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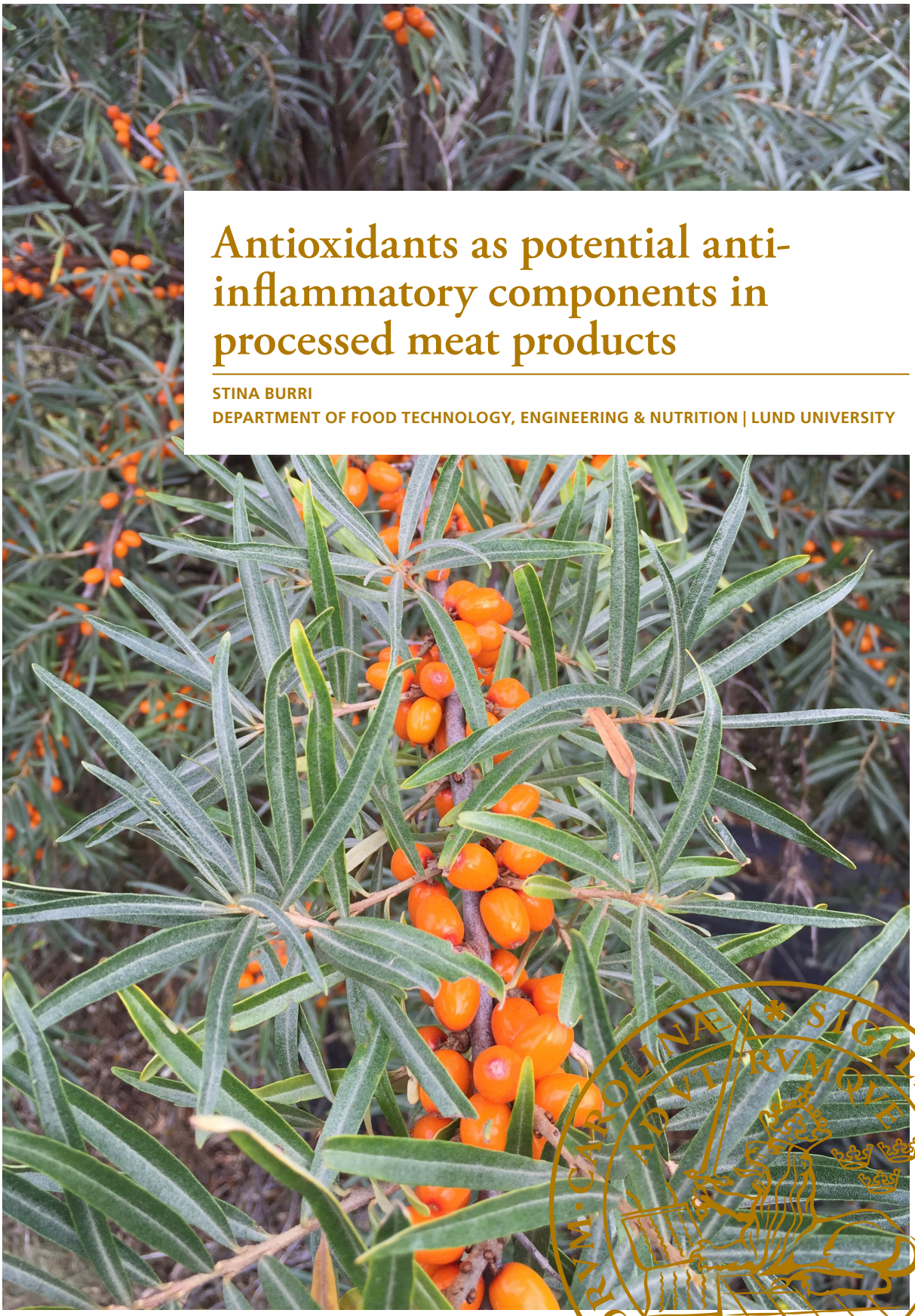
2019



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LUND
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DOCTORAL DISSERTATION

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To be defended at The Chemical Centre, Friday 6th of December 2019 at 9.15.

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Organization
LUND UNIVERSITY
Lund University, Faculty of Engineering
Department of Food Technology, Engineering and Nutrition

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Document name Doctoral Thesis

Date of issue 2019-12-06

Sponsoring organization

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Abstract

In 2015, the World health organisation (WHO) released a press statement together with the International Agency for Research in Cancer (IARC) classifying processed meat as carcinogenic. Since then, research in this area has been focused on finding the underlying mechanism(s) responsible for the link between processed meat and the increased prevalence of colorectal cancer. One of the main hypotheses is that the heme iron present in the meat catalyses lipid oxidation which increases the levels of Reactive Oxygen Species (ROS) and toxic lipid oxidation secondary products which are harmful to our health.

This thesis focuses on this lipid oxidation reaction, and the inhibition thereof, to gain further knowledge in the etiology of colorectal cancer caused by consumption of processed meat products. First, sustainable plant antioxidants were screened for their efficiency and content. Next, a commonly consumed meat product was manufactured based on its proneness to oxidise. When this was done, the most efficient antioxidants previously screened were added to these meat products for inhibition of lipid oxidation. And finally, the five most efficient antioxidants were tested in meatballs, together with relevant controls, for health evaluating purposes in an *in vivo* trial.

We found several natural antioxidants which effectively decreased the level of lipid oxidation in our meat product. For instance, a freeze-dried summer savory (*Satureja hortensis L.*) powder, decreased the level of lipid oxidation to 13.8 % compared to the meat product with no added antioxidants at 200 ppm concentration. A water extract of sea buckthorn (*Hippophae rhamnoides L.*) leaves and sprouts lowered the oxidation to 22.9 % at 100 ppm concentration. However, we also found some antioxidants with highly pro-oxidant properties in a meat model pre-trial. This is of importance, showing the great complexity of both antioxidants and matrices studied. As for the *in vivo* trial, some differences between trial groups were found. However, the results from this screening were unfortunately not as conclusive as we expected, but gave nonetheless great inspiration and knowledge for further studies in the area. It is clear that lipid oxidation is not the sole mechanism behind the increase in colorectal cancer prevalence amongst processed meat consumers, but important differences could be seen in mice after only four months of trial in terms of microflora composition and certain immunological reactions between trial groups.

Key words:Plant phenols, processed meat, colorectal cancer, inflammation

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language eng

ISSN and key title

ISBN:
978-91-7895-300-4 (print)
978-91-7895-301-1 (digital)

Recipient's notes

Number of pages 96

Price

Security classification

Signature  Date 2019-10-28

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Cover photo by Stina Burri

Back cover by Jonas Andersson

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Department of Food Technology, Engineering and Nutrition

ISBN 978-91-7895-300-4

Printed in Sweden by Media-Tryck, Lund University
Lund 2019

To my family

Table of Contents

List of papers

This thesis is based on the following papers, and will be referred to by their respective Roman numbers

I) **Burri, C. M. S.**, Ekholm, A., Håkansson, Å., Tornberg, E., & Rumpunen, K. (2017). Antioxidant capacity and major phenol compounds of horticultural plant materials not usually used. Journal of Functional Foods, 38, 119-127.

II) **Burri, C. M. S.**, Ekholm, A., Bleive, U., Püssa, T., Jensen, M., Hellström, J., Mäkinen, S., Korpinen, R., Mattila, H. P., Radenkovs, V., Segliņa, D., Håkansson, Å., Rumpunen, K., Tornberg, E. Lipid oxidation inhibition capacity of plant extracts and powders in a processed meat model system. (Manuscript under review in Meat Science).

III) **Burri, C. M. S.**, Granheimer, K., Rémy, M., Ekholm, A., Håkansson, Å., Rumpunen, K., Tornberg, E. (2019). Lipid oxidation inhibition capacity of 11 plant materials and extracts evaluated in highly oxidised cooked meatballs. Foods, 8 (406) 1-14.

IV) **Burri, C. M. S.**, Granheimer, K., Rémy, M., Rumpunen, K., Tornberg, E., Canaviri Paz, P., Uhlig, E., Oscarsson, E., Rahman, M., Håkansson, Å. Meatballs with added plant antioxidants affect the microbiota and immune response in C57BL/6JRj mice with cyclically induced chronic inflammation (Unpublished manuscript)

Author's contributions to the papers

I) Stina Burri, Kimmo Rumpunen and Anders Ekholm planned the experimental design. Stina Burri acquired plant materials and prepared samples for analysis. Stina Burri performed all analyses with the assistance of Anders Ekholm except for analysis of ascorbic acid. Stina Burri performed ABTS analysis. Stina Burri performed data analysis together with Kimmo Rumpunen. Stina Burri wrote the first draft of the manuscript, which was then revised by co-authors.

II) Stina Burri and Eva Tornberg planned the experimental design. Stina Burri and Anders Ekholm prepared and analysed samples. Stina Burri analysed results and conducted statistical analyses. Stina Burri wrote the first draft of the manuscript, which was then revised by co-authors.

III) Stina Burri and Eva Tornberg planned the experimental design. Stina Burri performed the experimental work with assistance of Anders Ekholm, Kajsa Granheimer and Marine Rémy. Stina Burri analysed results and conducted statistical analyses. Stina Burri wrote the first draft of the manuscript which was then revised by co-authors.

IV) Stina Burri and Åsa Håkansson planned the experimental design. Stina Burri and Elisabeth Uhlig acquired necessary materials for the *in vivo* trial. Stina Burri and Marine Rémy prepared samples for the *in vivo* trial. Stina Burri and Kajsa Granheimer conducted the *in vivo* trial. Stina Burri, Kajsa Granheimer, Pamela Canaviri Paz and Elin Oscarsson conducted the FACS analysis with technical support of Milladur Rahman. Stina Burri conducted MPO and cytokine analysis with the technical support of Anne-Marie Rohrstock. Vandana Tannira conducted DNA extractions. Stina Burri and Yunjeong So conducted T-RFLP analysis. Stina Burri, Åsa Håkansson and Kimmo Rumpunen evaluated and analysed the results. Stina Burri wrote the first draft of the manuscript which was then revised by co-authors.

