ESTONIAN UNIVERSITY OF LIFE SCIENCES
Institute of Veterinary Medicine and Animal Sciences

Minna Latva-Somppi

ANTIMICROBIAL EFFECT OF PLANT POWDERS IN RAW AND COOKED MINCED PORK

TAIMSETE LISANDITE ANTIMIKROOBNE EFEKT TOORES JA KÜPSETATUD SEAHAKKLIHAS

Final Thesis in Veterinary Medicine

Supervisors: Professor Mati Roasto, *PhD*
Associate professor Kadrin Meremäe, *PhD*

Tartu 2019

LÜHIKOKKUVÕTE

<table>
<tr><td colspan="2">Eesti Maaülikool
Kreutzwaldi 1, Tartu 51014</td><td colspan="2">Loomaarstiõppe lõputöö
lühikokkuvõte</td></tr>
<tr><td colspan="2">Autor: Minna Liisa Kaarina Latva-Somppi</td><td colspan="2">Õppekava: Veterinaarmeditsiin</td></tr>
<tr><td colspan="4">Pealkiri: Taimsete lisandite antimikroobne efekt toores ja küpsetatud seahakklihas</td></tr>
<tr><td>Lehekülgi: 49</td><td>Jooniseid: 3</td><td>Tabeleid: 4</td><td>Lisasid: 0</td></tr>
<tr><td colspan="4">Osakond / Õppetool: Toiduhügieeni ja rahvatervise
ETIS-e teadusvaldkond ja CERC S-i kood: B680
Juhendaja(d): Professor Mati Roasto ja dotsent Kadrin Meremäe
Kaitsmiskoht ja -aasta: Tartu, 2019</td></tr>
<tr><td colspan="4">Tarbijad on järjest enam huvitatud, et toit, mida endale valmistatakse või mida poest valmiskujul ostetakse, oleks mikrobioloogiliselt ja keemiliselt ohutu ning ei sisaldaks erinevaid sünteetilisi toidu lisaaineid. Seetõttu on paljud toiduainetööstused otsimas looduslikke alternatiive, seni toidus kasutatud sünteetilistele toidu lisaainetele, mis aitaks pikendada toiduainete säilimisaegu. Uurimistöö eesmärgiks oli valitud taimsete lisandite mikroorganismide kasvu pidurdava toime välja selgitamine toores ja kuumtöödeldud hakklihas. Uuringutes kasutatavad taimed olid in-vitro eelkatsetes näidanud mikroobide kasvu pärssivat toimet ning nendeks olid rabarber (Rheum rhaponticum L.), must sõstar (Ribes nigrum L.), söödav kuslapuu (Lonicera caerulea L. var. edulis), aroonia (Aronia melanocarpa) ning tomat (Solanum lycopersicum). Laboratoorsete katsete tulemustest selgus, et kõige enam pärssisid mkroorganismide kasvu rabarberi varred ning keedusool kombinatsioonis nitritiga. Kahe erineva taime kombinatsioonidest oli toores hakklihas parima antimikroobse toimega 1% rabarberi varred kombinatsioonis 1% tomatiga.
Kuumtöödeldud hakklihas avaldasid mikroobide kasvu pärssivat toimet ka mõned teised taimsed pulbrid. Lõppkokkuvõttes osutasid kestvuskatsetel kõige paremat mikroobide kasvu pärssivat toimet tomat, rabarberi varred, gallushape, rutiin ning keedusool kombinatsioonis nitritiga.</td></tr>
<tr><td colspan="4">Märksõnad: taimsed pulbrid, mõned taimsed polüfenoolid, seahakkliha, mikroobide üldarv, kestvuskatsed</td></tr>
</table>

SUMMARY

Estonian University of Life Sciences Kreutzwaldi 1, Tartu 51014		Abstract of veterinary medicine thesis	
Author: Minna Liisa Kaarina Latva-Somppi		Curriculum: Veterinary medicine 2013	
Title: Antimicrobial effect of plant powders in raw and cooked minced pork			
Pages: 49	Figures: 3	Tables: 4	Appendixes: 0
Department / Chair: Food hygiene and veterinary public health Field of research and (CERC S) code: B680 Supervisors: Professor Mati Roasto and associate professor Kadrin Meremäe Place and date:			
In present study the aim was to study microbial growth inhibiting effect of selected plant powders by counting microorganism's general numbers in raw and cooked minced pork at the end of defined shelf-life. Plants used in study were siberian rhubarb *(Rheum rhaponticum*), black currant (*Ribes nigrum*), blue honeysuckle (*Lonicera caerulea* var edulis), black chokeberry (*Aronia melanocarpa)* and tomato (*Solanum lycopersicum*). The most efficient microbial growth inhibiting effect compare to control were found for rhubarb petioles and the combination of sodium chloride with sodium nitrite. Among the combinations of two different plant additives, the most efficient combination of the additives in raw minced pork were 1% rhubarb petioles in combination with 1% tomato. Compared to the other findings, the number of microorganisms in raw minced pork with 1% rhubarb petioles combined with 1% tomato increased most slowly for 6 day of storage. The inhibitory effect in cooked minced pork was also observed on the several other plant additives. The most efficient antimicrobials both in raw and cooked minced pork were tomato, rhubarb petioles, gallic acid, rutin and sodium chloride + sodium nitrite.			
Keywords: plant powders, some plant polyphenols, minced pork, total numbers, durability studies			

TABLE OF CONTENTS

INTRODUCTION

Microorganisms are always associated with harvested plants and slaughtered animals (Negi 2012). Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. The use of plant extracts with known antimicrobial properties can be of great significance in food preservation. The value of plants is in some chemical substances that produce a definite action on the microbiological, chemical and sensory quality of foods (Cowan 1999). Plant extracts have shown a considerable range of applications in the food industry e.g. food natural antimicrobials can cause delay in microbial growth or kill the bacteria. Substances with antimicrobial properties can be robustly divided as natural and synthetic. Some synthetic food additives, benzoic acid, are also naturally found in some berries e.g. in cranberries (Negi 2012).

The use of natural antimicrobials such as organic acids, essential oils, plant extracts, and bacteriocins could be a good alternative to ensure food safety. Spoilage by bacteria in meat causes off-odor, off-flavor, discoloration, gas production, slime production, and reduced pH, leading to significant economic losses (Papuc *et al.* 2017).

The antimicrobial activities of plant extracts may reside in a variety of different components, and several extracts owing to their phytochemical constituents have been shown to have antimicrobial activity. Plant extracts also have shown antifungal activity against a wide range of fungi. Also, antimutagenic activities and inhibition of lipid oxidation in foods have been reported (Negi 2012).

Berries are a great source of bioactive compounds such as polyphenols (i.e., phenolic acids, flavonols, anthocyanins, tannins) and ascorbic acid. They may act as antimicrobials and antioxidants. Plant extracts are incorporated in meats as water-soluble and water insoluble extracts and powders. Type of delivery agents include juices, hulls, essential oils, decoctions, hydrolysates, and grinded residues. They are able to affect the self-life, quality, various sensorial and health related aspects of enriched meat products. Some antimicrobials and antioxidants are well known for their antioxidant potential and are

available commercially in crude or active ingredient form, such as rosemary and grape seed extract (Lorenzo *et al.* 2017).

The concentration of antibacterial and antioxidant compounds in plant materials varies considerably and hence their dosage application in diets and meat products varies from plant to plant. Taking into account many beneficial properties, and their juices/extracts are rich with polyphenols are reported to contain health benefit compounds, hence suitable for the use in the meat and meat products (Lorenzo *et al.* 2017).

Numerous studies have been done *in-vitro* to evaluate the antimicrobial activity of plant extracts, very few studies are available for food products.

ACKNOWLEGMENTS

I thank my supervisor’s Professor Mati Roasto and Associate Professor Kadrin Meremäe. Additionally, I thank Professor Tõnu Püssa and other members of the Chair of Food Hygiene and Veterinary Public Health, namely Dea Anton, Piret Raudsepp and Julia Koskar who were the researchers of the related project, therefore also participated in experimental work.

The research was financially supported by:
EU ERA-NET SUSFOOD project "Sustainable plant ingredients for healthier meat products – proof of concepts” (SUSMEATPRO);
Basic financing project “The study of the effect and related mechanisms of natural bioactive substances in food matrices” of Estonian University of Life Sciences P180279VLTR.

1. REVIEW OF THE LITERATURE

1.1 Antimicrobial activity of plants and their metabolites

There are present chemical compounds called phytochemicals in plants. These compounds are secondary metabolites meaning that they do not affect growth of the plant, but impact for example the colour of plants (Ahmad *et al.* 2015). These compounds have been developed by plants to defend their organisms from the effects of free radicals, viruses, bacteria and fungi, but also against herbivores and insects. Phytochemicals can be classified into several major groups based on their chemical structure: including polyphenols, flavonoids, tannins, alkaloids, terpenoids, isothiocyanates, lectins, polypeptides or their oxygen substituted derivatives (Barbieri *et al.* 2017, Cowan 1999). Some groups of phytochemicals, like terpenoids give plants their odors, others (quinones and tannins) are responsible for plant pigments. Another compound groups are responsible for plant flavour, for example the terpenoid capsaicin from chili peppers. Latter are used as spices or medical herbs (Cowan 1999). There have been isolated at least 12,000 phytochemicals in the world.

Plant secondary metabolites, most of which are phenols or their oxygen-substituted derivatives possess various benefits including antimicrobial properties against pathogenic and spoilage microbes. Major groups of compounds that are responsible for antimicrobial activity from plants include phenolics, phenolic acids, quinones, saponins, flavonoids, tannins, coumarins, terpenoids, and alkaloids. Variations in the structure and chemical composition of these compounds result in differences in their antimicrobial action (Gyawali, Ibrahim 2014).

During food processing, often large amount of by-products is generated including fruit pomace, seeds, peels, pulps, and husks. These are promising sources of valuable food component and have several functionalities including antimicrobial activity (Guil-Guerrero *et al*. 2016).

The effectiveness of antimicrobial compound depends on pH of the food, type and number of contaminating microorganisms, and type and concentration of antimicrobial. Storage temperature may also influence the effectiveness of antimicrobial because the diffusion of compounds is related to the temperature. Phytochemicals present in many foodstuffs are often lost by thermal processing such as sterilization, pasteurization, and dehydration. The use of combinations of natural antimicrobials is usually more effective than adding just one antimicrobial, because some microorganisms are not inhibited or killed by the commonly used doses of single natural antimicrobials (Negi 2012).

