Survival (log CFU/100 mL) of lactic acid bacteria (LAB), Acetic acid bacteria (AAB) and yeast cells present in spray-dried RWK powder (SD), freeze-dried RWK powder (FD) and Russian olive water kefir drink (RWK) during exposure to simulated conditions of the gastrointestinal transit. (a-c): Time zero (0), upon 1-hour exposure to simulated gastric fluid (SGF) (1), 2 hours exposure to SGF (2). (d-f): Time zero (0), upon 1-hour exposure to simulated intestinal fluid (SIF) (1) and 2 hours exposure to SIF (2).*



(a) (b) (c)

Figure 7.6 *Reduction rate (log CFU) of a) lactic acid bacteria present (LAB), b) Acetic acid bacteria (AAB) and c) yeast cells in spray-dried RWK powder (SD), freeze-dried RWK powder (FD) and Russian olive water kefir drink (RWK) in different stages of exposure to simulated conditions of the gastrointestinal transit.*

The viability of water kefir microorganisms in the spray-dried sample decreased by 0.25–2.9 log cycles after sequential exposure to SGF and SIF, which is comparable and within the range of survival rate of suggested probiotic bacteria such as lactic acid bacteria strains isolated from different sources including fruits and fermented foods, which are reported to have up to 5.56 log cycle reduction after exposure to simulated GI track (Musikasang et al., 2009; Shekh et al., 2016; Boricha et al., 2019; Joghataei et al., 2019). As it can be seen in these figures, water kefir microorganisms (including LAB, AAB and yeast cells) survived within the range of 10^6–10^8 CFU per 100 mL serving size of a rehydrated RWK powder, showing a satisfactory level to overcome the gastro-intestinal tract (GIT) chemical stress barrier, which is among the *in vitro* tests commonly suggested by FAO/WHO (2002) for investigating the potential probiotic properties of products. The results confirm previous studies on the potential probiotic properties of probiotic strains present in water kefir (Magalhães et al., 2010; Schneedorf, 2012; Diosma et al., 2014; Laureys & Vuyst, 2014a; Zanirati et al., 2015; Romero-Luna et al., 2020).

Figure 7.6A-C presents the overall loss of water kefir microorganisms during different stages of a 4 hours exposure to simulated gastric liquids. As can be seen in these figures, all samples showed the highest rate of reduction of viable cells during the second and first hour of exposure to SGF for water kefir bacteria (LAB and AAB) and yeasts, respectively. The results show that the yeast

cells, among water kefir microorganisms, are the most resistant cells with the highest survival rate upon exposure to gastric fluids. They showed some level of recovery at the end of the gastrointestinal passage, which might be attributed to the neutral pH of the intestinal phase (Picot & Lacroix, 2004). Comparing the two methods of encapsulation for RWK samples shows that except for AAB cells, in which the freeze-dried powder showed a lower microbial loss, no significant difference was observed in LAB and yeast cells loss during the gastric transit. Also, comparing the resistance of water kefir microorganisms in RWK sample and encapsulated samples (spray-dried and freeze-dried), in the simulated gastric environment, shows that LAB and AAB cells tend to retain higher viability in RWK. This can be explained first by the encapsulation properties of RWK and second, by the stress applied to the cells during the drying process. Similarly, the *in vitro* studies by Desmond et al. (2002), reported a limited loss of viability of probiotics using carrier agents such as gum Arabic during spray-drying, while our results show that spray drying encapsulation using Gum Arabic/Maltodextrin can protect probiotics in RWK against stress during digestion, which indicates the successful application of spray drying for producing synbiotic microparticles. Comparing two methods of encapsulation, both spray-dried and freeze-dried RWK samples showed closely related viability loss at the end of the gastrointestinal transit, supporting the use of spray drying as an economical method compared to freeze drying for encapsulation of RWK microorganisms.