Contributions to conferences and workshops

Burri, C. M. S., Ekholm, A., Rumpunen, K., Tornberg, E. (Oral presentation), Screening local horticultural plant material for antioxidant capacity in an *in vitro* meat model. International Conference of Meat Science and Technology (ICoMST) 2016, August 14-19th 2016, Bangkok, Thailand.

Burri, C. M. S., Ekholm, A., Rumpunen, K., Tornberg, E. (Workshop) Sustainable plant ingredients for healthier meat products (SUSMEATPRO), September 1-2nd 2016, Aarhus, Denmark.

Burri, C. M. S., Ekholm, A., Rumpunen, K., Håkansson, Å., Tornberg, E (Oral presentation), Sustainable plant ingredients for healthier meat products (SUSMEATPRO), Pufendorf institute – interdisciplinary protein shift advanced study group, May 8th 2019, Lund, Sweden.

Burri, C. M. S., Ekholm, A., Rumpunen, K., Håkansson, Å., Tornberg, E (Oral presentation), Är antioxidanter lösningen på kött- och tarmcancersambandet? (Are antioxidants the solution to the connection between meat and colorectal cancer?) Biomedical analysist day, October 17th 2019, Malmö, Sweden.

Burri, C. M. S., Ekholm, A., Rumpunen, K., Håkansson, Å., Tornberg, E. (Accepted poster presentation), Sustainable plant waste material antioxidants in meatballs – The answer to healthier meat products? 33rd EFFoST International Conference 2019, November 12-14th 2019, Rotterdam, Netherlands.

Abbreviations

4-HNE	4-Hydroxynonenal
ABTS	[2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)]
CAC	Colitis-Associated Cancer
CRC	Colorectal Cancer
DW	Dry Weight
FACS	Fluorescence-Activated cell-Sorting
FC	Folin-Ciocalteu
FRAP	Ferric Reducing Ability of Plasma
GAE	Gallic Acid Equivalent
Hb	Hemoglobin
HCA	Heterocyclic Amines
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IBD	Inflammatory Bowel Disease
Mb	Myoglobin
MDA	Malondialdehyde
MetMb	Metmyoglobin
MPO	Myeloperoxidase
Neu5Gc	N-glycolylneuraminic Acid
NK	Natural Killer (cell)
NOC	N-nitroso Compound
OxyMb	Oxymyoglobin
PAH	Polycyclic Aromatic Hydrocarbons
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Specie
SCFA	Short-Chain Fatty Acid
SUSMEATPRO	Sustainable Plant Ingredients for Healthier Meat Products
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Reactive Substances
TCA	Trichloroacetic Acid
TMP	1,1,3,3,-tetramethoxypropane
T-RFLP	Terminal Restriction Fragment Length Polymorphism
WCRF	World Cancer Research Fund

Abstract

In 2015, the World health organisation (WHO) released a press statement together with the International Agency for Research in Cancer (IARC) classifying processed meat as carcinogenic. Since then, research in this area has been focused on finding the underlying mechanism(s) responsible for the link between processed meat and the increased prevalence of colorectal cancer. One of the main hypotheses is that the heme iron present in the meat catalyses lipid oxidation which increases the levels of Reactive Oxygen Species (ROS) and toxic lipid oxidation secondary products which are harmful to our health.

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Popular science summary

Can natural antioxidants in meatballs decrease the risk of colorectal cancer?

In June 2015, a project was started at Lund University with the goal of evaluating if oxidation, a chemical reaction releasing free oxygen radicals harmful to the intestine, is the underlying mechanism linking processed meat consumption to the rising prevalence of colorectal cancer among meat consumers. This theory is one of the more prevalent ones in the field since red meat contains a lot of iron which is easily oxidised and may oxidise other food components such as proteins or fat. Except for the approximately 75 percent water contained in a piece of meat, precisely these other two components are present, why this theory is one of the more popular ones. Processed meat products are regarded as even more problematic, seeing as they are often smoked and additionally usually contain nitrite salts and a higher amount of fat.

To study this matter, we started by producing a processed meat product easily subjected to oxidation. Meatballs, one of our most common Swedish dishes, were therefore chosen for the project. A range of properties such as; type of meat, fat content, salt content, cooking type, and storage time were studied before the optimal oxidised meatball was found.

The background for this project was that the U.N. World Health Organisation (WHO) published a report in October 2015 together with the International Agency for Research on Cancer (IARC) placing processed meat in the same carcinogenicity group as smoking or alcohol consumption (Group I carcinogens). The statistics in the report show that you have an 18 percent increased risk of colorectal cancer for every 50 grams daily consumption of processed meat products. This is a number that we in Sweden exceeded in 2016 with our average of 52 grams daily consumption per person. The corresponding numbers for red meat was reported to be 17 percent increased risk for colorectal cancer for every 100 grams daily consumption of red meat. In Sweden, we average 145 grams per day.

The project at LTH, Lund University, was a part of an EU financed research project called SUSMEATPRO (Sustainable Plant Ingredients for Healthier Meat Products – Proof of concept). A collaboration between scientists in five different countries, namely; Sweden, Denmark, Finland, Estonia, and Latvia whose common goal was to find antioxidants powerful enough to counteract the harmful oxidation in the meat products.

In total, 28 different antioxidant extracts were analysed from all collaborating countries. These were first tested in a so called meat model system made of the part of the meat containing the iron, together with an oil that easily oxidises. These two components were mixed with one antioxidant at a time, before the mixture was heated, to resemble

an industrial heating process of processed meat products. When the mixture had cooled down, the level of oxidation was measured in the mixtures both with and without antioxidants after specific periods of time. The antioxidants with the best effects were then added to the previously manufactured oxidation meatball to see if they were powerful enough to counteract the oxidation in a real product. Which they could.

In the next step of the project, we wanted to see if the consumption of these meatballs with antioxidants lead to a decrease in colorectal cancer compared to meatballs with no antioxidants. This we did by feeding mice a diet containing 20 percent of our meatballs (with and without antioxidants) since this number approximately corresponds to the average amount of meat consumed by an adult in a balanced diet. Since colorectal cancer almost always is preceded by an inflammation, we induced a low grade one using a substance slightly irritating to the intestines. To not induce an inflammation would have made the study far too long, due to that tumours take long to develop, and it would have increased the long-term suffering of the trial animals.

We know that oxidation is counteracted by antioxidants. However, the results of the animal trial are not conclusive in if these special meatballs were truly healthier than regular meatballs. Some differences were noticed, in for instance the gut microflora, but the study should probably have had to be longer for clearer results to be seen. It is also reasonable to believe that the oxidation is not the only factor in meat linked to colorectal cancer, but rather an interplay between many mechanisms and environmental circumstances. The mice did also consume their meatballs together with a feed containing fibre, which has a protective effect against colorectal cancer. In summary, natural and relatively unexplored antioxidants were found to efficiently inhibit lipid oxidation in both meat model and processed meat product. Moreover, these antioxidant-enriched meatballs were then found to show some health-related differences in the *in vivo* trial.

Populärvetenskaplig sammanfattning

Kan naturliga antioxidanter i köttbullar minska risken för tjocktarmscancer?

I juni 2015 påbörjades ett projekt på Lunds Tekniska Högskola som skulle utreda huruvida oxidationen, en kemisk reaktion som frisätter syreradikaler skadliga för tarmen, ligger till grund för sambandet mellan konsumtionen av processade köttprodukter och den stigande siffran av tjock- och ändtarmscancer hos köttätare. Denna teori är en av de mer befästa inom forskningsområdet eftersom rött kött innehåller mycket järn, en metall som gärna oxiderar och/eller oxiderar andra beståndsdelar omkring sig såsom protein eller fett. Förutom de cirka 75 procent vatten som finns i en köttbit hittar man även just dessa beståndsdelar, varför denna teori är en av de mest betrodda. Processade köttprodukter anses än mer problematiska eftersom dessa dessutom ofta blir bl.a. rökta, behandlade med nitritsalter och har högre fetthalt.

För att undersöka saken började vi därför med att ta fram en vanlig processad köttprodukt som lätt utsätts för oxidation. Köttbullarna, en av våra populäraste husmanskosträtter, valdes för projektet. En rad olika egenskaper såsom typ av kött, fetthalt, salthalt, tillagningssätt och lagringstid undersöktes innan den optimala oxidationsköttbullen för ändamålet var framtagen.

Bakgrunden till detta projekt är att FNs Världshälsoorganisation (World Health Organisation, WHO) publicerade en rapport i oktober 2015 tillsammans med IARC (International Agency for Research on Cancer) som placerade processat kött i samma kategori av cancerframkallande kategori som rökning eller alkoholkonsumtion (Grupp 1 karcinogener). Statistiken i rapporten visar att man löper 18 procent högre risk att insjukna i tjock- och ändtarmscancer per 50 gram dagligt intag av processade köttprodukter. Detta är en siffra som vi i Sverige år 2016 överskred enligt Sveriges officiella statistik från Jordbruksverket med våra i genomsnitt 52 gram per person och dag. Motsvarande siffra för rött kött rapporterades vara 17 procent ökad risk för tjock- och ändtarmscancer för varje 100 gram som konsumeras dagligen. I Sverige ligger vi på ett dagligt genomsnittligt intag av 145 gram per dag.

Projektet på LTH var en del av det EU-finansierade projektet SUSMEATPRO (Sustainable Ingredients for Healthier Meat Products – Proof of concept). Ett samarbete mellan forskare från fem olika länder, Sverige, Danmark, Finland, Estland och Lettland, vars gemensamma mål var att hitta antioxidanter effektiva nog att motverka den skadliga oxidationen i köttprodukterna.

Totalt analyserades 28 olika antioxidanter från samtliga länder. Dessa testades först på ett så kallat köttmodellsystem bestående av den proteindel i köttet som innehåller järnet, blandat med en olja som lätt oxiderar. De två komponenterna blandades ihop med en antioxidant i taget innan blandningarna hettades upp för att efterlikna en industriell värmebehandling av processade köttprodukter. När blandningarna svalnat

mättes hur mycket de oxiderat med och utan antioxidanter under en given tid. Antioxidanterna med bäst effekt tillsattes sedan till den tidigare framtagna oxidationsbenägna köttbullstypen för att se om reaktionen kunde motverkas i en riktig produkt. Vilket den kunde.