1.2 The mechanisms behind of antibacterial effect on plants

In plant and in their by-products antibacterial effects are mostly caused by secondary metabolites like tannins, terpenoids, alkaloids, and phenolics. They have a great number of subclasses of active compounds, so that the list of compounds to check in is almost inexhaustible (Guil-Guerrero *et al.* 2016). The exact target for natural antimicrobials are often not known or not well defined, as it is difficult to identify a specific action site where many interacting reactions take place simultaneously (Negi 2012). To arrest the spread of pathogens, plants possess an innate immunity that involves different layers of defence responses. Some of these defences are preformed and others are activated after recognition of pathogen elicitors and include reinforcement of the cell wall, biosynthesis of lytic enzymes and production of secondary metabolites and pathogenesis related proteins (González-Lamothe *et al.* 2009).

Most of the bacterial plant pathogens are Gram negative and most of the biologically active purified plant products show low activity against such organisms. Gram positive bacteria are often nevertheless susceptible to plant products and this suggests that the fundamental morphological differences in the cell wall and membrane organization of Gram negative and Gram positive organisms modulate their susceptibility to purified phytoanticipins and phytoalexins (González-Lamothe *et al.* 2009).

The outer membrane of Gram-negative bacteria acts as a permeability barrier and is responsible for the intrinsic resistance of these micro-organisms to antimicrobial compounds. The effect is mainly due to the presence and features of lipopolysaccharide molecules in the outer leaflet of the membrane, resulting in many Gram-negative bacteria in an inherent resistance to hydrophobic antibiotics. Besides lipopolysaccharide molecules various multidrug efflux pumps also contribute to the resistance of the cells (Puupponen-Pimiä *et al.* 2005).

Alkaloids: With great structural diversity, alkaloids have no single classification. Among plant foods, such compounds occur mainly in *Solanaceae* and *Fabaceae*, and in larger or smaller amounts in their by-products. The action mechanism of highly unsaturated planar quaternary alkaloids is attributed to their ability to intercalate with DNA (Guil-Guerrero *et al.* 2016).

Essential oils: these include volatile compounds of terpenoid or non-terpenoid origin, all being hydrocarbons and oxygenated derivatives. They act like other phenolics, for example, disturbing the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport, and coagulation of cell contents (Guil-Guerrero *et al.* 2016).

Glycosides: are molecules in which a sugar is bound to another functional group via a glycosidic bond, and they are classified according to the chemical nature of the aglycone. Many plants store chemicals in the form of inactive glycosides, which can be activated by enzyme hydrolysis. Products from glucosinolate hydrolysis have been evaluated as antimicrobial agents in Gram-positive and Gran-negative bacteria (Guil-Guerrero *et al.* 2016).

Phenolics: are molecules having one or more unsaturated rings with one or more hydroxyl groups, constituting a ubiquitous group of secondary metabolites that occur profusely in species of the plant kingdom with wide pharmacological activities. Phenolic acids occur in most plant foods, mainly in seeds, fruit peels, and leaves. Polyphenols are not involved in the normal growth and development of plants but do have important roles in plant defence mechanisms against viruses, bacteria, fungi, and herbivores. Polyphenols

can be subdivided into 3 main classes, flavonoids, stilbenoids, and phenolic acids. Flavonoids are the most prevalent of these (Papuc *et al.* 2017). Low-molecular-weight phenolic acids exert antimicrobial effects by the diffusion of the undissociated acid across the membrane, leading to the acidification of the cytoplasm and, in some cases, cell death. For example, tannins mechanisms of action are the inhibition of extracellular microbial enzymes, and deprivate the substrate needed for microbial growth, and inhibition of oxidative phosphorylation, which affects microbial metabolism (Guil-Guerrero *et al.* 2016). Another example is the antimicrobial effect of tea polyphenols by damaging bacterial cell membranes, including having increased outer and inner membrane permeability and disrupted cell membranes. Many studies have reported that polyphenols can exhibit antibacterial activity via anti-biofilm agents (Papuc *et al.* 2017).

Saponins: These are structurally diverse compounds derived from steroids or triterpenoid glycosides, which occur in many plant foods and plant-food by-products. Their activity has been linked to their membrane-permeabilizing properties, being immunostimulant and affecting growth, feed intake, and reproduction in animals (Guil-Guerrero *et al.* 2016).

The effectiveness of antimicrobial compound depends on pH of the food, type and number of contaminating microorganisms, and type and concentration of antimicrobial. Storage temperature may also influence the effectiveness of antimicrobial as the diffusion of compounds is related to the temperature. Phytochemicals present in many foodstuffs are lost by heat processing such as sterilization, pasteurization, and dehydration. The use of combinations of antimicrobials is usually more effective than adding just one antimicrobial, because some microorganisms are not inhibited or killed by the commonly used doses of antimicrobials (Negi 2012).

1.3 Bioavailability

Bioavailability is defined as the fraction of an ingested nutrient that is available to the body through absorption for utilization in normal physiogical functions and for metabolic processes (Shi, Le Maguer 2000). It is very important concept when assessing beneficial effect of polyphenols (Pineda-Vadillo *et al.* 2016). Factors that has effect to bioavailability are plants grow environment and weather, food processing, like thermal treatment and storage time and food matrix. The polyphenols and their metabolites can bind to proteins. It has been reported the existence of intermolecular bonds between serum albumin and quercetin metabolites (D'Archivio *et al.* 2010).

The composition and structure of food have an impact on the bioavailability of lycopene (Shi, Le Maguer 2000). Cooking or fine grinding of foods can increase the bioavailibility of lycopene by disrupting or softening plant cell wall. Several studies have found out that lycopene bioavailability is higher from the product than fresh tomatoes. Lycopene bioavailability in paste and processed tomato juice was significantly higher than from unprocessed fresh tomatoes (Shi, Le Maguer 2000).

The proportion of polyphenols released from the food matrix and solubilized into the digestive fluids (bioaccessibility) is a key step that has to be accomplished in all cases since only bioaccessible polyphenols can be further absorbed and remain bioavailable (Pineda-Vadillo *et al.* 2016). Structure and composition of the food matrix in which polyphenols are included are factors that can either enhance or prevent the release and stability of these compounds during digestion and hence, their effectiveness. The effect of the co-digestion of polyphenols with different food components, matrices or diets has been proven to affect their digestibility, bioaccessibility or antioxidant activity (Pineda-Vadillo *et al.* 2016). Various types of dietary fiber can reduce the bioavailability of carotenoids in foods. Absorption of lycopene seemed to be more efficient at lower dosages, and lycopene ingested with β-carotene was absorbed more than when ingested alone (Shi, Le Maguer 2000). Pineda-Vadillo *et al.* (2016) showed that the inclusion of the grape extracts into the different egg and dairy food matrices greatly impacted the release and solubility of anthocyanins and proanthocyanidins during digestion, especially in the solid food matrices and during the oral and gastric phases of digestion.

1.4 Overview of different studies on antibacterial effect of plant additives

Mostly, the antibacterial effect of plant additives is measured by using *in-vitro* methods like agar-diffusion methods, broth microdilution method as a fast screening method for MIC determination and the macrodilution method at selected MIC values to confirm bacterial inactivation (Klančnik *et al.* 2010). However, the plant additives (extracts, powders etc.) may have not the same kind of antimicrobial effect *in vitro* and in products, because the concentrations on foods are usually not very high, additives may be bonded into the food matrix, and because of other possible reasons.

The most frequently used way is to study antibacterial effect of plant additives in foods to compare the shelf-life of enriched foods and non-enriched foods. This is similar to classical durability study which usually include both microbiological and chemical food analyses. One possible way is to perform Challenge-testing with targeted micro-organisms e.g. *Listeria monocytogenes* growth in enriched and non-enriched RTE-foods. In classical food products durability study the general numbers of microorganisms are measured, because these are reflecting microbiological food quality. Also, are quite non-expensive, therefore preferred by food enterprises.

Challenge tests aim to provide information on the behaviour of *L. monocytogenes* which have been artificially inoculated into a food, under given storage conditions. They may take into account the variability of the batches, of the food samples and of strains. The level of contamination, the heterogeneity of the contamination and the physiological state of the bacteria are difficult to mimic in a challenge test study; the contamination method cannot always enable to fully imitate the natural contamination (EURL 2014).

Durability studies allow an assessment of the shelf-life of the food regarding *L. monocytogenes* in a naturally contaminated food during its storage according to reasonably foreseeable conditions. Durability studies may be considered more realistic than a challenge test, as the contamination is naturally occurring. But the implementation

of durability studies is limited in case of low prevalence and low level of contamination (EURL 2014).

Aerobic colony count is useful test for various categories of ready-to-eat food. It counts organisms which grow under aerobic conditions at mesophilic temperatures on a particular growth medium. This provide useful information to assess a food's quality or its remaining shelf-life (Food Safety Authority of Ireland 2016), but cannot used to assess the safety of food. An unsatisfactory result for the test does not mean that the batch of food is unsafe, but the result represents unsatisfactory levels of microbial contamination. It does not differentiate aerobic microorganisms or indicate the presence of pathogens. The aerobic colony count result should be assessed against the limits presented for the category into which the food best fits, based on the type of product, the processing it has received and the potential for microbiological growth during storage (Food Safety Authority of Ireland 2016).

1.4.1. Overview of different *in-vitro* studies

Phenolic berry extracts have been found to inhibit the growth of *Salmonella*, *Escherichia*, *Staphylococcus*, *Helicobacter*, *Bacillus*, *Clostridium* and *Campylobacter* species but not *Lactobacillus* and *Listeria* species. *Salmonella*, *Staphylococcus*, *Helicobacter and Bacillus* strains were the most sensitive bacteria for the berry extracts. The phenolic extract of cloudberry possessed the strongest antimicrobial activity, followed by raspberry and strawberry. The weakest antimicrobial effects were measured with chokeberry, rowanberry, crowberry and buckthorn berry. Cranberry extract was effective against *Bacillus cereus* and *Clostridium perfringen* (Puupponen-Pimiä *et al.* 2005).

A lot of interested has put into cranberry. Wu *et al.* (2008) tested American cranberry (V*accinium macrocarpon*) concentrate effect against some common foodborne pathogens. In the study, cranberry concentrate showed antibacterial effects on both Gram-positive (*L. monocytogenes* and *S. aureus*) and Gram-negative (*E. coli* O157:H7 and *S.* Typhimurium) bacteria. Gram-positive bacteria were less sensitive to the cranberry concentrate than the Gram-negative bacteria when nutrients were abundant in a suitable growth environment, BHI broth.

In another study Côté *et al.* (2011) investigated the antimicrobial effect of cranberry juice and of three cranberry extracts: water-soluble, apolar phenolic compounds, and anthocyanins was investigated against seven bacterial strains. Each cranberry sample was analyzed to determine the minimum inhibitory concentration (MIC) and the maximal tolerated concentration (MTC) at neutral pH. The results, reported in mg phenol/mL, indicated that all the bacterial strains, both Gram-positive and Gram-negative, were selectively inhibited by the cranberry phenolic compounds. The extract rich in water-soluble phenolic compounds caused the most important growth inhibitions. The ERV bacteria (*Enterococcus faecium* resistant to vancomycin), and to a lesser degree, *P. aeruginosa, S. aureus* and *E. coli* ATCC 25922, were the most sensitive to the antimicrobial activity of water soluble extract. The growth of *P. aeruginosa* and *E. coli* ATCC was also affected by the presence of the anthocyanin-rich cranberry extract of anthocyanins, even the observed antibacterial effect was not as important as with water soluble extract. In general, *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium were the most resistant to the antibacterial activity of the cranberry extracts.