7.5 Conclusion

Microencapsulation through spray drying, as an economical method for the preservation of RWK powder bioactive compounds for extended periods, is of interest to the production of RWK. Based on our results, the storage temperature was not found to be significantly important for better retention of total phenolic content and antioxidant activity in the encapsulated RWK powder,

therefore considering better stability of bioactive compounds of encapsulated RWK powder, associated extra costs for refrigeration seem unnecessary. However, it showed a significant effect on the survival of water kefir microorganisms. Refrigeration of RWK powder can potentially extend its shelf life from two weeks to a minimum of 3 months through prolonged preservation of water kefir microorganisms above 10^7 CFU per serving size of the product, which is the minimum number of probiotics required for imparting health benefit from consumption. Comparing the two methods of encapsulation (SD and FD), results showed that the optimized spray drying encapsulation can protect the antioxidant capacity and total phenolics of the samples similar to freeze drying. However, considering the survival of water kefir bacteria, when encapsulated powders were stored at room temperature, the freeze-dried powder showed superior quality; whereases, in refrigerated storage, SD samples showed related viability compared to FD samples within the first two months of storage but lower for the last month of storage. Therefore, our findings support the storage stability of refrigerated SD for a minimum of three months with preserved antioxidant activity and sufficient water kefir microorganisms. In addition, our results indicate that microencapsulation of RWK through spray drying can produce dry RWK while protecting the live probiotic cells against the adverse gastrointestinal conditions including highly acidic conditions of the stomach and the enzymes and bile salts in the small intestine, to impart the expected beneficial effects for human health, when administered in adequate amounts. Therefore, the optimized spray drying process using wall materials for the production of RWK powder is suggested as a fast and cost-effective drying method to help the commercialization of a high-quality RWK product for responding to the increasing trend in the consumption of water kefir related products. This study also provided insight into how properties of the RWK powder can be affected as a consequence of storage time and temperature; however, to optimize the long-term

storage stability of encapsulated RWK product, a variety of other factors affecting the quality of the product such as oxygen content and relative humidity in the package as well as other quality factors of the product such as its sensory properties and retention of other bioactive compounds need to be further investigated.

7.6 Availability of data and materials

Data analyzed during this study are included in this published article [and its supplementary information file].

7.7 Acknowledgments

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CHAPTER VIII

OVERALL SUMMARY & CONCLUSION, CONTRIBUTION TO KNOWLEDGE AND RECOMMENDED FUTURE STUDIES

8.1 Overall Summary and Conclusion

The initial intention of this thesis was to evaluate and confirm the potential of Russian olive fruits, leaves and flowers to be utilized for the development of functional food products. The main goal of this project was to develop and optimize processes (extraction, fermentation, encapsulation), which help the development of Russian olive-based products and will facilitate their successful commercialization. The literature review and our preliminary test results showed that due to the high antioxidant activity and various health properties of Russian olive, it can be considered to be used as a functional ingredient (Chapters II and III). Therefore, developing new Russian olive-based products and optimization of associated post-harvest processes for valorizing this plant were evaluated in the experiments of this study (Chapters IV-VII).

Chapter IV demonstrated that Russian olive leaves and flowers are rich in phytochemicals with high antioxidant properties and highlighted microwave-assisted extraction as an efficient extraction method for producing antioxidant-rich extracts from this plant. This chapter evaluated the effects of microwave-assisted extraction process conditions on the quality of produced extracts from Russian olive and optimized the extraction process using response surface methodology. Among the MAE experimental factors, the concentration of ethanol in the solvent and the ratio of sample to solvent were identified as the most significant factors affecting the recovery of bioactive compounds from Russian olive leaves and flowers. The optimized recovery of antioxidant

compounds, total phenolic content and total flavonoids contents in the extracts was observed using 2 M citric acid, 1:7.5 (v/w) solid to solvent ratio, 66.4 and 59.8% ethanol concentration and at a temperature of 97.5 and 97.4 °C for MAE of Russian olive flowers and leaves, respectively. Application of antioxidant-rich extracts with beneficial health effects for the development of functional foods are of great interest in the food industry, and the optimized MAE extraction process developed in this study could be the first step for the preparation of antioxidant-rich extracts from Russian olive leaves and flowers at larger scales in future. Moreover, due to the potential therapeutic properties of this plant, which is attributed to its antioxidant compounds, the produced antioxidant-rich Russian olive extracts could have functional food applications for potential health benefits.

Chapter V utilized Russian olive fruit to develop Russian olive water kefir (RWK), as a new non-dairy beverage with enhanced bioactivities, and investigated the effects of fermentation factors on bioactive properties of the developed product. This chapter reported the result of optimized process conditions, in which a RWK product with a maximized number of water kefir microorganisms, TPC and antioxidant properties, is developed. The optimized fermentation condition was observed at the temperature of 31.2ºC for a duration of 24 hours and using 30 % Russian olive juice concentration. This study holds high significance from an industrial perspective as this is the first study that has been carried out using advanced bioreactors and response surface methodology for developing Russian olive water kefir. The developed RWK in this study can provide kefir's beneficial health properties for non-dairy consumers and create diversification in providing a wider range of kefir-like products for responding to increasing demand in non-dairy functional foods. Also, the optimized fermentation conditions in this study can facilitate the production of RWK at future industrial scale and the commercialization of this product.