I nästa steg ville vi se om konsumtionen av köttbullarna med antioxidanter ledde till att man i lägre omfattning drabbades av tjock- och/eller ändtarmscancer än köttbullarna utan. Detta gjorde vi genom att mata möss med en diet bestående av 20 procent av köttbullarna ifråga, då denna andel kött ungefär motsvarar en vuxen människas köttkonsumtion. Eftersom cancer i tjock- och ändtarm nästan alltid föregås av en inflammation, framkallade vi en låggradig sådan med hjälp av ett ämne som är lätt irriterande för tarmslemhinnan. Att inte framkalla en inflammation hade lett till att studien hade varit långt för långdragen med tanke på den tid det tar att inducera cancer utan föregående inflammation kontra djurens totala levnadstid. Dessutom hade det bidragit till ökat lidande för försöksdjuren.

Att oxidationen motverkas av de naturliga antioxidanterna, det vet vi. Däremot var resultaten av djurstudien inte säkra angående huruvida dessa specialköttbullar var mer hälsosamma än vanliga köttbullar. Vissa skillnader kunde ses, såsom skillnad i tarmflora, men troligtvis hade studien behövt vara längre för att kunna urskilja tydligare resultat på fler av de undersökta parametrarna. Dessutom är det troligt att det inte bara är oxidationen som ligger till grund för insjuknandet i tjock- och ändtarmscancer, utan snarare ett samspel mellan många fler reaktioner och förutsättningar. Mössen åt dessutom köttbullarna tillsammans med ett foder innehållande fibrer, vilket har motverkande effekter på insjuknandet. Sammanfattningsvis fann vi att naturliga och relativt outforskade antioxidanter effektivt kunde minska lipidoxidationen i både köttmodellsystem och i processad köttprodukt. Dessutom fann vi att köttbullar berikade med dessa antioxidanter visade sig ge olika hälsorelaterade effekter hos djuren i vår *in vivo*-studie.

1 Introduction

The consumption of red and processed meat products has been positively correlated epidemiologically with an increased risk of colorectal cancer (CRC) [1, 2]. The knowledge about dietary patterns and their correlation to different types of cancers is increasing, where we get recommendations to stay as lean as possible, consume plenty of fruits, whole grains, vegetables and pulses, eat low amounts of red meats, avoid processed meat entirely, and limit salt intake, to avoid different types of cancers [3]. The correlations are there, but the mechanisms underlying some of these remain unknown, although hypotheses are many.

Meat is considered a healthy and nutritious food element when consumed in low amounts, thanks to its proteins of high biological value, important minerals, and vitamins. However, the amount of meat that is included in the western diet of today is excessive [4] when compared to the above-mentioned recommendations, and does not only have health implications, but also environmental and ethical implications.

Meat consists largely of water and protein, with low amounts of fat and carbohydrates. It is a complex food matrix since it originates from living animals with their own metabolisms and life cycles. When muscle foods become meat products, this allows for a number of reactions to take place, both positive and negative, e.g. tenderisation and rancidification. A major issue in the production of meat products is oxidation, due to its deteriorating effect on meat lipids and proteins, causing off-flavours and discoloration. Lipid oxidation is a cascade reaction known to increase the level of reactive oxygen species (ROS) in our bodies that may be harmful to us. These ROS may further inflict more reactions, giving rise to a number of end products also harmful to us.

One of the greatest challenges for the meat industry is to tackle the problem with oxidation in meat products. This has previously been done using synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) or propyl gallate (PG), to name a few. However, it is now known that these synthetic antioxidants are toxic to us [5, 6], why the demand for natural antioxidants is increasing.

1.1 SUSMEATPRO

Sustainable Plant Ingredients for Healthier Meat Products – Proof of concepts (SUSMEATPRO) was a project funded by the European Union as a part of the ERA-NET SUSFOOD program which was initiated to promote for healthier foods until the year of 2020. SUSMEATPRO started in 2015 as a response to the previously mentioned increased demand for healthier meat additives for antioxidant and antimicrobial purposes. The project was a collaboration between six universities in five countries, namely; Sweden, Denmark, Finland, Latvia and Estonia, where each university had been assigned their specific sub-topic to study. The overall goal for the project was to collect local horticultural plant materials and by-products to be screened for their antioxidant and antimicrobial properties. Next, complex natural food additives were manufactured for incorporation in a range of different meat products. In the last step of the project, *in vivo* trials were performed in order to test the health effects of these natural additives.

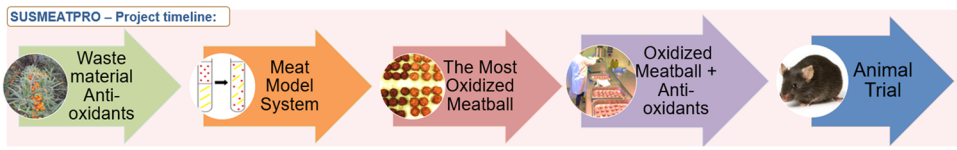


Figure 1.
Timeline of the doctoral thesis work in the frame of SUSMEATPRO.

2 Background

2.1 Chemical composition of meat

Whenever we talk about meat, it is often the skeletal muscles we address. They are the ones anchored to our bones using tendons, and the muscle type that moves us forward and keeps us upright. As opposed to the smooth muscles (found in e.g. the digestive system and the arteries) and the cardiac muscles (found in the heart) skeletal muscles are mostly moved voluntarily [7]. Generally, the muscle is said to contain about 75 % water, 19 % proteins, 3.5 % Soluble non-protein substances and 2.5 % fat [8] (Figure 1), however this of course depends on the specific muscle, nutrition, and genetic predisposition of the live animal.

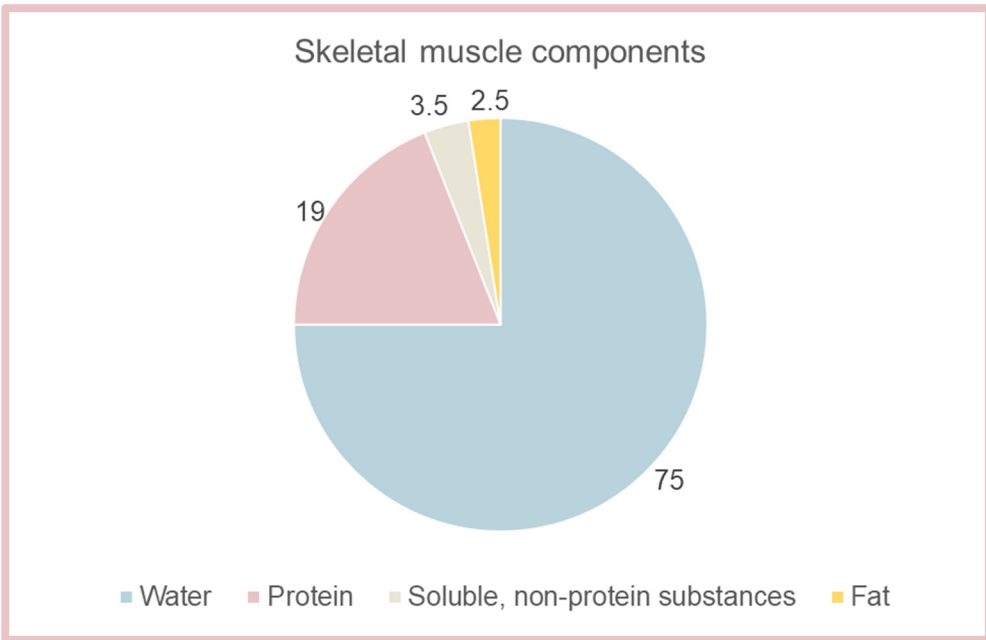


Figure 2.
Skeletal muscle components as a percentage of the total muscle weight.

2.1.1 Meat proteins

The muscle contains over 50 different proteins, of which there are three major groups which can be classified depending on their solubility [7] (Table 1). The fibrous parts of the muscle consist of myofibrillar proteins which are soluble in concentrated salt solutions. Myosin and actin, i.e. the thick and the thin filaments, are crucial for muscle contraction and mobility. Stromal proteins, which are neither salt nor water soluble, mostly constitute the connective tissue where collagen maintains structure, and elastin maintains flexibility. The last sub-group of muscle proteins are sarcoplasmic proteins. These are proteins soluble in water or dilute salt solutions situated in the sarcoplasm, i.e. the cellular fluid of the muscle.

Table 1.
Skeletal muscle protein sub-groups and contents in an adult mammal. The number in brackets is based on a 19 % protein content in the skeletal muscle. Table modified from Barbut [7].

Group	Protein	%
Sarcoplasmic		(5.5)
	Myoglobin	0.2
	Hemoglobin	0.6
	Cytochromes	0.2
	Glycolytic enzymes	2.2
	Creatine kinase	0.5
Myofibrillar		(11.5)
	Myosin	5.5
	Actin	2.5
	Tropomyosin	0.6
	Troponin	0.6
	C-protein	0.3
	α-actinin	0.3
	β- actinin	0.3
Stromal		(2.0)
	Collagen	1.0
	Elastin	0.05
	Mithocondrial	0.95

Myoglobin (Mb) and hemoglobin (Hb) are two important sarcoplasmic proteins with the main task to transport oxygen in the blood and into the muscle. Hb is composed of four Mb units and is mostly found in red blood cells. Both Hb and Mb are complex molecules that consist of two main parts; a protein part (globin) and a heme part (heme-ring) [8]. The heme ring, commonly known as the heme group, is responsible for

binding water and oxygen to the molecule, which is made possible thanks to an iron molecule situated in the centre (Figure 3) [7]. When Mb is exposed to oxygen, i.e. oxymyoglobin (OxyMb), the iron molecule is in its reduced ferrous state (Fe^{2+}) which manifests as a bright red coloration of the meat. OxyMb oxidises to metmyoglobin (MetMb), where oxygen is released and exchanged with a water molecule [9]. The iron molecule is now in its ferric (Fe^{3+}) state which manifests a brown coloration of the meat [7]. Upon heating, i.e. cooking, the globin-part of the protein is denatured, separating the globin from the heme-group, adding the heme-group to the non-heme iron pool in meat, and the ability to bind oxygen is lost [8]. The oxidation of OxyMb to MetMb produces ROS and occurs due to various factors e.g. increased temperature, lower pH, the presence of non-heme iron, MetMb reducing activity, and lipid oxidation [8, 9].