Lu *et al.* (2011) tested rhubarb crude extract effect against *Aeromonas hydrophila*, which is important pathogenic bacteria in fish. It showed excellent antibacterial activity against *A. hydrophila* and was positively related anthraquinone content. Minimum inhibitory concentration of five rhubarb anthraquinone against *A. hydrophila* ranged 50-200 μg/ml. Study showed that anthraquinone emodin inhibit cellular function by binding DNA after penetrating the cell membrane, so the cell will die.

Smolarz *et al.* (2013) examined roots and petioles from *Rheum rhaponticum* for antimycobacterial activity. Extract from the roots of *R. rhaponticum* were found to have activities both against *M. Tuberculosis* H_{37}Ra and *M. bovis*. The antimicrobial effect was shown by minimum inhibitory concentration and minimal bactericidal concentration tests. Values of minimum inhibitory concentration and, minimal bactericidal concentration were generally the same or similar for both species of the mycobacteria. The anthraquinones were found to have a significant antibacterial activity.

Alaadin *et al*. (2007) examined ethanol, aqueous, and organic extracts from the root of *Rheum ribes Linn (Polygonaceae)*. The Minimum Inhibitory concentration values of the biologically active extracts, aloe emodin, and emodin, were 500, 125, 250, and 63 mg = mL against *Staphylococcus aureus*. The extracts and compounds did not inhibit *Pseudomonas aeruginosa* and *Escherichia coli* at the highest concentration tested, 4000 and 250 mg/mL.

Rigano *et al.* (2012) investigated antibacterial activity of a synthetic peptide derived from the tomato defensin family. Defensins are small, basic, highly stable proteins with antifungal and antibacterial properties. They synthesized chemically its g-motif (peptide) and tested its antimicrobial activity. They demonstrate in the study that the synthetic peptide exhibits potential antibacterial activity against Gram-positive bacteria, such as *Staphylococcus aureus* A170, *Staphylococcus epidermidis*, and *Listeria monocytogenes*, and Gram-negative bacteria, including *Salmonella enterica* serovar Paratyphi, *Escherichia coli*, and *Helicobacter pylori*.

1.4.2 Antimicrobial effect studies in products (*in vivo*)

Sanchez-Escalante et al. (2003) did study by lycopene-rich tomato pulp and extract of tomato rich in lycopene effect to beef patties. The patties were stored 20 days at temperature of +2 ° C in the dark. Control samples and those with lycopene-rich tomato pulp showed no significant differences in inhibition; counts were above 7 $\log_{10}(CFUg^{1})$ at day 12 of storage. Beef patties with extract of tomato rich in lycopene showed a significant inhibition of psychrotrophic bacteria growth; in fact, they did not reach a count of 7 $\log_{10}$(CFU/g) even at the end of the storage period. The shelf-life of treated beef patties ranged between 8 and 12 days. Inhibition was not as effective as treatment with Cayenne hot pepper and red sweet pepper.

Palmeri *et al.* (2018) studied prickly pear fruit extract and self-life of sliced beef. In their study *in vivo* application of extract effectively reduced microbial growth during refrigerated storage; total mesophilic count was maintained below the limit established by Commission Regulation (EC), 5 × 10log CFU/g of beef up to 8 days, in comparison to

control sample that reached the mentioned limit after 4 days. Moreover, extract addition preserved beef color and texture over the considered storage period, supporting the potential prospect to utilize the extract to improve overall quality and to prolong domestic shelf-life of sliced beef.

In Delgado-Adámez *et al.* (2016) study the effectiveness of the olive leaf extract in sliced pork loin found *in vitro* was not confirmed in the *in vivo* assays, even assays confirmed the antioxidant and antibacterial activity of fresh and freeze-dried olive leaf extract *in vitro*. Only, at day 1, the levels of mesophilic and *E. coli* counts were lower in olive leaf extract than in control. The application of the extracts into the packaging did not show antioxidant and antimicrobial effects on meat even applying high dose of the lyophilized extract. Delgado-Adámez *et al.* (2016) suggest that this could be because of the fact that extracts are not properly incorporated to the meat when the active compounds are in the plastic package.

Mhalla *et al.* (2017) studied *Rumex tingitanus* leave extracts for meat preservation and *in vivo* against *L. monocytogenes* in minced beef meat during storage. The *R. tingitanus* ethyl acetate fraction showed a bactericidal effect in a dose dependent manner against the foodborne pathogens *L. monocytogenes*. Thus, it was applied as a natural preservative in inoculated minced beef meat stored at 4 °C for 30 days. This fraction was found to be effective in controlling *L. monocytogenes* and inhibit the microbial growth during refrigerated storage.

In Hsouna *et al.* (2011) study the inhibitory effect of *Ceratonia siliqua* pods essential oil was evaluated *in vivo* against a foodborne pathogens *Listeria monocytogenes*, experimentally inoculated in minced beef meat (2×10 CFU/g of meat) amended with different concentrations of the *Ceratonia siliqua* pods essential oil and stored at 7 °C for 10 days. The antibacterial activity of *Ceratonia siliqua* pods essential oil in minced beef meat was clearly evident and its presence led to a strong inhibitory effect against the pathogens at 7 °C.

Gniewosz and Stobnicka (2018) studied extracts from American cranberry pomace (*Vaccinium macrocarpon*). They prepared three forms: water, ethanol, and a

water/ethanol and observed antimicrobial activity in relation to 18 strains of bacteria, and three strains of fungi. Extracts contained organic acids, flavonols, terpenes (ursolic acid), and stilbenes (resveratrol). The inhibition of pathogens growth (*Escherichia coli, Salmonella ser. Enteritidis, Listeria monocytogenes,* and *Staphylococcus aureus)* was determined in minced pork containing 2.5% cranberry pomace in water. The results showed a significant ($p < 0.05$) growth inhibition for all pathogens in the minced pork meat with cranberry extract, compared to the control sample (Gniewosz, Stobnicka 2018).

Dhanze et al. (2013) studied effect of extract of sea buckthorn leaves to chicken legs. They observed on sensory and microbiological quality of chicken legs at days 0, 1, 3, 5 and 7. No significant difference ($p < 0.05$) was observed for sensory attributes in the control and treated groups; however, scores were higher for the treated groups compared with the control group. All three concentrations of aqueous extract of sea buckthorn leaves lowered the ($p < 0.05$) standard plate count, psychrophilic count, coliform count and yeast and mold count significantly on chicken leg as compared with the control.

1.5 Description of plants used in present study

1.5.1 Siberian rhubarb (*Rheum rhaponticum)*

Rhubarb refers to any of several species of the genus *Rheum L.* in the family Polygonaceae. The genus *Rheum L.*, consisting of about 60 herbaceous perennial plants growing from short and thick rhizomes, is distributed in the temperate and sub-tropical regions. Most common subspecies are garden rhubarb *Rheum rhabarbarum L.* and chinese rhubarb *Rheum palmatum* (ITIS 1999). The roots and rhizomes of *R. officinale Baill, R. palmatum L. and R. tanguticum* have been used for medicinal purposes in China for over 2000 years, and are still used for the treatment of constipation, inflammation and cancer (Takeoka 2013). The *R. rhaponticum* root is very potential as natural antioxidant, antimicrobial or functional additive in foods due to its high content of polyphenols. A special extract of the roots of *R. rhaponticum* has been used as a medication to treat menopausal symptoms.

The safety studies of the extract of th*e R. rhaponticum* root in the concentrations of 100, 300, and 1000 mg of ERr 731/kg body weight (bw)/day in the long term toxicity studies in the beagle dogs of the both sexes have been performed (Raudsepp 2013). Studies in the eighteen species of the genus Rheum L. led to the isolation of two hundred constituents including anthraquinone, anthrone, stilbene, flavonoids, acylglucoside, and pyrone (Zheng *et al.* 2013).

1.5.2 Black currant (*Ribes nigrum*)

A wide range of nutritional compounds like carbohydrates, minerals, vitamins, and organic acids and especially polyphenols, make black currants *(Ribes nigrum* L. family *Grossulariaceae*) one of the most investigated species in the berry kingdom. The use of black currant has spread from food and beverages (colors and flavors), to functional food and beyond, through additives in the form of antioxidants in meat as well as meat products preservers. Recent trends have moved toward the testing and potential use of black currants buds and leaves as a rich source of natural antioxidants (Miladinovic *et al.* 2014).

Compounds contained in fruits and leaves of blackcurrant are known as agents acting preventively and therapeutically on the organism due its high polyphenol content. Polyphenols are bioactive secondary plant metabolites widely present in commonly consumed foods of plant origin. They are powerful antioxidants in vitro and they are considered to carry many potential beneficial health effects (Mattila *et al.* 2016). Anthocyanins, in particular derivatives of cyanidin and delphinidin, are the main polyphenols in fruit extract. Leaves of blackcurrant, which contain quercetin derivatives, have a range of activities, including antimicrobial, anti-inflammatory, antiviral, antitoxic, antiseptic, and antioxidant effects (Bonarska-Kujawa *et al.* 2014).

The black, blue and red colouration of the fruits (berries) can be attributed to high contents of anthocyanins, making especially blackcurrants good sources of these compounds. The other phenolic components in currants include flavonols, proanthocyanidins and phenolic acids. In Mattila *et al.* (2016) study of currant varieties the anthocyanin profile of black currant consisted of four major anthocyanins, namely

dephinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside. The total anthocyanin content was on average 467 ± 114 mg/100 g FW flavonol contents in 32 blackcurrant genotypes studied. The total contents varied from 9.6 to 21.6 mg/100 FW (43.6 to 89.9 mg/100 g DW).

1.5.3 Blue honeysuckle (*Lonicera caerulea* var edulis)

Lonicera caerulea L., also called blue honeysuckle, is a traditional crop belonging to the Caprifoliaceae family. This long-lived and deciduous shrub is one of the 180 species of the genus *Lonicera* and is native to the Northern Hemisphere. The berries contain high levels of vitamin C, anthocyanins, phenolic acids and flavanols (Caprioli 2016). Honeysuckle is commonly used as folk medicine, but are less known as edible fruits because of their bitterness and astringency. Polyphenolic compounds, especially anthocyanins, are the prominent functional components in *L. caerulea* berries. The anthocyanins in *L. caerulea* berries include glucoside and rutinoside of cyanidin peonidin, and delphinidin, along with 3,5-dihexoside of cyanidin and peonidin that have not been found in some other berries such as blueberry. This may be one reason why *L. caerulea* berries have a higher antioxidant capacity than blueberries (Wang *at al*. 2016).