In Chapter VI, carrier materials were used to encapsulate RWK microorganisms and bioactive compounds using spray drying, and a synbiotic RWK powder was developed. The effects of spray drying parameters on the quality of microencapsulated RWK were assessed using RSM. Among water kefir microorganisms, yeasts showed the highest susceptibility during the dehydration process. Application of drying aids showed significant improvement in the survival of water kefir microorganisms and preservation of antioxidant compounds during the spray drying process. CCD was employed to find the optimal conditions of the spray drying process, as the encapsulation method, for maximizition of the retention of bioactive properties of RWK while improving survival of its water kefir microorganisms. The optimized encapsulation conditions were observed at an inlet air temperature of 120ºC, 35 % feed flow rate, and using a 7% drying aid concentration. Under the selected experimental conditions, spray drying showed to be an efficient encapsulation method for preserving the quality of RWK, and the spray-dried RWK powder showed promising microbial and physicochemical properties when compared with the freeze-dried powder. These results support the application of spray drying for encapsulation of water kefir-related products in future. Considering water kefir as a non-dairy product with high antioxidant properties and probiotics beneficial to health, the optimized spray drying process in this study could help to respond to increasing consumer's demand for functional food products. It provides an alternative format of Russian olive water kefir with a longer shelf life, and facilitates commercialization of RWK through improving storage stability and reducing storage and transportation associated costs of this product.

Finally, in chapter VII, the storage stability of encapsulated water kefir samples was investigated. Results proved the spray drying process optimized in the previous chapter is an efficient encapsulation method for the preservation of RWK bioactive properties during storage, and

refrigerated RWK powder can have a minimum of 90 days of storage stability. Spray-dried RWK samples were exposed to the simulated gastrointestinal conditions and the results showed high survival of the water kefir microorganisms, with the yeast cells showing the highest resistance and survival rate among all. The effect of storage conditions on the quality of encapsulated RWK powders was assessed and compared between spray-dried (SD) and freeze-dried (FD) samples. The results showed that even though cold storage did not significantly affect the retention of total phenolic content and antioxidant activity in the encapsulated RWK powder, it significantly improved the survival of Russian olive water kefir microorganisms. Concerning the retention of water kefir microorganisms during storage, above the minimum number of probiotics required for imparting health benefits (10^7 CFU per serving size), freeze drying showed to produce RWK powder with superior stability compared to spray drying, unless SD samples are stored refrigerated. In cold storage, SD samples showed prolonged storage which was closely related to the viability of water kefir microorganisms in FD samples within the first two months of storage, but lower for the third month of storage. Overall, this study showed good functional properties such as a sufficient number of water kefir microorganisms and preserved antioxidant activity in refrigerated spray-dried RWK for a minimum of three months.

8.2 The important contributions of this study to knowledge are as follows:

1) The potential application of Russian olive leaves, flowers and fruits as functional food ingredients is investigated.

2) An optimized microwave-assisted extraction process is developed to obtain antioxidant-rich extracts from Russian olive leaves and flowers for their application in the development of functional foods.

3) A novel water kefir beverage is formulated using Russian olive with high antioxidant activity and known therapeutic properties to introduce a non-dairy probiotic beverage.

4) The fermentation process of Russian olive water kefir is optimized for maximizing the bioactive properties of this product.

5) Russian olive water kefir powder is produced for improving the stability and commercialization of this product and providing an opportunity for potential application of the developed powder in different food products.

6) The encapsulation process of Russian olive water kefir is optimized based on maximum retention of its bioactive compounds protecting its functional properties.

7) Storage stability of spray-dried powder of Russian olive water kefir for 3 months is confirmed.

Overall, this thesis contributes to knowledge by demonstrating how Russian olive could be employed for extracting value-added compounds and developing functional products for the valorization of the Russian olive leaves, flowers and fruits with various functional health properties. This study is promising from an industrial perspective as it developed and optimized processes that widen the application of Russian olive as a functional ingredient, with various functional nutritional health properties, in the food industry.