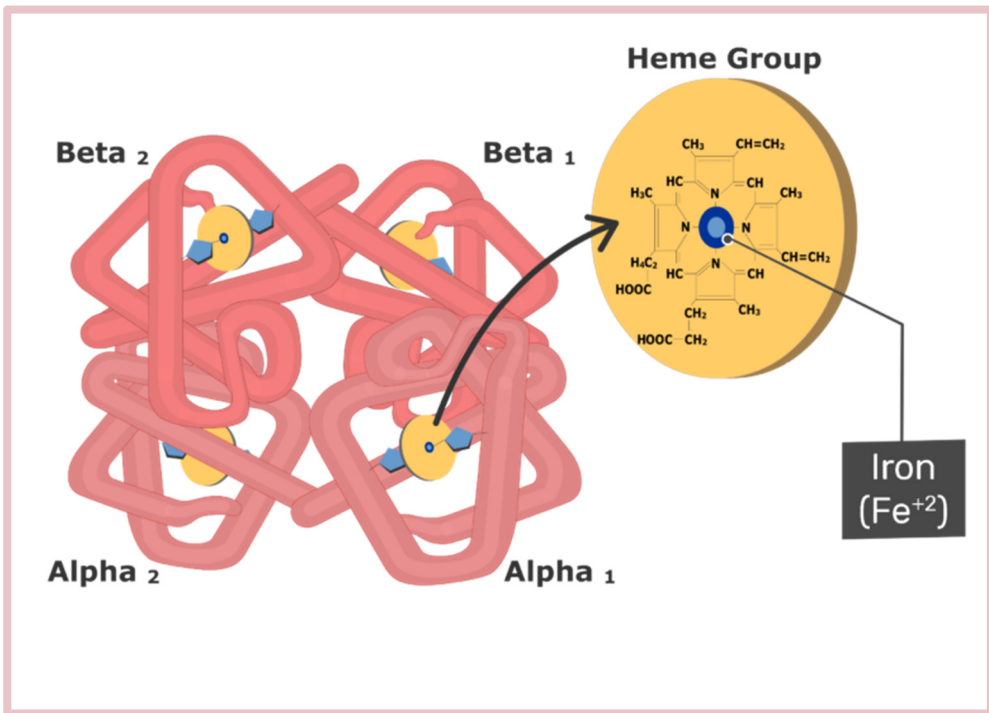


Figure 3.
Schematic representation of hemoglobin (left) and heme group (right) (from imgbin.com).

2.1.2 Meat lipids

Adipose tissue is a protecting and insulating type of tissue for sensitive organs, as well as an energy storage for animals [7]. Adipose tissue consists to more than 99 % of true fat, i.e. esters of glycerol with fatty acids, as opposed to the fat in muscles, which instead consists of considerable amounts of phospholipids and unsaponifiable substances such

as cholesterol [8]. The fatty acid composition between beef and pork is very similar, except for levels of linoleic acid (C18:2) and levels of polyunsaturated fatty acids (PUFA) which was shown when they were measured in beef sirloin steak and pork chops by Enser, Hallett [10] (Table 2).

Table 2.
Fatty acid composition of beef and pork fats and muscular tissue as a % of total fatty acids. Table modified from Lawrie and Ledward [8].

Fatty acid	Structure	Beef fat	Beef muscle	Pork fat	Pork muscle
Palmitic	C16:0	26.1	25.0	23.9	23.3
Stearic	C18:0	12.2	13.4	12.8	12.2
Oleic	C18:1 (n-9)	35.3	36.1	35.8	32.8
Linoleic	C18:2 (n-6)	1.1	2.4	14.3	14.2
α-Linolenic	C18:3 (n-3)	0.5	0.7	1.43	1.0
Arachidonic	C20:4 (n-6)	Not detected	0.6	Not detected	2.2
Eicosapentaenoic	C20:5 (n-3)	Not detected	0.3	0.36	0.3
Dodecosahexanoic	C22:6 (n-3)	Not detected	0.05	Not detected	0.4

2.2 Lipid oxidation

Lipid oxidation is a reaction between PUFA and different types of ROS, such as; hydroxyl radicals (HO•), superoxide radicals (O^{-}_2), singlet oxygens (O^{1}_2), and hydroperoxide acid ($HO•_2$), which, due to the chain reaction nature of the process, leads to lipid degradation and deterioration of, for instance, meat and meat products [11, 12]. There are three stages in lipid oxidation; initiation, propagation and termination [13]. In the initiation phase, a free radical reacts with an organic substrate (RH) due to e.g. thermal decomposition from heating, hydroperoxide decomposition, exposure to light and/or catalysis by metals [11, 13]. RH (depicted as an unsaturated lipid in Figure 4) loses its hydrogen to the reactive hydroxyl radical, yielding water and an unsaturated lipid radical (RH + •OH = R• + H_2O). In the propagation step, molecular oxygen (O_2) reacts with the lipid radical by attaching to it, forming a lipid peroxyl radical (ROO•). This lipid peroxyl radical can then react with another unsaturated fatty acid (RH + ROO• = R• + H_2O), perpetuating the cycle of the lipid oxidation. The reaction ends in the termination step, when radicals react with each other (depicted in Figure 4 as lipid peroxide), stabilising the molecules into peroxides. These lipid oxidation primary products (peroxides) degrade to volatile and non-volatile secondary products such as; carbonyls, hydrocarbons, alcohols and furans [11] of which the reactive carbonyl species malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are most commonly debated in the field of meat science [6, 12].

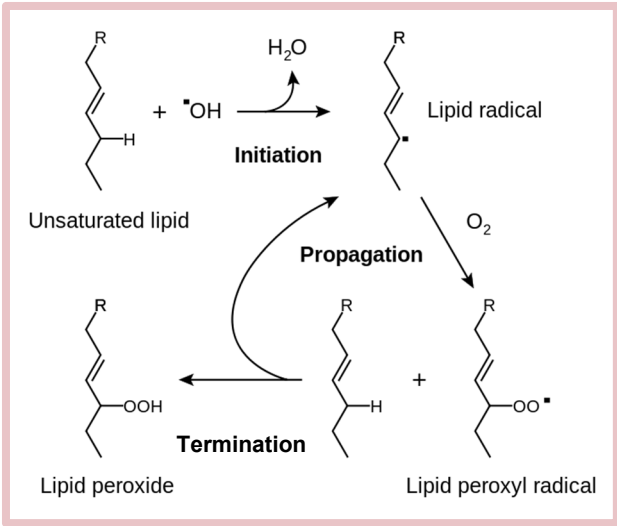


Figure 4.
Illustration of lipid oxidation chain reaction (from imgbin.com).

2.3 The meat we eat

Meat is considered to be one of the most important sources of proteins in the human diet, containing e.g. all essential amino acids of high biological value. Meat also provides B-group vitamins and essential minerals [14], and is considered a healthy food ingredient when consumed in moderate amounts. However, red meat contains heme and free iron, and more so than other types of fresh meat [15]. Heme iron from Mb or Hb is one of the components commonly hypothesized to promote oxidation, either as it is or when combined with nitrite in processed meat [16] particularly when in presence of PUFA [17]. Processed meat refers to products typically made of red meat or fowl, often containing high amounts of fatty tissues together with endogenous phospholipids [18]. Other ingredients might include internal organs, blood, or skin. The processed meat product has undergone one or more physical and/or chemical treatment(s), such as comminution/cutting, tumbling/mixing, curing/salting, fermentation, drying, smoking and/or stuffing into varying casings (Figure 5). These factors, together with high-temperature cooking, make them very susceptible to oxidative reactions potentially contributing to harmful health hazards [6]. The worlds' average intake of processed meat was 13.7 g/day in 2010, but 26.4 g/day in Western Europe, and 34.6 g/day for North America [4]. This is of interest given that the western diet is growing in popularity globally [19] and since the incidence of colorectal cancers are increasing in eastern parts of Europe, Australia/New Zealand, North Americas and Eastern Asia where incidences reflect western dietary patterns, life style factors and obesity [20, 21].

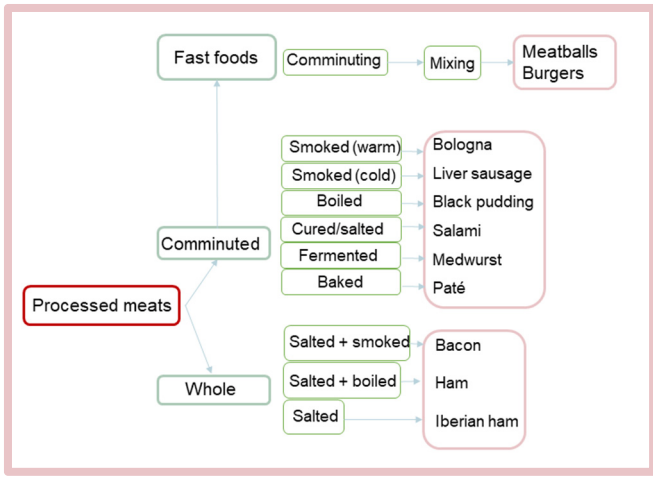


Figure 5.
Schematic overview of examples of processed meat products and processes.

2.4 Meat and colorectal cancer

Since the International Agency for Research on Cancer (IARC) released their monograph stating that red and processed meats are linked to colorectal cancer (CRC) [22], research regarding this topic has grown in popularity, particularly since the mechanisms leading to these links remain partly unknown. Meta-analyses have previously reported an increased risk of CRC by 17 % per 100 g of daily red meat intake and 18 % per 50 g of daily processed meat intake [2]. Furthermore, CRC is the second most prevalent cancer type in Europe with its 500 000 cases, and second most common cause of death from cancer with its 243 000 cases estimated in 2018 [23]. Hypotheses regarding the possible mechanism(s) underlying the link between red meat and CRC are many. Most certainly, there will not be one isolated underlying mechanism, but rather a number of overlapping mechanisms, together with genetic predispositions and environmental factors. The currently proposed mechanisms will be presented in the section to follow.