1.5.4 Black chokeberry (*Aronia melanocarpa*)

Aronia with the common name chokeberry, originates from the eastern parts of North America. The genus *Aronia, Rosaceae f*amily includes two species of shrubs. Native to eastern North America and Eastern Canada: *Aronia melanocarpa* known as black chokeberry and Aronia arbutifolia, also known as red chokeberry (Wangensteen *et al*. 2014). Around 1900 it was transferred to Europe and in the 1960s the plant was stablished as a cultivar in the former Soviet Union.

Aronia berries are distinctive with a high content of polyphenols and possess one of the highest antioxidant activities among plant species. Chokeberries are a rich source of anthocyanins, proanthocyanidins, and hydroxycinnamic acids. The total amount of anthocyanins in fresh berries varies in the range 357 to 1790 mg/100 g fresh weight.

Compared to other berries the aronia anthocyanin profile is very simple consisting almost exclusively of cyanidin glycosides, namely cyanidin-3-arabinoside, cyanidin-3-galactoside, cyanidin- 3-glucoside, and cyanidin-3-xyloside (Denev *et al.* 2012).

1.5.5 Tomato (*Solanum lycopersicum*)

Tomato was originally in genus *Lycopersicon* (Asamizu, Ezura 2009), but nowdays belongs to genus *Solanum*, the nightshade family, which includes eggplant (*Solanum melongena*) and potato (*Solanum tuberosum*) as well as 1400 other species. Botanically tomato is a fruit and it is rich in lycopene red colour owing to pigments that are synthesize during fruit ripening (Perveen *at al.* 2015). Tomatoes are not only mainly consumed as a raw staple food due to their desirable nutritional properties but they are also being increasingly used in many popular tomato products. More than 80% of tomatoes grown are consumed in the form of processed products such as juice, soup, concentrate, dry-concentrate, sauce, salsa, puree, dry-tomato, ketchup, or paste (Viuda-Martos 2013).

Tomato and tomato product contains number of carotenoids such as phytoene, phytofluene, α-carotene, β-carotene, gammacarotene, and neurosporene. Carotenoids like lycopene are important pigments found in plants, photosynthetic bacteria, fungi, and algae. They are responsible for the bright colours of fruits and vegetables and protection of photosynthetic organisms from excessive light damage (Perveen *at al.* 2015).

Most important pigment in tomato is lycopene. It is the red colored pigment abundantly found in red fruits and vegetables particularly in tomatoes and tomato products. Lycopene is ranked as the most potent among the following antioxidants: lycopene > α-tocopherol > α-carotene > β-cryptoxanthin > β-carotene > lutein. Lycopene is a lipid soluble antioxidant member of the carotenoid family of phytochemicals. It is synthesized by many plants and microorganisms to absorb light during photosynthesis and to protect them against photosensitization (Viuda-Martos 2013).

2. AIMS OF THE STUDY

The aim of the study was to investigate microbial growth inhibition (antimicrobial effect) of some plant powders such as rhubarb, tomato, black currant, blue honeysuckle and black chokeberry in raw and cooked minced pork.

Also sodium chloride, sodium nitrite, rutin and gallic acid were studied for their microbial growth inhibition effect in raw and cooked minced pork.

3. MATERIALS AND METHODS

3.1 The plant material

The plant materials were selected in accordance with previous *in-vitro* study results for which the plant material of rhubarb varieties was obtained from the collection of Pure Horticultural Research Centre, Latvia. All studied plants were grown in the plantation of Polli Horticultural Research Centre, Estonia. The samples were collected in 2015. Berries of chokeberry (selected among three seedlings, according to the content of anthocyanins); blue honeysuckle i.e. haskap berry cultivar (cv.) ′Tomitška′ (selected among five cv.-s, according to the content of anthocyanins) and berries of black currant cv. ′Ben Alder′ (selected among 37 cv.-s according to the content of anthocyanins); leaves of black currant cv. ′Pamyati Vavilova′ and petioles of garden rhubarbs were freeze-dried with VirTis AdVantage 2.0 EL freeze dryer (SP Industries, Warminster, USA) and kept at the temperature -40°C until powdering. Two dark-rooted rhubarbs (cv. ′Victoria′ and seedling no 303) and one light-rooted (cv. ′Ogres′) were previously selected among 16 different cultivars or seedlings, according to their content of anthraquinones – the darker the roots, the higher the content of various hydroxyanthraquinones and their glycosides: aloe emodin, emodin and chrysophanol (Püssa *et al*. 2009). The roots of garden rhubarb varieties and seedling were washed, diced and dried at 50 °C in a drying oven (Binder FED 101, Binder GmbH, Tuttlingen, Germany) and kept at room temperature.

3.2 Sample preparation for meat studies

Pork minced meat (max 30% fat) was purchased from local supermarket and was packed in modified atmosphere. Different mixtures (Table 1) with plant powders (Photo) and other components were made in two batches, raw and cooked. Plant materials used in mixtures were lyophilized except for rhubarb roots and tomato, which were dried thermally. Minced meat without any added components added was used as a blank sample. Positive control was prepared with sodium nitrite and sodium chloride.

Additionally, rutin and gallic acid were used as representatives of flavonoids and fenolic acids.

The components were added to minced meat and mixed with hand mixer during three minutes for uniform distribution. Samples for cooking were formed like meatloaves, wrapped into baking paper and aluminium foil and cooked at 225 ºC for 20 minutes, then cooled down and thoroughly homogenized. All samples were divided into screw cap jars for storage in a refrigerator at 4 ± 1 ºC. Analyses were performed at days 0, 2, 4, 6 and 8 (microbiological analyses of raw samples only up to the 6th day). Microbiological analyses were performed simultaneously in duplicate.

Table 1. Raw and cooked minced meat mixtures with additives

No	Samples
1	Minced pork without additives as "Control"
2	Minced pork with 1% sodium chloride (Fluka)
3	Minced pork with 1% sodium chloride and 150 mg/kg sodium nitrite (Sigma-Aldrich)
4	Minced pork with 1% rhubarb root + 1% black currant berries
5	Minced pork with 1% rhubarb root + 1% black currant leaves
6	Minced pork with 1% rhubarb root + 1% chokeberry berries
7	Minced pork with 1% black currant leaves + 1% blue honeysuckle berries
8	Minced pork with 1% rhubarb petioles + 1% tomato
9	Minced pork with 2% blue honeysuckle berries
10	Minced pork with 2% rhubarb petioles
11	Minced pork with 2% tomato
12	Minced pork with 48 mg/600 g rutin (Sigma)
13	Minced pork with 48 mg/600 g gallic acid (Sigma)

No 2 - salt

No 3 - rhubarb root + black currant berries

No 4 - rhubarb root + black currant leaves

No 5 - rhubarb root + chokeberry berries

No 6 - black currant leaves + blue honeysuckle

No 7 - rhubarb petioles + tomato

No 8 - haskap berries (blue honeysuckle)

Photo: Plant materials and sets selected as additives minced meat (photo by Piret Raudsepp)

3.3 Microbiological analyses

In present study the antimicrobial effect of plant powders was estimated by counting microorganism's general numbers in enriched and non-enriched raw and cooked minced pork. For raw minced pork and cooked minced pork products the lengths of the experiment period were six and eight days, respectively.

3.3.1 Yeasts and moulds count and aerobic plate count

For the preparation of the initial suspensions and further decimal dilutions the EVS-EN ISO 6887-1:2017 was used. For the enumeration of yeasts and moulds the EVS-ISO standard 21527-1:2009 was followed. For colony count at 30 °C the surface plating technique was used following the instructions of the ISO 4833-2:2013 standard. Shortly, 10 gram of minced meat was weighted into the stomacher bag, and diluted with 90 ml of sterile buffered peptone water ISO (LAB204, Lab M, Lancashire, UK) to get initial ten-fold dilution. Samples were blended using Stomacher™ 400 Circulator (Seward, UK) within one minute at 230 rpm. For the enumeration of microorganisms at 30 °C (the aerobic plate count) the Plate Count Agar (LAB010, Lab M, Lancashire, UK) was used. For enumeration of yeast and moulds the DRBC Agar ISO (LAB217, Lab M, Lancashire, UK) was used. For both enumerations surface plating technique was used transferring 100 µl (0.1 ml) of initial dilution and further decimal dilutions onto the surface of the agar plate. By using spreading spatula, the inoculum was spread evenly over the agar surface. Before incubation at appropriate temperatures the plates were kept 15 minutes at room temperature. PCA plates were incubated at 30 °C for 72 hours, and DRBC agar plates were incubated at 25 °C for 5 days. After incubation the colonies were counted to get total counts per gram of product in accordance with instructions given in ISO standard 7218:2018+A1:2013 General requirements and guidance for microbiological examinations. All enumeration analyses were performed in duplicate series. Results were expressed as $\log_{10}$ numbers of colony forming units/gram (cfu g^{-1}).

3.4 Statistical analyses

The statistical significance of treatment and storage time effects on studied variables were tested with two-way analysis of variance followed by Dunnett' *post-hoc* test comparing other treatments with meat as control. P values less than 0.05 are representing the treatments with statistically significant difference from meat on an average level of microbial counts over storage times ($p<0.05$, Dunnett' *post-hoc* test).

4. RESULTS

Raw minced pork, aerobic plate count

In raw minced pork samples, the number of microorganisms increased from 3.46 at 0 day to 4.57 log cfu g^{-1} at 6 days and 3.54 at 0 day to 5.20 log cfu g^{-1} at 6 days (Figure 1A). It was found that the most efficient antibacterials in raw minced pork were rhubarb petioles and the combination of sodium chloride with sodium nitrite. Among the combinations of two different plant additives, the most efficient combination of the additives in raw minced pork were 1% rhubarb petioles in combination with 1% tomato (Figure 1B). Compared to the other results in accordance with Figure 1B, the number of microorganisms in raw minced pork with 1% rhubarb petioles combined with 1% tomato increased most slowly on average from 4.22 at 0 day to 4.81 log cfu g^{-1} for 4 days followed by a rapid increase in numbers from 4.81 to 7.62 cfu g^{-1} for 6 day of storage. While comparing the parts A and B of Figure 1 it can be seen, that the most efficient antibacterials were rhubarb petioles and the combination of rhubarb petioles and tomato. Also, rutin and gallic acid as single additives were found to be efficient antibacterials.