To conclude, there is no doubt that Russian olive with various functional health properties has the great potential to be used as a functional food ingredient and the developed products and optimized processes in this study will play a major role and could be the first step in the valorization of this plant.

8.3 Future studies

The following are the recommendations for future research based on the current study:

1) Scale-up studies such as MAE of Russian olive leaves and flowers and fermentation of Russian olive water kefir should be carried out based on the optimized process conditions specified in the study.

2) Evaluation of the sensory properties of developed RWK and RWK powder is required for the successful commercialization of this product.

3) Investigation of appropriate packaging for RWK: Appropriate packaging is an important issue for maintaining probiotics viability and bioactive properties of the product during storage, which is affected by packaging's permeability, material and technique (Barbosa and Teixeira 2017). In addition, as yeast growth continues after packaging, it is important for the kefir product containers to withstand the buildup pressure or to be a flexible container capable of retaining the produced gas (Sarkar 2008).

4) Assessment of the therapeutic properties of Russian olive kefir powder: According to studies on kefir and Russian olive, both share different bioactivities and therapeutic properties including antioxidant, anti-inflammatory, anti-ulcerogenic, and antimicrobial activities (Rodrigues et al., 2005; Alsayadi et al., 2013; Alsayadi et al., 2014; Farzaei et al., 2015; Rodrigues et al., 2016). Therefore, when water kefir grains and Russian olive are combined for producing RWK, evaluating the combined therapeutic properties of the developed Russian olive water kefir needs to be investigated.

5) Evaluation of the antibacterial activity of RWK: The production of various types of antimicrobial compounds as beneficial metabolites is important in fermentation processes. It is expected that the fermented RWK will show antibacterial activity, which can be primarily

attributed to the increased production of acetic acid and also biosynthesis of other metabolites during the water kefir fermentation process (Ayed et al. 2017).

6) Future studies on potential applications of Russian olive water kefir powders in food products:

6.1 Application of Russian olive water kefir powder in candy formulation: It can provide a flavorful option for developing a candy with kefir health benefits for non-dairy customers, which is valuable to be investigated.

6.1.2 Application of Russian olive water kefir powder in bread: There are several advantages of mixed starter in baking over yeasts including development of better flavour and increased preservation time (because of in situ production of antimicrobial compounds). Water kefir has a natural mixed culture including yeasts, LAB and AAB and its application as a baker's yeast can result in the production of a water kefir sourdough bread with improved spoilage resistance, taste and aroma (Harta et al. 2004). Also, Russian olive has a unique aroma and taste and high water holding capacity. Therefore, the application of RWK powder as an alternative starter for sourdough bread is recommended to be investigated in future studies.

6.2 Application of Russian olive water kefir powder for yogurt supplementation: Low viscosity and syneresis in yogurt are among the most typical problems in the production of this product. Particularly in low-fat yogurt, in which fat globules with an important role in retaining water in yogurt are decreased (Kroger 2003). In traditional yogurt, stabilization of protein gel is mainly through weak and non-covalent interactions, whereas the addition of phenolic compounds can create new covalent bonds to proteins leading to gel formation with a different structure (Vital et al. 2015; Kroger 2003). RWK powder, as a source of bioactive compounds such as phenolic compounds with antioxidant activity (and other functional health properties of interest), can be

considered as a suitable food constituent in the production of low-fat yogurt, in order to overcome the problem of syneresis, especially during the storage. In addition, due to the high water holding capacity of Russian olive, supplementation of yogurt with RWK is expected to enhance the water holding capacity of yogurt leading to decreased syneresis. Therefore, investigating the microbiological, antioxidant, functional and rheological properties of low-fat yogurt supplemented with Russian olive water kefir powder (RWK) is valuable in future studies.

6.3 Supplementation of ice cream with Russian olive water kefir powder: Russian olive with high water holding capacity can modify viscosity and texture of the products and consequently bulking, thickening and gelling effects of the ingredients (Sahan et al. 2015). The application of Russian olive as a food additive for optimization of flavour and viscosity in ice cream has shown good results in an experiment by Cakmakc et al. (2015). Therefore, the application of spray-dried Russian olive water kefir powder in different products such as ice cream not only is expected to improve the rheological and sensory properties of these products, but also can introduce the health benefits of water kefir in their formula, which needs to be investigated in future studies.

CHAPTER IX

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