2.4.1 Proposed mechanisms for links between meat and colorectal cancer

The currently proposed mechanisms underlying the link between red/processed meat and CRC are depicted in Figure 6 and could be attributed to the following, partly overlapping, mechanisms:

- An increase in oxidative load leading to lipid oxidation and thus, lipid oxidation secondary products, such as MDA or 4-HNE, which are known to be cytotoxic and genotoxic to the adenomatous polyposis coli (APC) gene [24, 25]. A mutation in the APC gene is known to potentially result in CRC [26].
- An increase in N-nitrosation load, i.e. a reaction between nitrite in processed meats and amino compounds, leading to DNA adducts (a cancer-causing chemical bound to a segment of DNA in the intestinal epithelium) [19, 27].
- Stimulation of proliferation of the epithelium by heme or other food metabolites acting either directly or following conversion to, e.g. heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs) through high temperature cooking [19, 27].
- Pro-malignant processes triggered by a higher inflammatory response where N-glycolylneuraminic acid (Neu5Gc), a glycan containing a type of sialic acid present in red meats, which provokes xenosialitis, i.e. an inflammatory syndrome inducing cancer formation and progression [24, 28].

All of these mechanisms could lead to an increased risk of CRC due to inflammation and a lack of intestinal cell repair caused by DNA mutations and disruption of normal cell structure and proliferation of gut epithelial cells.

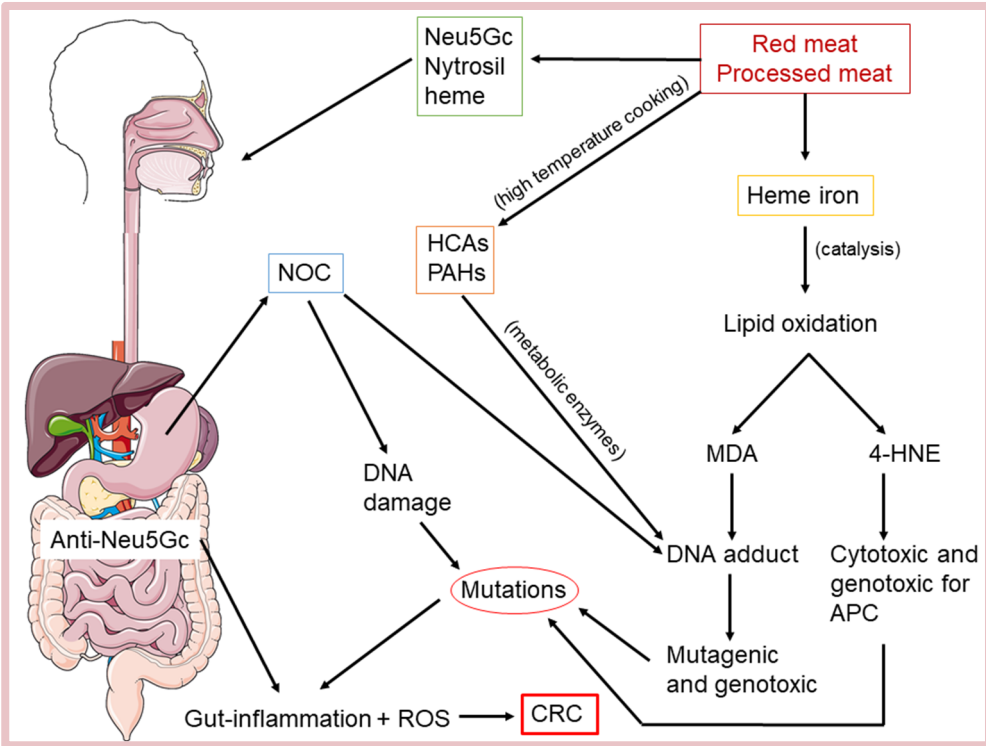


Figure 6.
Illustration of mechanisms involved in linking red and processed meat to colorectal cancer (CRC) where Neu5Gc = N-glycolylneuraminic acid, HCA = heterocyclic amines, PAH = polycyclic aromatic hydrocarbons, NOC = N-nitroso compounds, MDA = malondialdehyde, 4-HNE = 4-hydroxynonenal, APC = adenomatous polyposis coli gene, and ROS = reactive oxygen species. Modified from Cascella, Bimonte [24].

2.5 Gut-associated lymphoid tissue and immune system

The lymphoid tissue of the gastrointestinal tract is commonly known as gut-associated lymphoid tissues (GALT) which include e.g. Peyer's patches (PP) lymphoid follicles and mesenteric lymph nodes (MLN) enriched with different phenotypes of T-helper cells (T_H-cells), cytotoxic T-cells (T_C-cells) B-cells, natural killer cells (NK-cells) and natural killer T-cells (NKT-cells) [29]. Via receptors such as toll-like receptors and MHC class II expressed on epithelial cells, antigen-presenting cells (APC), and lymphocytes, the microbiota can interact with the immune system, and through dendritic cells (DC), macrophages and M-cells, bacterial antigens can be captured from the lumen side and be presented to B-cells and T_H-cells [30]. Through the subsequent production of cytokines, this results in the differentiation and activation of T-, NK-, and B-cells as well as IgA production [31]. Cytokines are immunological mediators able

to initiate differentiation of innate T-cells to different phenotypes. They can be either anti-inflammatory such as interleukin (IL)-4, IL-6, IL-10, IL-11, and IL-13 or pro-inflammatory such as IL-1, IL-12, interferon gamma (IFNγ), or tumour necrosis factor alpha (TNF-α) [32]. An example of how cytokines are directing different phenotypes of T-cells is given in Figure 7, where IL-1β promotes the differentiation of T_H2-cells, IL-12 promotes the differentiation of T_H1-cells (initiating a pro-inflammatory response via TNF-α and IFN-γ), and NK-cells [33]. An example of the adaptive immune regulation is also shown in Figure 7, where DCs promote the production of Immunoglobulin (Ig)A producing B-cells or promote the maturation of Naïve T-cells to the anti-inflammatory forkhead box P3 (FoxP3) regulatory T-cells [29].

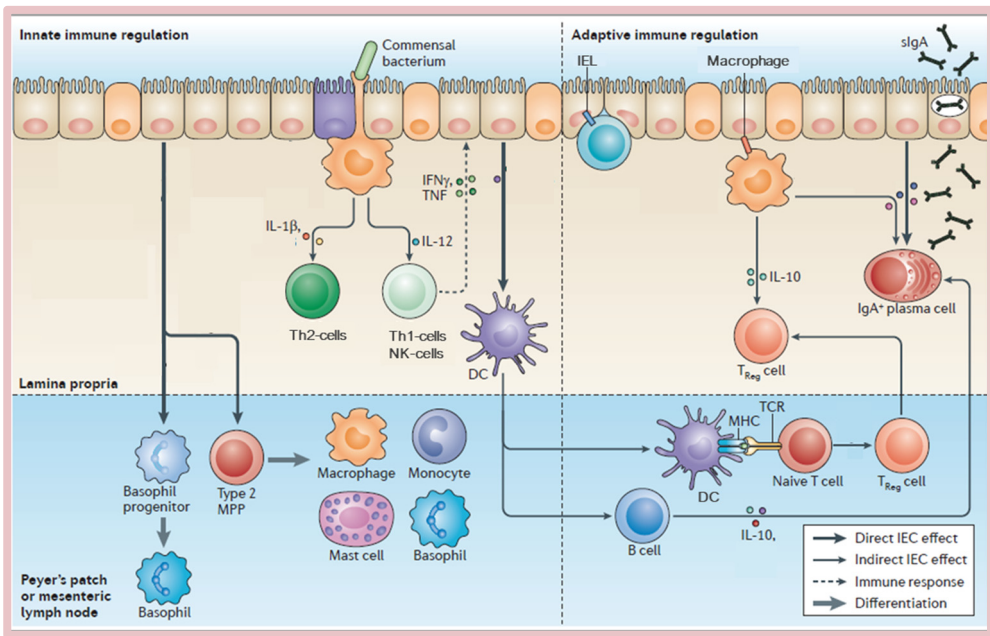


Figure 7.
Schematic figure of intestinal epithelial cell regulation of both innate and adaptive immunity where IL = Interleukin, a cytokine protein with a certain role in regulationg lymphocyte function, MPP = multi-potent progenitor. Reprinted by permission from Springer Nature, Nature Reviews Immunology, Intestinal epithelial cells: Regulators of barrier function and immune homeostasis, (Peterson & Artis, 2014), © Macmillan Publishers Limited 2014.

The epithelial and mucous layers play an important role in regulating the immune response from both commensal and pathogenic bacteria [34] where the response needs to be weak towards commensal bacteria and strong towards pathogenic bacteria. Most of the commensals are non-pathogenic, however, there are some potential pathogenic bacteria which might act as pathogens if the homeostasis is disrupted [33]. To avoid a constant bacterial stimulation, the epithelial layer of the intestines is covered in one or two layers of mucous [31] (Figure 8). The mucous coating of the epithelial cells is of

great importance since it protects against bacterial stimulation and penetration [31]. The mucin is secreted by goblet cells which, except for absorptive enterocytes, Paneth cells (excreting antimicrobial proteins (AMP)) and neuroendocrine cells, are included in the epithelium [33]. Colonic mucous consists of a dense inner layer and a looser outer layer, the latter containing a large number of bacteria, whereas the small intestinal mucous is thinner and unevenly distributed (Figure 8). It has been shown that an impaired mucous layer is linked with spontaneous development of colitis and inflammation-associated cancers [33].