In present study, the largest increase in number of microorganisms on average from 3.40 to 7.43 log units cfu g^{-1} for 6 day of storage was observed in raw minced pork containing 1% black currant leaves in combination with 1% blue honeysuckle berries followed by 1% rhubarb root + 1% black currant leaves (3.70 to 7.60 log cfu g^{-1}) and 1% rhubarb petioles + 1% tomato (4.22 to 7.62 log cfu g^{-1}) during the storage period.

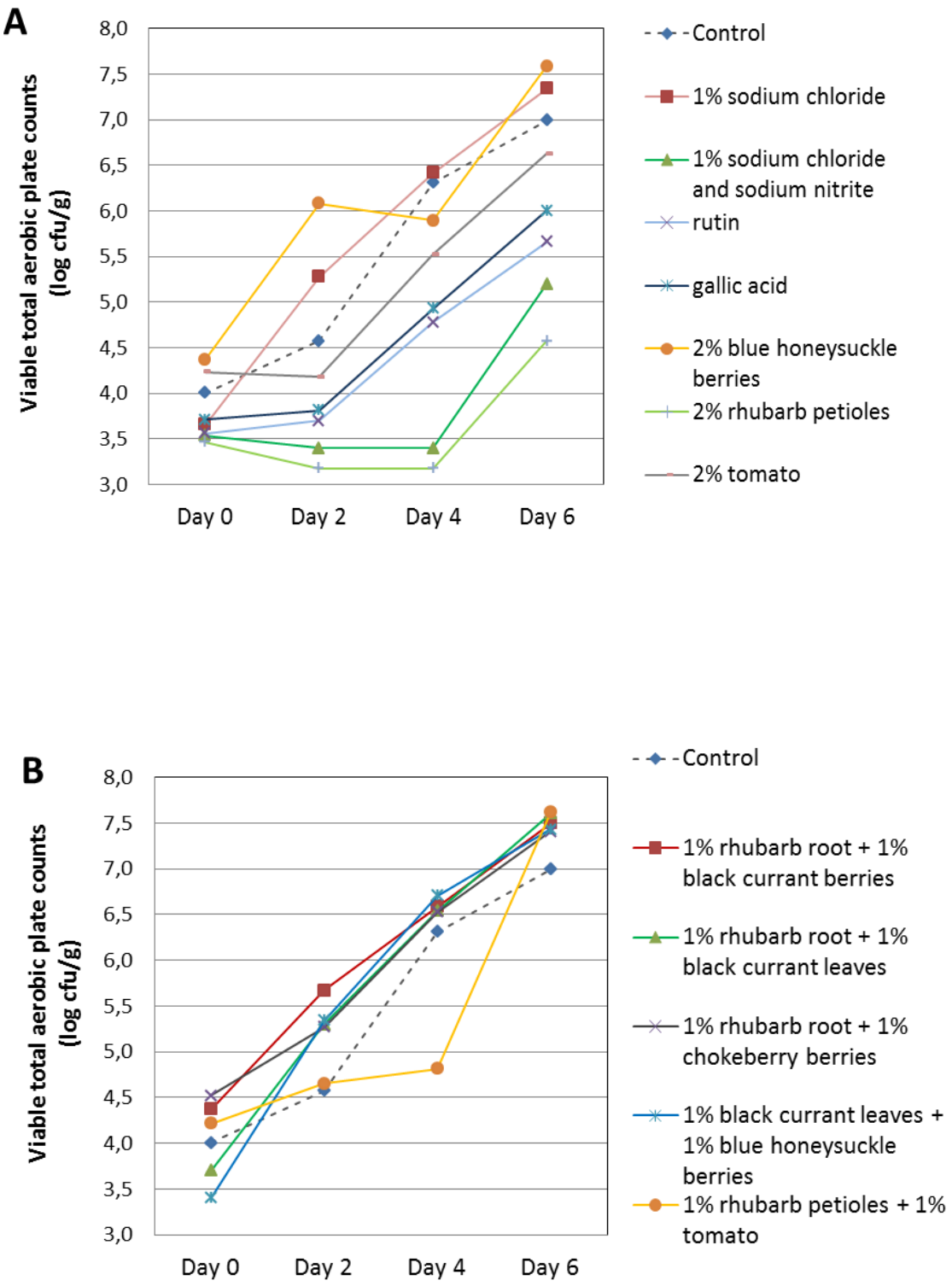
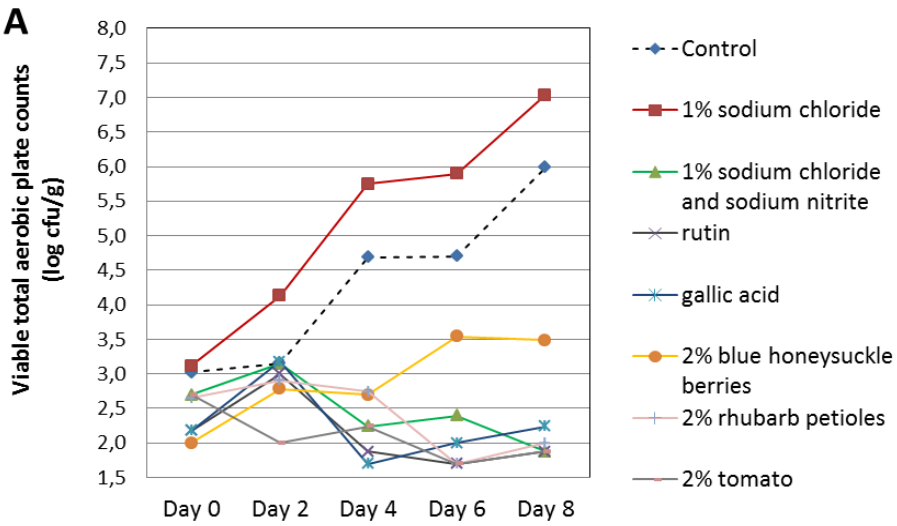


Figure 1: Dynamics of aerobic plate counts during storage period (days) of raw minced pork without any additives ("Control") and with different plant additives. **Series A:** with one additive. **Series B:** mixture of two plant additives.

Cooked minced pork, aerobic plate count

Compare to the day 0 the number of microorganisms decreased in average from 3.32 to 2.54 log cfu g^{-1} and 2.70 to 1.88 log cfu g^{-1} in cooked minced pork with 1% rhubarb root combined with 1% black currant berries (Figure 2B) and with 2% tomato, respectively (Figure 2A). The inhibitory effect of additives on the number of microorganisms in cooked minced pork was also observed for the several other plant additives, used in present trial, depending on the storage days of tested samples. Compared to the initial levels (0 days), in the presence of 2% tomato, 1% sodium chloride with sodium nitrite, 1% rhubarb root with 1% black currant berries and 2% rhubarb petioles in cooked minced pork, the average decrease in cfu-s of the number of microorganisms varied from 0.65 to 0.82 log units (on average from 2.84 log cfu g^{-1} to 2.08 log cfu g^{-1}) at the end of storage trial (day 8). Some decrease on microbial numbers after 2 days of storage was observed for minced pork enriched with 2% of tomato as a single additive. Same effect was observed in combination of two plant additives for 1% rhubarb roots + 1% black currant berries and for 1% rhubarb petioles + 1% of tomato.

The largest increase in microbial numbers from 3.24 (day 0) to 7.93 log cfu g^{-1} (day 8) was observed in cooked minced pork containing 1% rhubarb root + 1% black currant leaves followed by 1% sodium chloride (3.11 to 7.03 log cfu g^{-1}) and 1% black currant leaves + 1% blue honeysuckle berries (2.74 to 6.49 log cfu g^{-1}).



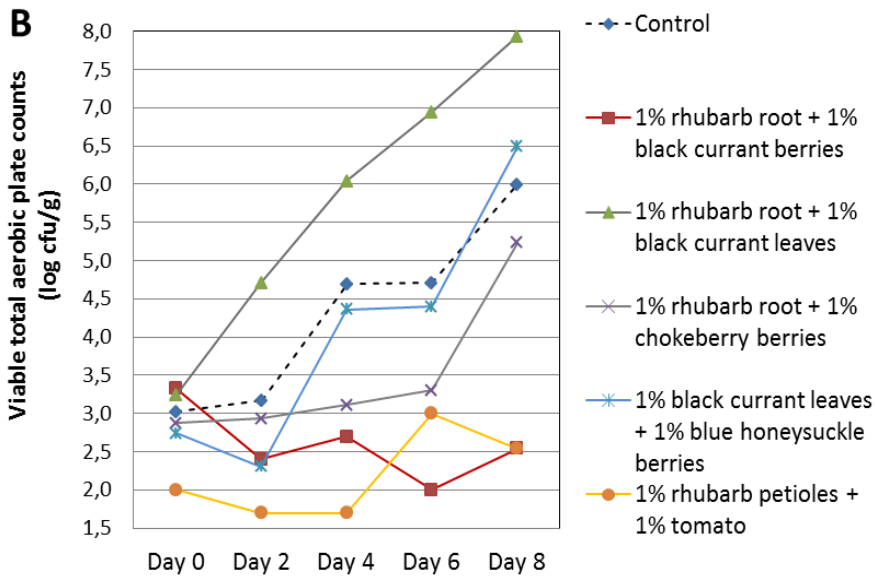


Figure 2: Dynamics of aerobic plate counts during storage period (days) of cooked minced pork without any additive ("Control") and with different plant additives. **Series A:** with one additive. **Series B:** mixture of two plant additives.

Yeasts and moulds in raw minced meat samples

In Figure 3AB it can be seen that the initial numbers of yeasts and moulds for all raw minced pork samples without and with additives was between 2.30 and 3.70 log cfu g^{-1} at 0 day. It can be named as initial contamination level.

These numbers increased in most tested samples steadily reaching 3.00 to 5.62 log cfu g^{-1} for 6 days of storage. However, compared to the initial contamination levels at 0 days, small decrease of the cfu-s of yeasts and molds in raw minced pork was observed for samples with 2% tomato (from 2.60 to 2.0 log cfu g^{-1}) up to second storage day; 1% sodium chloride + sodium nitrite (from 2.30 to 2.0 log cfu g^{-1}) up to the fourth storage day, also for minced pork samples with gallic acid (from 2.48 to 2.0 log cfu g^{-1}) up to the fourth storage day.