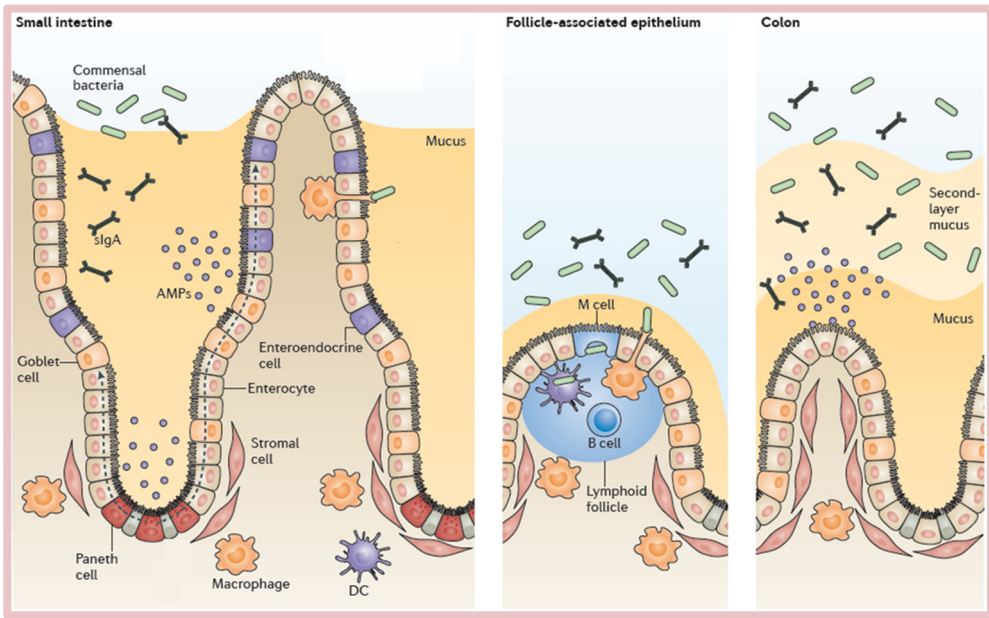


Figure 8.
Illustration of the epithelial cell barrier of small intestine, follicle-associated epithelium, and colon where AMPs = antimicrobial proteins, and sIgA = secretory immunoglobulin A. Reprinted by permission from Springer Nature, Nature Reviews Immunology, Intestinal epithelial cells: Regulators of barrier function and immune homeostasis, [33], © Macmillan Publishers Limited 2014.

2.6 Inflammation and colorectal cancer

Inflammation is promoted by an accumulation of immune and inflammatory cells as well as inflammatory mediators such as cytokines/chemokines, growth factors, ROS, lipid molecules, and reactive nitrogen species [35, 36]. Inflammatory bowel disease (IBD) has been shown to lead to high-mortality linked colitis-associated cancer (CAC) [37] and it has been reported that more than 20 % of IBD patients develop cancer of which 50 % suffer a lethal outcome [38, 39]. The link between inflammation and CRC

is clear, since the interplay between immune and inflammatory cells and mediators generate autocrine and paracrine signals which foster tumour cell progression, growth and metastases [35]. Patients with CRC show an infiltration of various innate immune cells, such as neutrophils, natural killer cells, dendritic cells, mast cells, and tumour-associated macrophages [40]. In an acute colitis, these cells induce an anti-tumour response which supress tumour growth and angiogenesis. This innate immune response, together with an adaptive immune response, helps in an early detection of aberrant crypt foci (ACF) and help the elimination of aberrant cells which could potentially turn into CRC adenomas [35]. In a chronic inflammation however, an environment, unfavourable for this immune surveillance mechanism, is created over time, which favours inhibition of anti-tumour immunological responses. This leads to the formation of tolerogenic dendritic cells, i.e. a pool of dendritic cells with immune-suppressive properties, and infiltration of regulatory T-cells which may help in promoting tumour cell growth [35].

2.7 Gut microbiota, inflammation and cancer

All gut-resident microorganisms commonly go under the collective name of gut microbiota. These microorganisms, including certain fungi and parasites, collaborate in symbiosis to keep host homeostasis stable. In the small intestine, the most prevalent bacterial phyla are *Firmicutes* and *Proteobacteria* (jejunum), *Bacteroidetes* and *Firmicutes* (ileum) [30], where pathogenic bacteria such as *Salmonella* and *Campylobacter* spp. may be asymptomatically present in small numbers [41]. In the colon, *Firmicutes* and *Bacteroidetes* are the two largest phyla [30, 36]. Of these, species of the genus *Bifidobacterium*, *Eubacterium*, *Enterococcus*, *Bacteroides* and species from the *Enterobacteriaceae* family are most commonly found, where *Escherichia coli* and *Bacteroides fragilis* are potential pathogenic bacteria encountered in small amounts from the latter [41]. The diversity of the microflora is of importance since different types of bacteria are able to produce various types of metabolites and bi-products which affect host homeostasis [42]. An eubiotic (balanced) and diverse microflora successfully interacts and regulates host physiology via GALT [43]. It is however worth noting that the composition of bacteria constituting the diversity may be of higher importance than the actual diversity, i.e. should the diversity be high whilst constituted of pathogenic bacteria, a high diversity would evidently not be health-promoting [44]. In the case of dysbiosis, pathogenic and opportunistic bacteria are able to reproduce and produce types of metabolites and bi-products toxic to the host, potentially triggering inflammation and carcinogenesis [42] as well as induce various reactions via receptors. The following sections will divide microorganisms in anti-tumoural and pro-tumoural microorganisms respectively. It should however be noted that the effects of some

microorganisms change as a function of their environmental condition (eubiosis vs dysbiosis).

2.7.1 Anti-tumoural and anti-inflammatory microorganisms

Short-chain fatty acids (SCFAs), produced by many enteric bacteria from non-digestible carbohydrates, are known for their anti-cancer effects due to their ability to inhibit the host's tumour cells histone deacetylation [42]. Examples of microorganisms producing SCFAs include *Coprococcus catus* and *Phascolarctobacterium succinatutens* producing propionate, *Eubacterium rectale* and *Faecalibacterium prasnitzii* producing butyrate and *Ruminococcus bromii* and *Blautia hydrogenotrophica* producing acetate [36]. Histone deacetylation occurs when an enzyme (histone deacetylase) cleaves off an acetyl group from a lysine amino acid on a histone, which is not desirable since DNA thus is wrapped more tightly around histones, preventing regular transcription [36], this is however desirable when considering transcription of host tumour cells. Moreover, butyrate and propionate have shown to play a crucial role in controlling intestinal inflammation since they induce differentiation of regulatory T-cells expressing transcription factor FoxP3 [36]. Another anti-tumoural component is bacterial lipopolysaccharide (LPS), which is an endotoxin abundant in the outer membrane of gram-negative bacteria. LPS itself is pro-inflammatory, but activates one of the pattern recognition receptors (PRRs), toll-like receptor 4 (TLR4), which activates anti-tumoural responses via T-cells [42]. Finally, certain strains of *Lactobacillus* and *Bifidobacterium* are known to be anti-inflammatory organisms by contributing to the microbial balance (commonly known as probiotics) [30].

2.7.2 Pro-tumoural and pro-inflammatory microorganisms

There are several known types of pathogenic microorganisms specifically involved in inflammatory diseases such as IBD, e.g. *Mycobacterium avium* subsp. *paratuberculosis*, *Listeria monocytogenes*, *Campylobacter concisus* as well as *Clostridium difficlie* and *E. coli* [45]. For the onset of CRC, some of the most common pathogens believed to be involved in the etiology include enterotoxigenic *Bacteroides fragilis* (generating ROS), *Escherichia coli* strain NC101 (generating DNA double strand breaks in epithelial cells) [36], *Shigella flexneri* (inducing loss of DNA damage control and repair), *Helicobacter pylori* (generating ROS and potentially increasing mutation incidences), and *Fusobacterium nucleatum* (inhibiting NK-cells) [42]. Moreover, three types of bacteria are known to be pro-tumoural due to their involvement in the β -catenin pathway activation, namely; *Fusobacterium nucleatum*, *Bacteroides fragilis,* and *Salmonella enterica* [42]. The activation of the β -catenin pathway and mutation of the tumour-suppressive APC gene induces a loss of barrier function in the colonic epithelium, resulting in translocation of cells and products to the tumoural microenvironment, in

turn inducing the production of tumour-promoting cytokines and hence, tumour growth [36]. Although many pro-inflammatory and pro-tumoural microorganisms have been identified, recent trends in research lead towards emphasising the role of dysbiosis in microbiota for cancer initiation and progression [46].

2.8 Red meat and inflammation

It has been established that inflammation is highly linked to CRC, the question to follow is why an inflammation is induced, and what part the red meat plays. Red meat contains various risk elements that could be held accountable for inducing gut inflammation, as previously mentioned. For instance, the Neu5Gc, which is non-naturally occurring in human tissues, and is incorporated in the epithelial cell surface glycol-conjugates upon consumption of red meats. Antigens then induce an immune response against this newly recruited self-antigen, i.e. a type of molecule commonly known as a xeno-autoantigen, which plays a big part in inducing the previously mentioned xenosialitis [47]. The microbiota is affected by meat consumption in several ways, including: temporal changes in bacterial composition and substrates by direct ingestion from the food source, variability in transit times through the gastrointestinal tract, changes in pH, and changes in regulation of gene expression of microbiota and/or host [48]. Moreover, it has been shown that gut-inflammation is generated by a number of lipid oxidation products induced by heme and ROS in red and processed meat [24], as well as products of protein fermentation (N-nitroso compounds (NOCs), polyamines, and bioactive compounds such as phenylacetic acid, phenols and indols) [36]. Lastly, a diet rich in animal proteins has shown to limit the carbohydrate intake, reducing the number of SCFA producing bacteria which could lead to pro-inflammatory responses and an increased risk of CRC [48].

2.9 How could we reduce the risk of colorectal cancer?

As previously reviewed, there are many potential factors involved in increasing the risk of CRC. In addition to these factors, between five and ten percent of CRCs are linked with hereditary predispositions such as familial adenomatous polyposis (FAP) and hereditary non-polyposis CRC (HNPCC), and another 20 percent of cases are linked to patients with a family history of CRC, according to the World Cancer Research Fund [49]. The hereditary predisposition is of course hard to battle, but there are some factors that have been found to be inversely correlated to CRC, such as a low intake of processed meat products, a high intake of dietary fibre, a high intake of dairy products, and a high intake of fruits and berries for women [50], together with physical activity

[49]. An approach often seen in research concerning red and processed meat products is the use of antioxidants to prevent oxidation [5, 6, 51].