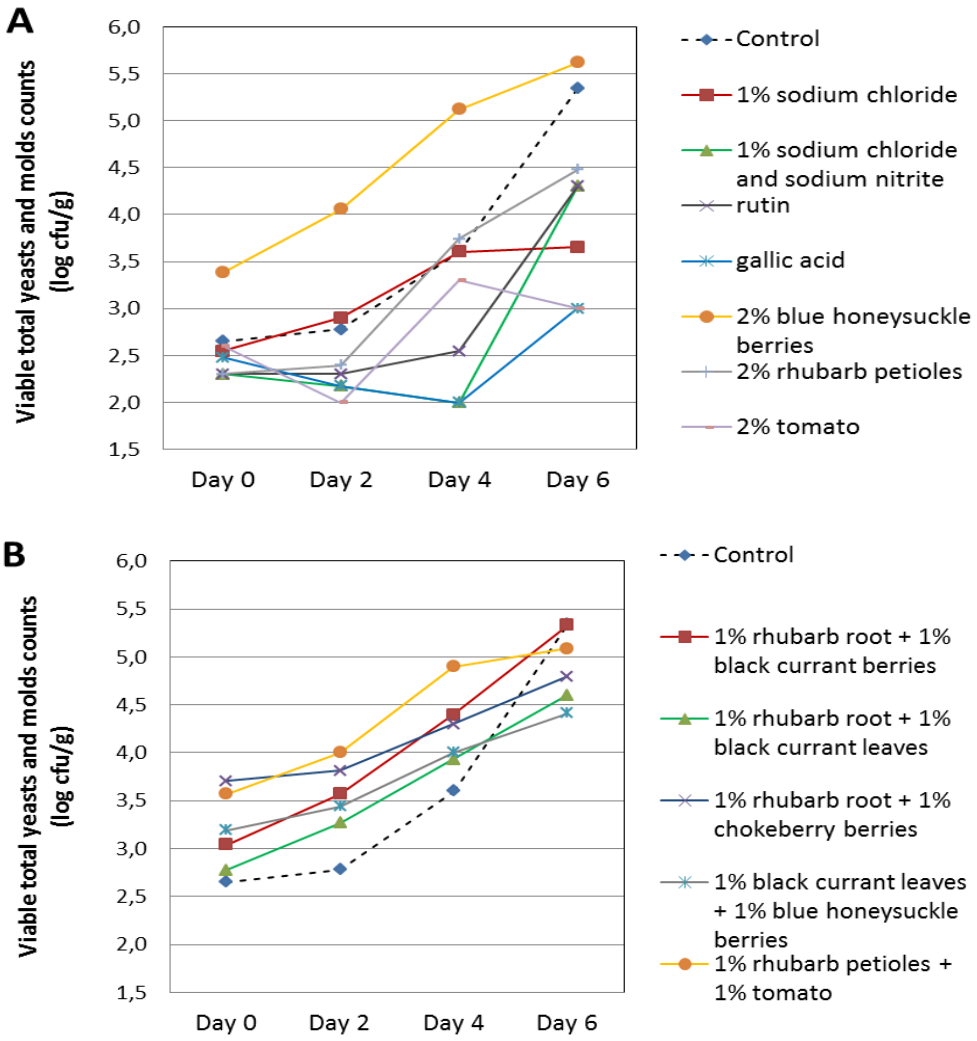


Figure 3. Dynamics of yeasts and molds counts during storage period (days) of raw minced pork without any additive ("Control") and with different plant additives. **Series A:** with one additive. **Series B:** mixture of two plant additives.

Yeasts and moulds in cooked minced meat samples

Generally, the counts of yeasts and moulds in cooked minced pork (with and without additives samples) remained under detection limit (Table 2). Table 2 shows that in cooked minced pork samples enriched with rhubarb petioles, tomato and rutin the initial contamination in decreasing during study period. Generally, the yeasts and mould numbers stayed under detection limit (the threshold of 100 CFU/g).

Table 2. Yeasts and molds counts* of cooked minced pork with different additives

Analysed food matrix	Day 0	Day 2	Day 4	Day 6	Day 8
Control	0	0	0	0	0
Minced pork + 1% sodium chloride	100	100	100	100	100
Minced pork + 1% rhubarb root + 1% black currant berries	0	0	0	0	0
Minced pork + 1% rhubarb root + 1% black currant leaves	0	0	0	0	0
Minced pork + 1% rhubarb root + 1% chokeberry berries	100	0	0	0	0
Minced pork + 1% black currant leaves + 1% honeysuckle berries	0	0	0	0	0
Minced pork + 1% rhubarb petioles + 1% tomato	100	0	0	0	0
Minced pork +2% honeysuckle berries	0	0	0	0	0
Minced pork + sodium chloride + sodium nitrite	200	100	100	100	100
Minced pork + 2% rhubarb petioles	100	100	0	0	0
Minced pork + 2% tomato	100	100	0	0	0
Minced pork + rutin	100	100	0	0	0
Minced pork + gallic acid	0	0	0	0	0

*Note: in table results are presented in CFU numbers per gram (not in log units)

Statistical analyses

The Tables 3 and 4 shows the differences of average total microbial counts as well as the differences of yeasts and mould counts between control (meat) and treatments (meat with powders), respectively. Compared to raw minced pork as control, four treatments such as raw minced pork with 1% sodium chloride + sodium nitrite (p=0.000), 2% rhubarb petioles (p=0.000), gallic acid (p=0.007) and rutin (p=0.000) were significantly more effective against microbial growth during storage period.

In the present study, the use of 1% rhubarb root + 1% black currant berries (*p=0.000*), 1% rhubarb root + 1% black currant leaves (*p=0.001*), 1% rhubarb root + 1% chokeberry berries (*p=0.499*), 1% rhubarb petioles + 1% tomato (*p=0.000*), 2% blue honeysuckle berries (*p=0.015*), 1% sodium chloride + sodium nitrite (*p=0.000*), 2% rhubarb petioles, 2% tomato, gallic acid (p=0.000) and rutin (p=0.000) in treatments were found to give a strong microbial growth inhibition effect compared to meat sample without additives (control).

Table 3. The differences of average total microbial counts between control (meat) and treatments (meat with powders)

Samples	Raw minced meat samples		Cooked minced meat samples	
	Average microbial counts (log cfu/g)	*p*-value (comparing with meat)	Average microbial counts (log cfu/g)	*p*-value (comparing with meat)
Control	5.46	1.000	4.15	1.000
Minced pork + 1% sodium chloride	5.66	0.989	5.14	0.115
Minced pork + 1% rhubarb root + 1% black currant berries	5.90	0.454	2.32	0.000*
Minced pork + 1% rhubarb root + 1% black currant leaves	5.76	0.856	5.73	0.001*
Minced pork + 1% rhubarb root + 1% chokeberry berries	5.92	0.400	3.47	0.499*
Minced pork + 1% rhubarb petioles + 1% tomato	5.07	0.586	2.18	0.000*
Minced pork + 2% blue honeysuckle berries	5.94	0.368	2.86	0.015*
Minced pork + 1% sodium chloride + sodium nitrite	4.05	0.000*	2.36	0.000*
Minced pork + 2% rhubarb petioles	3.70	0.000*	2.27	0.000*
Minced pork + 2% tomato	4.92	0.219	2.05	0.000*
Minced pork + gallic acid	4.59	0.007*	2.16	0.000*
Minced pork + rutin	4.35	0.000*	2.08	0.000*

*Stars in table denote the treatments with statistically significant difference from meat (control) on an average level of microbial counts over storage times ($p<0.05$, Dunnett' *post-hoc* test).

Table 4 shows that the use of 1% rhubarb root + 1% chokeberry berries (p=0.000), 1% rhubarb petioles + 1% tomato (p=0.022), 2% blue honeysuckle berries (p=0.003), 1% sodium chloride + sodium nitrite (p=0.003), 2% tomato (p=0.004), rutin (p=0.030) and gallic acid (p=0.000) in treatments were found to give a strong total yeasts and mould counts growth inhibition effect compared to meat sample without additives (control).

Table 4. The differences of average total yeasts and mould counts between control (meat) and treatments (meat with powders)

Samples	Raw minced meat samples	
	Average yeasts and mould counts (log cfu/g)	*p*-value (comparing with meat)
Control	3.49	1.000
Minced pork + 1% sodium chloride	3.15	0.638
Minced pork + 1% rhubarb root + 1% black currant berries	4.07	0.076
Minced pork + 1% rhubarb root + 1% black currant leaves	3.64	0.997
Minced pork + 1% rhubarb root + 1% chokeberry berries	4.14	0.037*
Minced pork + 1% black currant leaves + 1% blue honeysuckle berries	3.75	0.860
Minced pork + 1% rhubarb petioles + 1% tomato	4.18	0.022*
Minced pork + 2% blue honeysuckle berries	4.33	0.003*
Minced pork + 1% sodium chloride + sodium nitrite	2.66	0.003*
Minced pork + 2% rhubarb petioles	3.19	0.767
Minced pork + 2% tomato	2.67	0.004*
Minced pork + rutin	2.82	0.030*
Minced pork + gallic acid	2.38	0.000*

*Stars in table denote the treatments with statistically significant difference from meat on an average level of yeast and mould counts over storage times ($p<0.05$, Dunnett' *post-hoc test*).

5. DISCUSSION

The shelf-life of most of foods can be extended by using cold storage. Low temperatures slow down chemical changes in food as well as the growth of many moulds, yeasts and spoilage bacteria. In accordance with general responsibilities laid down in legislation as well as in self-control programs, the food enterprises are obliged to carry out a durability studies which determine the shelf-life of food. It can be done by analysing the growth of microorganisms in the manufactured food, under previously determined reasonable conditions of distribution, storage and use (FSAI 2017). Self-life can also be extended by using new technologies, like activated films and non-thermal treatments, but these may cause loss of organoleptic properties (Negi 2012).

In present study for microbial enumeration aerobic plate count at 30 °C and colony count technique for the enumeration of yeasts and moulds were used, because these are generic microbiological tests that counts organisms which grow under aerobic conditions at defined temperatures on a particular growth medium. The total counts of microorganisms provide useful information to assess a food's quality or its remaining shelf-life (Roasto, Laikoja 2017), but does not differentiate aerobic microorganisms or indicate the presence of pathogens. Powdered plants are well suited for minced meat, because powders can be easily mixed uniformly into meat matrix. Plant powders have lower toxicity compared to synthetic ones. Also, plant powders can be collected from plant by-products which could be quite cheap raw material for food industry (Palmeri *et al* 2018).

5.1 Aerobic Plate Count in raw minced pork

Compared to the control and samples with other additives, the most efficient plant additives in inhibition of the growth of microorganisms in raw minced pork were rhubarb petioles, sodium chloride with sodium nitrite and tomato.

Compare to the initial number of microorganisms in raw minced pork samples not significant decrease in microbial numbers was reported in second storage day for raw minced pork enriched with 2% rhubarb petioles and for raw minced pork with 1% sodium chloride + sodium nitrite.

Taking into account the entire 6 days long storage period, the number of microorganisms in raw minced pork samples was mostly affected by the presence of rhubarb petioles, sodium chloride with sodium nitrite and tomato. Comparing to the control sample significantly lower number of microorganisms at the end of storage trial was reported for raw minced pork samples enriched with 2% rhubarb petioles and for 1% sodium chloride and sodium nitrite.

Among the combinations of two different plant additives, the most efficient combination of the additives in raw minced pork were 1% rhubarb petioles in combination with 1% tomato.