2.10 Antioxidants

Antioxidant is a broad term which includes numerous types of molecules counteracting oxidation in various ways. First, antioxidants are divided into enzymatic and non-enzymatic antioxidants, where the enzymatic subgroup is divided into primary enzymes, such as catalase and glutathione peroxidase, and secondary enzymes such as glutathione reductase and glucose 6-phosphatase dehydrogenase (Figure 9) [52]. The non-enzymatic subgroup of antioxidants include minerals (e.g. zink and selenium), vitamins (e.g. vitamin A, C, E, and K), carotenoids (e.g. lycopene, lutein, zeaxanthin, and β -carotene), organosulphur compounds (e.g. allium, allyl sulphide and, indoles), low molecular weight antioxidants (e.g. uric acid and glutathione), antioxidant cofactors (e.g. coenzyme Q_{10}), and phenols (Figure 9) [52]. Phenols include three major groups; phenolic acids, flavonoids and tannins, and two minor groups; stilbenes and lignans of which flavonoids are most abundant in our diets [53]. Natural antioxidants primarily consist of phenols, which are ubiquitous in all parts of the plant [54]. Due to different molecular structures, flavonoids are divided into six subgroups:

1) Flavonols, where e.g. quercetin is found in onions, broccoli and apple
2) Flavanols, where e.g. catechin is found in several fruits and teas
3) Flavanones, where e.g. naringenin is found in grapefruit
4) Anthocyanins, where e.g. cyanidin-glycoside is found in berries
5) Isoflavones, where e.g. genistein and glycetin is found in soybean
6) Flavons, where e.g. chrysin is found in honey [53]

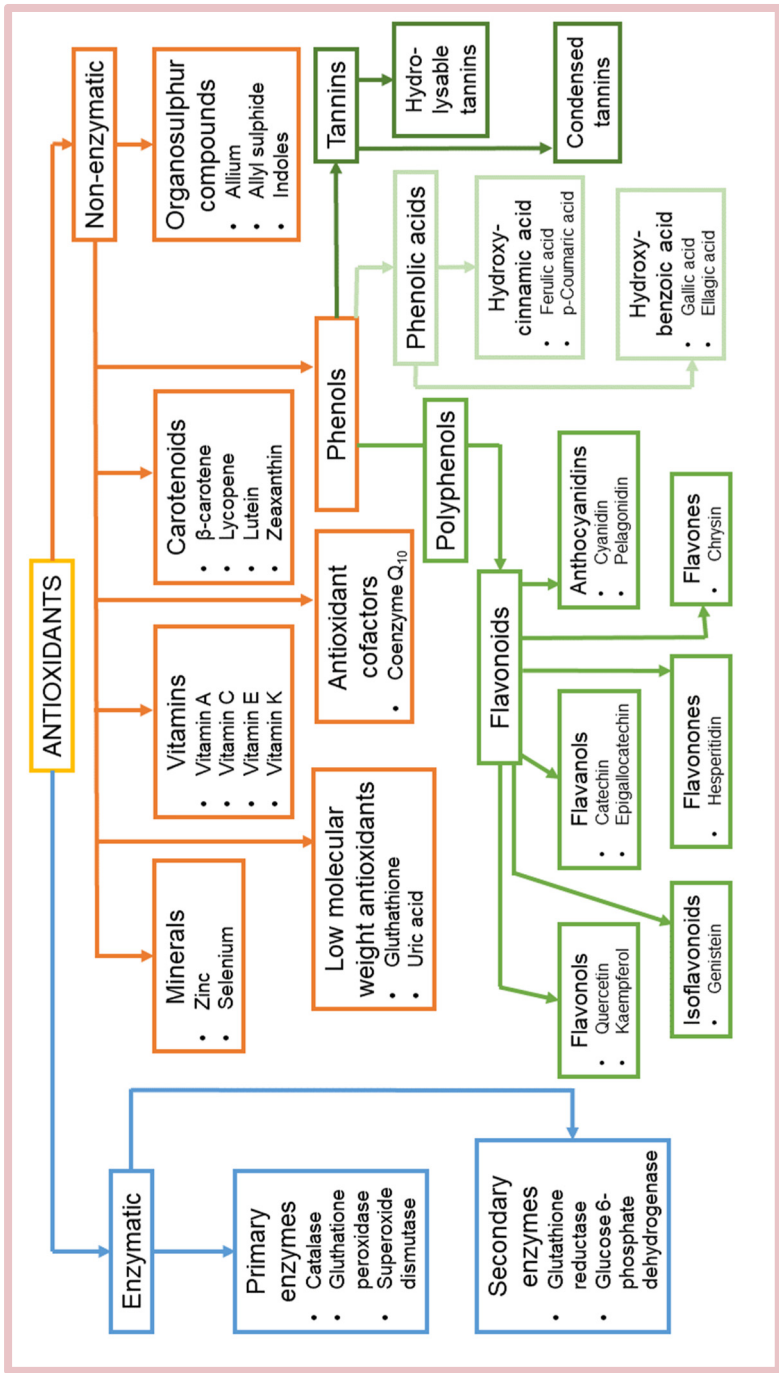


Figure 9.
Scheme of antioxidant classification modified from Bunaciu, Aboul-Enein [52].

2.10.1 Plant phenols and extraction methods

Numerous antioxidants may be extracted from different plant parts including: leaves, roots, stems, barks, seeds, flowers, fruits, aerial parts and rhizomes, of which leaves are the most common source to extract from [55]. Moreover, essential oils, used across centuries for their medicinal properties, may be extracted from aromatic plants, of which oils are synthesised by all of the above mentioned plant organs [56]. Plant materials are typically prepared before extraction of antioxidants by drying, grinding/milling, and homogenisation [53]. Common extraction methods include, maceration, decoction, distillation, pressurised liquid extraction, supercritical fluid extraction, microwave liquid extraction, and ultrasound assisted extraction [55]. Pressurized liquid extraction (PLE) also known as accelerated solvent extraction (ASE), provides high-pressure and -temperature extraction of antioxidants in a faster way using lower amounts of solvents than normal extractions [53]. The use of new emerging extraction techniques are however increasing, such as Pulsed Electric Field (PEF) in which neither high temperatures nor organic solvents are needed [57]. The most common ways of extracting essential oils include high or low pressure distillation, microwave extraction or using liquid carbon dioxide [56]. Solvents mixed with different proportions of water are typically used to facilitate and increase efficacy of extractions such as ethanol, methanol, and ethyl acetate [53] of which ethanol is a safe extraction solvent for human consumption. Essential oils are lipophilic and soluble in organic solvents [56]. Some plant crude extracts contain significant amounts of unwanted lipoid materials and/or carbohydrates why purification and/or fractionation might be necessary. Based on the extracts' polarity and acidity, liquid-liquid partitioning, sequential extraction and/or solid phase extraction (SPE) might be used [53]. Once antioxidants are extracted, their properties are usually studied in terms of phenol content and identification of phenol profile, as well as identifying their mode of action(s).

2.10.2 Antioxidant mechanisms

Antioxidants are molecules that can interfere with the oxidation process already in the initiation step but also in the propagation step (Figure 4) either as oxygen scavengers, by reacting with free radicals, or by chelating catalytic metals [5, 54]. They can be divided into two groups in terms of mechanisms of action:

1) Primary antioxidants; reacting instantly as chain breakers and free radical acceptors, interfering with lipid radicals by donating a hydrogen atom, thereby stabilizing them.

2) Secondary antioxidants; reacting by either inhibiting enzymes, binding metal ions able to catalyse oxidative processes, absorbing UV-light, scavenging oxygen or decomposing enzymes [5].

Phenolic acids have the adequate chemical structure for being radical scavengers due to the phenolic hydroxyl groups. Important analytical methods include FRAP (Ferric Reducing Ability of Plasma), ABTS ([2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)]), DPPH (2,2-diphenyl-1-picrylhydrazyl), ORAC (Oxygen Radical Absorbance Capacity), and total phenols content using e.g. the Folin-Ciocalteu (FC) reagent. Both processes of oxidation and reduction thereof using a range of different antioxidants is naturally occurring in our cells as a part of regulation of body homeostasis in immune function and nutrient metabolism [6]. In a living animal, these processes function as required, whereas at slaughter, many factors lose their antioxidant potential, due to changed circumstances such as anaerobic conditions and presence of pro-oxidants [5].

2.10.3 The addition of plant phenols to meat

Antioxidants are typically added to meat and meat products for preservation purposes, increasing shelf-life and for enhancement of nutritional value [6]. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) are commonly used in the food industry for oxidation-inhibiting purposes, but are decreasing in use due to their suspected genotoxicity [5, 6]. This has now resulted in an increased demand of plant-derived natural antioxidants for prevention of oxidation in different meat products. The majority of these antioxidants consist of phenolic compounds of which flavonoids, tocopherols and phenolic acids constitute an important part. Some of these phenolic compounds may act as both primary and secondary antioxidants. Several of these natural antioxidants have shown to have stronger antioxidant properties than their synthetic equivalents, however, negative effects such as process property, flavour and colour alterations have been noticed in some cases [5]. Moreover, antioxidants may act as pro-oxidants depending on their mode of action, concentration and the presence of transitional ions (such as heme iron) [58, 59], as well as the individual chemical structure of each phenolic compound present in the antioxidant [60]. It is therefore of great importance to properly identify the active ingredients of the antioxidants, their respective mode of actions, as well as the matrix they should be applied into, prior to studying their effectiveness. Meat is a very complex food matrix which undergoes a large amount of processes prior to being processed in our bodies. The main research question of the project, in which this doctoral work has been performed, is therefore to study complex antioxidants incorporated into intricate meat matrices with the hypothesis to make meat products healthier to consume.

3 Aim and objectives

The overall aim of this doctoral thesis was to study a variety of natural antioxidants and their capacity to inhibit lipid oxidation, as well as evaluate their potential to make a processed meat product healthier and safer.