The Commission Regulation (EC) No 2073/2005 establishes microbiological criteria for foodstuffs. The process hygiene criteria for raw minced meat are set for aerobic colony count with the limit of 5×10^6 cfu/g^{-1} as a maximum number of microorganisms in two of five units comprising the sample. For the rest of three units (subsamples) the limit for aerobic colony count is 5×10^5 cfu/g^{-1}. Taking into account these limits it can be deduced that at the end of storage trial for the day 6 for most of the samples of the present study the official limits were exceeded except for minced meat samples with 2% rhubarb petioles, 1% sodium chloride and sodium nitrite and minced meat samples with rutin.

5.2 Aerobic Plate Count in cooked minced pork

In cooked minced pork the most efficient microbial growth inhibitors as single additives were rutin, gallic acid, rhubarb petioles, tomato and the combination of sodium chloride with sodium nitrite. Among combinations of two different plant additives, the most efficient antimicrobials were rhubarb petioles together with tomato, and rhubarb root in combination with black currant berries.

The inhibitory effect of additives on the number of microorganisms in cooked minced pork was also observed on the several other plant additives depending on the storage days of tested samples. Compared to the initial levels (0 days), in the presence of 2% tomato, 1% sodium chloride with sodium nitrite, 1% rhubarb root with 1% black currant berries and 2% rhubarb petioles in cooked minced pork, the average decrease in cfu-s of the number of microorganisms varied from 0.65 to 0.82 log units at the end of storage trial (day 8). Some decrease on microbial numbers after 2 days of storage was observed for minced pork enriched with 2% of tomato as a single additive. Same effect was observed

in combination of two plant additives for 1% rhubarb roots + 1% black currant berries and for 1% rhubarb petioles + 1% of tomato.

Taking into account both study series with raw and cooked minced pork it is noteworthy that 1% rhubarb petioles in combination with 1% tomato was able to inhibit the microbial growth most efficiently. In raw pork samples it was seen up to 4 days of storage and in cooked pork samples until the end of storage trial.

5.3 Yeast and moulds in raw minced pork

The most efficient additives in raw minced pork samples were gallic acid, rutin and tomato as single additives as well as sodium chloride together with sodium nitrite. Interestingly, there was no considerable difference in antimicrobial activities against the growth of yeasts and moulds between combinations of two different plant additives. It can be explained with the findings of Gyawali and Ibrahim (2014) who reported that in food matrices active biocompounds can bind to the hydrophobic moieties of proteins and lipids which restrict the availability of the natural antimicrobials. According to the present study, the higher efficiency in microbial growth inhibition for 2% of individual plant additives compare to the combinations were 1% + 1% of different additives were used could be the consequence of too low separate concentrations (1%) of plant additives for inactivation of the growth of microorganisms in minced pork. High concentrations of separate plant additives in meat products cannot be always accepted in accordance with sensory properties of food. Both antimicrobial and sensory properties have to be taken into account while selecting plant additives for the use in food industry as natural antimicrobials and antioxidants. Latter requires the series of tests, both analytical and sensory, to be performed to find the combinations and concentrations of natural food additives acceptable for industrial usage. It was found by Tiwari et al. (2009) that in certain concentrations natural antimicrobials can inactivate microorganisms without impairing organoleptic properties of food.

There are no official microbiological criteria for the number of yeasts and moulds in raw minced meat, but mostly the limit 5×10^3 cfu/g^{-1} at the end of shelf-life has been used by many Estonian meat industries. Taking into account this indicative limit it can be deduced that at the end of storage trial for the day 6 for most of the samples the limit was not exceeded.

Highest numbers of yeasts and moulds at day 6 were determined for the minced meat with 2% blue honeysuckle berries and for control (raw minced pork without any additives). Also, black current laves and berries were associated with high numbers of yeasts and moulds in present study. Lowest numbers of yeasts and moulds were determined for minced meat samples enriched with 2% tomato, gallic acid, rutin and 1% sodium chloride + sodium nitrite.

In case of enrichment of raw meat products with plant additives (e.g. powders) the initial contamination of plant materials sometimes may lead to serious microbiological contamination. It may also cause food safety problems with pathogenic microorganisms including those able to produce thermostabile toxins. Therefore, the control measures which eliminate or significantly reduces the microbial contamination loads of plant additives should be applied including high hygiene standards while collecting and processing of plant additives for the use in food matrices.

5.4 Yeast and moulds in cooked minced pork

Very low numbers of yeasts and moulds in cooked minced pork samples with and without additives can be explained with efficient thermal processing, which destroys most of the yeasts and moulds in cooked meat. Australian risk assessment study found that the most effective means of reducing the risk of listeriosis from processed meats was to reduce initial contamination levels of microorganisms, and to use in-pack pasteurisation technology (Ross *et al*. 2009). In present study because of relatively short durability, the numbers of *L. monocytogenes* were not determined, but the findings of Ross *et al.* (2009) apply also for total numbers of microorganisms in various ready-to-eat food products.

Finally, in present study the levels of microorganisms both in raw and cooked minced pork samples without and with additives did not reach the levels to cause radical spoilage changes such as slime production and unpleasant odour.

6. CONCLUSION

According to the results of present study it can be concluded that most efficient microbial growth inhibiting plant powders both in raw and cooked minced pork were tomato and rhubarb petioles. Therefore, these plant powders may provide meat industry with a useful ingredients to achieve better taste and colour for meat products as well as protection against microbial spoilage during reasonable shelf-life.

SUMMARY

The use of natural antimicrobials such as organic acids, essential oils, plant extracts, and bacteriocins could be a good alternative to ensure food safety. Berries are a good source of bioactive compounds such as polyphenols and ascorbic acid. Berries also contain other health benefit compounds and could be suitable for the use in the meat and meat products. Plants secondary metabolites, most of which are phenols or their oxygen-substituted derivatives, possess various benefits including antimicrobial properties against pathogenic and spoilage microbes

In present study the aim was to study antimicrobial effect of plant powders by counting microorganism's general numbers in enriched and non-enriched raw and cooked minced pork. For raw minced pork and cooked minced pork products the experiment period was six and eight days, respectively.

Plants used in study were siberian rhubarb (*Rheum rhaponticum),* black currant (*Ribes nigrum*), blue honeysuckle (*Lonicera caerulea* var edulis), black chokeberry (*Aronia melanocarpa),* and tomato (*Solanum lycopersicum).*

It was found that the number of microorganisms of samples in raw pork increased from 3.46 at 0 day to 4.57 log cfu g^{-1} at 6 day and 3.54 at 0 day to 5.20 log cfu g^{-1} at 6 day.

The most efficient antibacterials were rhubarb petioles and the combination of sodium chloride with sodium nitrite.

Among the combinations of two different plant additives, the most efficient combination of the additives in raw minced pork were 1% rhubarb petioles in combination with 1% tomato. Compared to the other findings, the number of microorganisms in raw minced pork with 1% rhubarb petioles combined with 1% tomato increased most slowly on average from 4.22 at 0 day to 4.81 log cfu g^{-1} for 4 days followed by a rapid increase in numbers from 4.81 to 7.62 cfu g^{-1} for 6 day of storage.

The inhibitory effect in cooked minced pork was also observed on the several other plant additives. Compared to the initial levels (0 days), in the presence of 2% tomato, 1% sodium chloride with sodium nitrite, 1% rhubarb root with 1% black currant berries and 2% rhubarb petioles in cooked minced pork, the average decrease in cfu-s of the number of microorganisms varied from 0.65 to 0.82 log units.

Finally, we can summarise that the most efficient microbial growth inhibitors both in raw and cooked minced pork were tomato, rhubarb petioles, gallic acid, rutin and sodium chloride together with sodium nitrite.

REFERENCES

Alaadin, M.A., Al-Khateeb, E.A., Jäger, A.K. (2007). Antibacterial activity of the Iraqi *Rheum ribes* root. Pharmaceutical Biology, 45(9), 688–690.

Ahmad, A., Kaleem, M., Ahmed, Z., Shafiq, H. (2015). Therapeutic potential of flavonoids and their mechanism of action against microbial and viral infections. Food Research International, 77, 221–235.

Asamizu, E., Ezura, H. (2009). Inclusion of tomato in the Genus Solanum as "*Solanum lycopersicum*" is evident from phylogenetic studies. Journal of the Japanese Society for Horticultural Science, 78 (1), 3–5.

Balouiri, M., Sadiki, M., Ibnsouda, S.K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis, 6, 71–79.

Barbieri, R., Coppo, E., Marchese, M., Daglia, M., Sobarzo-Sánchez, E., Nabavi, S.F., Nabavi, S.M. (2017). Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. Microbiological Research, 196, 44–68.

Bonarska-Kujawa, D., Cyboran, S., Žylka, R., Oszmianski, J., Kleszczynska, H. (2014). Biological activity of blackcurrant extracts (*Ribes nigrum* L.) in relation to erythrocyte membranes. BioMed Research International, 1–13.

Caprioli, G., Iannarelli, R., Innocenti, M., Bellumori, M., Fiorini, D., Sagratini, G., Vittori, S., Buccioni, M., Santinelli, C., Bramucci, M., Quassinti, L., Lupidi, G., Vitali, L.A., Petrelli, D., Beghelli, D., Cavallucci, C., Bistoni, O., Trivisonno, A., Maggi, F. (2016). Blue honeysuckle fruit (*Lonicera caerulea* L.) from eastern Russia: phenolic composition, nutritionallue and biological activities of its polar extracts. Food and Function, 7, 1892–1903.

Christofilogiannis, P. (2001). Current inoculation methods in MIC determination. Aquaculture, 196(3-4), 297–302.

Cowan, M.M. (1999). Plant products as antimicrobial agents. Clinical microbiology rewiews, October, 12(4), 564–565.

Côté, J., Caillet, S., Doyon, G., Dussault, D., Sylvain, J.-F., Lacroix, M. (2011). Antimicrobial effect of cranberry juice and extracts. Food Control, 22(8), 1413–1418.

D'Archivio, M., Filesi, C., Varì, R., Scazzocchio, B., Masella, R. (2010). Bioavailability of the polyphenols: status and controversies. International Journal of Molecular Sciences, 11(4), 1321–1342.

Delgado, J., Bote, E., Parra-Testal, V., Ramírez, R. (2016). Effect of the olive leaf extracts in vitro and in active packaging of sliced Iberian pork loin. Packaging Technology and Science, 29(12), 649–660.

Denev, P.N., Kratchanov, C.G., Ciz, M., Lojek, A., Kratchanova, M.G. (2012) Bioavailability and antioxidant activity of black chokeberry (*Aronia melanocarpa*) polyphenols: *in vitro* and *in vivo* evidences and possible mechanisms of action: A Review. Comprehensive Reviews in Food Science and Food Safety, 11(5), 471–489.

Dhanze, H., Khurana, S.K., Mane, B.G. (2013). Effect of Seabuckthorn leaf extract on microbiological quality of raw chicken during extended periods of storage. Journal of Food Quality, 36(1), 59–65.

EURL Lm technical guidance document for conducting shelf-life studies *on Listeria monocytogenes* in ready-to-eat foods (2014). Version 3 – 6 June.

Food safety of Ireland. (2016). Guidelines for the interpretation of results of microbiological testing of ready-to-eat foods placed on the market. Revision 2.

FSAI, Food Safety Authority of Ireland. (2017). Validation of Product Shelf-Life. Revision 3. ISBN: 1-904465-33-1. pp. 1-50.

González-Lamothe, R., Mitchell, G., Gattuso, M., Diarra, M.S., Malouin, F., Bouarab, K. (2009). Plant antimicrobial agents and their effects on plant and human pathogens. International Journal of Molecular Sciences, 10(8), 3400–3419.

Gniewosz, M., Stobnicka, A. (2017). Bioactive components content, antimicrobial activity, and foodborne pathogen control in minced pork by cranberry pomace extracts. Journal of Food Safety, 38(1), e12398.

Guil-Guerrero, J.L., Ramos, L., Moreno, C., Zúñiga-Paredes, J.C., Carlosama-Yepez, M., Ruales, P. (2016). Antimicrobial activity of plant-food by-products: A review focusing on the tropics Livestock Science, 189, July, 32–49.

Gyawali, R., Ibrahim, S.A. (2014). Natural products as antimicrobial agents. Food Control, 46 412–429.

Hsouna, A.B., Trigui, M., Mansour, R.B., Jarraya, R.M:, Damak, M., Jaoua, S. (2011). Chemical composition, cytotoxicity effect and antimicrobial activity of

Ceratonia siliqua essential oil with preservative effects against *Listeria* inoculated in minced beef meat. International Journal of Food Microbiology, 148(1), 66–72.

Integrated Taxonomic Information System (ITIS). (1999). *Rheum* L. ITIS Taxonomic Serial No.: 21318

ISO 21527-1:2008. Microbiology of food animals and feeding stuffs Horizontal method for the enumeration of yeasts and moulds. Part 1: Colony count technique in products with water activity greater than 0.95.

ISO 21527-1: 2008. Part 2: colony count at 30 °C by surface plate technique.

Jia, N., Kong, B., Liu, Q., Diao, X., Xia, X. (2013). Antioxidant activity of black currant (*Ribes nigrum* L.) extract and its inhibitory effect on lipid and protein oxidation of pork patties during chilled storage. Meat Science, 91(4), 533–539.

Klančnik, A., Piskernik, S., Jeršek, B., Možina, S.S. (2010). Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. Journal of Microbiological Methods, 81(2), 121–126.

Lorenzo, J.M., Pateiro, M., Domínguez, R., Barba, F.J., Putnik, P., Kovačević, D.B., Shpigelmand, A., Granato, D., Franco, D. (2017). Berries extracts as natural antioxidants in meat products: A review. Food Research International, 106, April, 1095–1104.

Lu, C., Wang, H., Lv, W., Xu, P., Zhu, J., Xie, J., Liu, B., Lou, Z. (2011). Antibacterial properties of anthraquinones extract from rhubarb against *Aeromanonas hydrophila.* Fish Science, 77, 375–384.

Mattila, P.H., Hellström, J., Karhu, S., Pihlava, J.-M., Veteläinen, M. (2016). High variability in flavonoid contents and composition between different North-European currant (*Ribes* spp.) varieties. Food Chemistry, 204, 14–20.

Mhalla, D., Bouaziz, A., Ennouri, K., Chawech, R., Smaoui, S., Jarraya, R., Tounsi, S., Trigui, M. (2017). Antimicrobial activity and bioguided fractionation of *Rumex tingitanus* extracts for meat preservation. Meat Science, 125, 22–29.

Miladinovic, B., Kostic, M., Savikin, K., Dordevic, B., Mihajilov-Krstev, T., Živanovic, S., Kitic, D. (2014). Chemical profile and antioxidative and antimicrobial activity of juices and extracts of 4 black currants varieties (*Ribes nigrum* L.). Journal of Food Science, 79(3), 301–309.

Negi, P.S. (2012). Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. International Journal of Food Microbiology, 156(1), 7–17.

Othman, M., Loh, H.S., Wiart, C., Khoo, T.J., Lim, K.H., Ting, K.N. (2011). Optimal methods for evaluating antimicrobial activities from plant extracts. Journal of Microbiological Methods, 84(2), 161–166.

Palmeri, R., Parafati, L., Restuccia, C., Fallico, B. (2018). Application of prickly pear fruit extract to improve domestic shelf-life, quality and microbial safety of sliced beef. Food and Chemical Toxicology, 118, 355–360.

Papuc, C., Goran, G.V., Predescu, C.N., Nicorescu, V., Stefan, G. (2017). Plant polyphenols as antioxidant and antibacterial agents for shelf-life extension of meat and meat products: classification, structures, sources, and action mechanisms. Comprehensive Reviews in Food Science and Food Safety, 16(6), 1243–1268.

Pineda-Vadillo, C., Nau, F., Dubiard, C.G., Cheynier, V., Meudec, E., Sanz-Buenhombre, M., Guadarrama, A., Tóth, T., Csavajda, E., Hingyi, H., Karakaya, S., Sibakov, J., Capozzi, F., Bordoni, A., Dupont, D. (2016). *In vitro* digestion of dairy and egg products enriched with grape extracts: Effect of the food matrix on polyphenol bioaccessibility and antioxidant activity. Food Research International, 8(Part B), 284–292.

Puupponen-Pimia, R. Nohynek, L., Alakomi, H.L., Oksman-Caldentey, K.M. (2005). The action of berry phenolics against human intestinal pathogens BioFactors, 23(4), 243–251.

Raudsepp P., Anton D., Roasto M., Meremäe K., Pedastsaar P., Mäesaar M., Raal A., Laikoja K., Püssa T. (2013). The antioxidative and antimicrobial properties of the blue honeysuckle (*Lonicera caerulea* L.), Siberian rhubarb (*Rheum rhaponticum* L.) and some other plants, compared to ascorbic acid and sodium nitrite. Food Control, 31(1), 129–135.

Roasto, M., Laikoja, K. (2017). Toidu säilimisaja määramine I osa (Food shelf-life determination, part one). Estonian University of Life Sciences. ISBN 978-9949-629-11-4 (pdf, web release), pp. 44.

Perveen, R., Suleria, H.A., Anjum, F.M., Butt, M.S., Pasha, I., Ahmad, S. (2015). Tomato (*Solanum lycopersicum*) carotenoidsand lycopenes chemistry;

metabolism, absorption, nutrition, and allied health claims - A comprehensive review. Critical Reviews in Food Science and Nutrition, 55(7), 919–929.

Rigano, M.M., Romanelli, A., Fulgione, A., Nocerino, N., D'Agostino, N., Avitabile, C., Frusciante, L., Barone, A., Capuano, F., Capparelli, F. (2012). A novel synthetic peptide from a tomato defensin exhibits antibacterial activities against *Helicobacter pylori*. Journal of Peptide Science, 18(12), 755–762.

Ross, T., Rasmussen, S., Sumner, J. (2009). Using a quantitative risk assessment to mitigate of *Listeria monocytogenes* in ready-to-eat meats in Australia. Food Control, 20(11), 1058–1062.

Shi, J., Le Maquer, M. (2000). Lycopene in tomatoes: chemical and physical properties affected by food processing. Critical Reviews in Biotechnology, 20(4), 293–334.

Smolarz, H.D., Swatko-Ossor, M., Ginalska, G., Medyńska, E. (2013). Antimycobacterial effect of extract and its components from *Rheum rhaponticum*. Journal of AOAC International, 96(1), 155–160.

Takeoka, G.R., Dao, L., Harden, L., Pantoja, A., Kuh, J.C. (2013). Antioxidant activity, phenolic and anthocyanin contents of various rhubarb (*Rheum* spp.) varieties. International Journal of Food Science and Technology, 48(1), 172–178.

Tiwari, B., Valdramidis, V., O' Donell, C., Muthukumarappan, K., Cullen, P., Bourke, P. (2009). Application of natural antimicrobials for food preservation. Journal of Agricultural Food Chemistry, 57, 5987–6000.

Viuda-Martos, M., Sanchez-Zapata, E., Sayas-Barbera, E., Sendra, E., Perez-Alvarez, J.A., Lopez, F. (2013). Tomato and tomato byproducts. Human health benefits of lycopene and its application to meat products: A Review Critical Reviews in Food Science and Nutrition, 54, 1032–1049.

Wangensteen, H., Braunlich, M., Nikolic, V., Malter, K.E., Slimestad, R., Barsett, H. (2014). Anthocyanins, proanthocyanidin phenolics in four cultivars of aronia and enzyme inhibitory effects. Journal of Functional Foods, 7, 746-752.

Wang, Y., Zhu, J., Meng, X., Liu, S., Mu, J., Ning, C. (2016). Comparison of polyphenol, anthocyanin and antioxidant capacity in four varieties of *Lonicera caerulea* berry extracts. Food Chemistry, 197, 522–529.

Wu, V.C.-H., Qiu, X., Bushway, A., Harper, L. (2008). Antibacterial effects of American cranberry (*Vaccinium macrocarpon*) concentrate on foodborne pathogens. LWT - Food Sc0ience and Technology, 41(10), 1834–1841.

Zheng, Q., Wu, H., Guo, J., Nan, H., Chen, S., Yang, J., Xu, X. (2013). Review of Rhubarbs: Chemistry and Pharmacology. Chinese Herbal Medicines, 5(1), 9–32.

Appendix

Non-exclusive licence for depositing the final thesis (indefinite restriction) and the supervisor's (supervisors') confirmation for allowing the thesis for the defence

Hereby I, **Minna Liisa Kaarina Latva-Somppi, (21/07/85)**

1. grant Eesti Maaülikool, the Estonian University of Life Sciences, a free-of-charge non-exclusive licence to store the final thesis titled "Antimicrobial effect of plant powders in raw and cooked minced pork", supervised by professor Mati Roasto and associate professor Kadrin Meremäe for preservation, including depositing a digital copy of the thesis in the archive of DSpace until the validity of the term of protection of copyright.
2. I am aware that the author retains the same rights as listed in point 1.
3. I confirm that by being issued the CC licence no rights deriving from the Personal Data Protection Act and the Intellectual Property Rights Act have been infringed.

Author of the final thesis _____
signature

In Tartu,

The core supervisor's approval for the final thesis to be allowed for defence

This is to confirm that the final thesis is allowed for defence.

……
……..

Supervisor's name and signature Date

……
……..

Supervisor's name and signature Date