This overall aim was divided into the following specific objectives:

- Screen and evaluate local horticultural plant materials for their antioxidant capacities and phenolic contents.
- Test the most promising antioxidants in different concentrations over time in a relevant meat model system.
- Evaluate which combination of a number of given parameters in a processed meat product that gives rise to most oxidation.
- Incorporate the best performing antioxidants to the most oxidised meat product for evaluation of capacity to inhibit lipid oxidation.
- Conduct an *in vivo* trial where mice were fed these meat products with and without antioxidants to evaluate potential health effects.

4 Methodology

4.1 Screening of plant materials and extracts (paper I)

Leaves from 15 cultivars of sea buckthorn (SBT) (*Hippophae rhamnoides*), black currant (BC) (*Ribes nigrum*), and red currant (RC) (*Ribes rubrum*) respectively, were collected at the Department of plant breeding, Balsgård, Swedish University of Agricultural Sciences, Sweden. Moreover, 3 cultivars of carrot (C) (*Daucus carota*) leaves, beetroot (BR) (*Beta vulgaris*) leaves and yellow onion (*Allium cepa*) peels (OP) and outer layer skins (OS) were collected from Nyskördade morötter AB, Fjälkinge, Alléns organic farm, Vittskövle and Åhus grönt AB, Horna gård, Åhus, Sweden, respectively. The plant materials were washed and dried in a ventilated convection oven at 30 °C for three days. A commercially available olive polyphenol powder (OPP) (Phenoliv AB) was used as a reference throughout all analyses.

After drying, the plant materials were ground into powders. A pre-trial was carried out for evaluation of the most advantageous particle size of powders to extract the most antioxidants. One cultivar of SBT, BC and RC respectively were ground both finely and coarsely before they were extracted with 50 % ethanol containing 0.05 M ortho-phosphoric acid, before their phenol contents were analysed with the Folin-Ciocalteu (FC) reagent using the protocol of Morgenstern, Ekholm [61] based on the work of Singleton, Orthoffer [62]. We chose to use the finely ground particles since they were found to yield most phenol content from the samples. Next, the antioxidant exchange of multiple extractions was studied to see how many extractions would be necessary to obtain the largest amounts of antioxidants possible per sample. Extractions were carried out using the solvent mentioned above, three times on the same sample. The first extraction yielded between 74 and 85 % of the total extracted phenols from three extractions which was regarded as a sufficient amount on which to perform following analyses.

4.1.1 Extraction of antioxidants

As previously mentioned, 50 % ethanol containing 0.05 ortho-phosphoric acid was used for extraction of all Swedish pant materials, of which 1.5 mL was added to 50 mg finely ground powder. Ethanol, 50 %, was chosen to extract as many phenols, both

hydrophilic and hydrophobic, as possible. Ortho-phosphoric acid was included in the solvent to increase the proton pressure in the extraction and hence give a higher exchange. Samples were then vortexed and incubated at room temperature (25 °C) in an ultrasonic bath for 15 minutes before they were centrifuged for 10 minutes at 16 g. Lastly, the supernatants of the samples were transferred to new tubes. Extraction methods for antioxidants from the other collaborating countries are presented in Paper II.

4.1.2 Folin-Ciocalteu

The Folin-Ciocalteu (FC) reagent is a mixture of colourless phosphotungstate and yellow phosphomolybdate in their fully oxidised states. When electrons are added, i.e. antioxidants, the reduction of molybdates result in "isostructural" molybdates of blue colour. The absorbance is measured at 760 nm and calculations are based on gallic acid total phenols for comparison. The total phenols content was analysed using the FC assay using the protocols mentioned above where results were calculated as mg gallic acid equivalents (GAE) per g dry weight (DW). All plant samples were analysed in duplicates and were re-analysed if the coefficient of variation between technical replicates exceeded 10 %. The six cultivars of SBT containing the most phenols, and the five cultivars from BC and RC respectively containing the most phenols, as well as all of the cultivars of C, BR, OP, OS, and OPP were further analysed for their antioxidant capacity using FRAP and ABTS.

4.1.3 ABTS

The ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] assay is a radical scavenging decolourisation assay used for both hydrophobic and hydrophilic antioxidants. The ABTS is oxidised with potassium persulfate to form the ABTS radical cation $ABTS^{\bullet+}$ which has a strong dark blue/green colour. The addition of antioxidants to this assay decolourises it when cations re-stabilise, and the absorbance is then measured at 734 nm from which an inhibition percentage is calculated. The radical scavenging capacity of all previously mentioned plant materials was measured using ABTS following the protocol of Re, Pellegrini [63]. The results from our plant materials were reported as mmol Trolox (E-vitamin analogue) equivalent antioxidant capacity (TEAC) per 100 grams DW.

4.1.4 FRAP

The ferric reducing ability of plasma (FRAP) assay measures the capacity of an antioxidant to reduce ferric (Fe^{3+}) ions into ferrous (Fe^{2+}) ions. This reaction takes place at a low pH, allowing the ferrous ion to form the blue ferrous-tripyridyltriazine

complex, of which the absorbance at 593 nm is measured and compared to a standard such as ascorbic acid. Results were reported as mmol Fe^{2+}/100 grams DW. FRAP was also measured on the previously mentioned set of samples, following the protocol of Morgenstern, Ekholm [61] based on the work of Benzie and Strain [64]. A plate reader (Tecan Sunrise) was used for meticulous measurement of colour change of samples over time.

4.1.5 HPLC-MS

When analyses of antioxidant contents and capacities were finalised, the next step was to identify the phenols in the samples using high performance liquid chromatography (HPLC) mass-spectrometry (MS) using a Sciex API 150EX Turbo Ionspray mass spectrometer with a Perkin-Elmer 200 auto-sampler with different separating columns for different sample types. All samples were analysed with methods developed for each species and the content was quantified using external standards (Paper I). Standards were gathered based on literature studies on the existing research of the different species and molar masses were studied accordingly. The amount of individual phenols were quantified as µg/gram DW.

4.1.6 Antioxidants in studies

The best performing antioxidants from each study moved on to the next, until there were only five remaining for the animal trial (Figure 10).

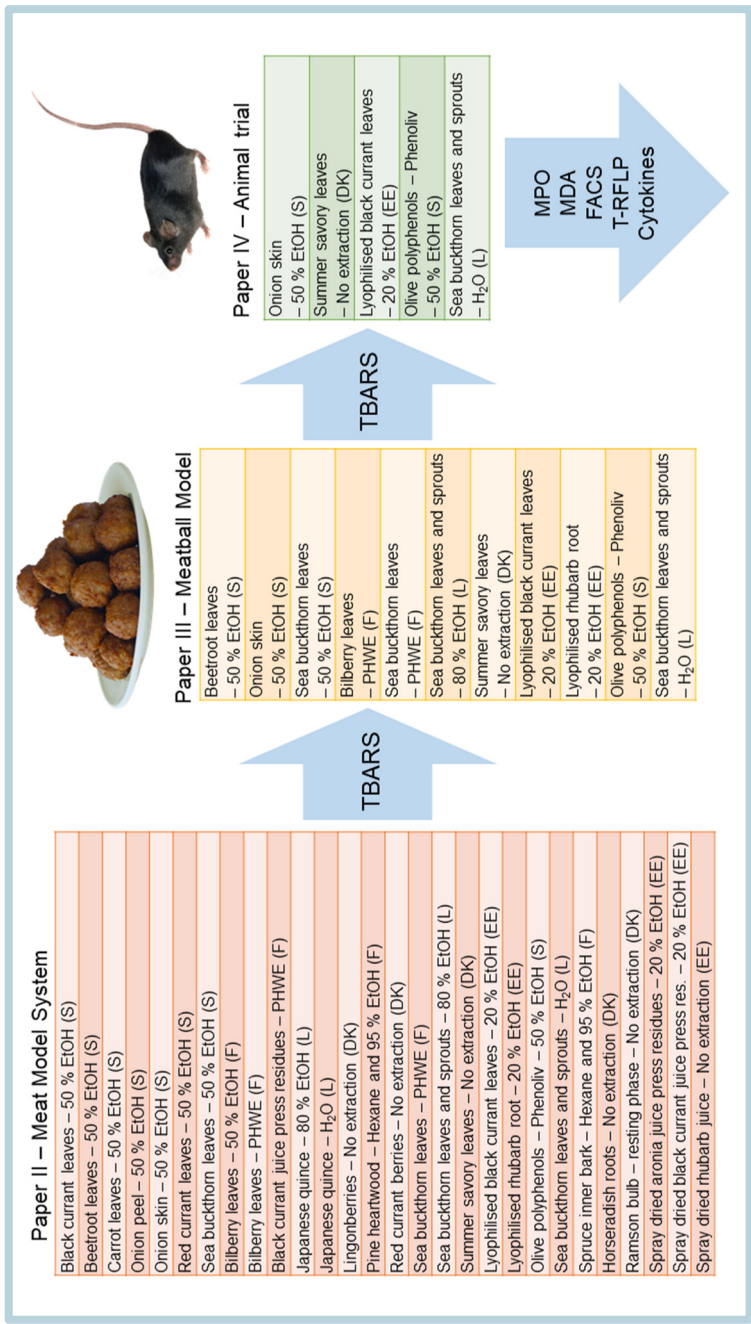


Figure 10.
Flowchart of samples from paper II – IV where TBARS was used as the determining analysis in model systems for further studies, and the analyses from the animal trial were carried out on animal samples. The letters in brackets signify the country of origin of the sample, where S = Sweden, F = Finland, L = Latvia, DK = Denmark, and EE = Estonia.

4.2 The meat model system (paper II)

The phenols with the highest antioxidant capacities from Sweden and the equivalent samples from the other collaborating countries were all to be tested for their capacity to inhibit lipid oxidation in meat products. Therefore, the meat model system was developed, due to the known complexity of a meat product matrix in terms of content standardisation. Hence, we extracted the proteins of value for studying lipid oxidation, namely the heme-containing sarcoplasmic proteins situated in the water phase of the muscular tissue. These proteins were homogenised with an easily oxidised oil, i.e. linoleic acid, resulting in a standardised meat model for testing of antioxidant lipid oxidation inhibitory capacity.

4.2.1 Emulsions

The model system consisted of 80 % sarcoplasmic proteins (protein content ~ 0.30 %, FlashEA 1112 N/Protein analyzer (Thermo Fisher Scientific)) extracted from pork knuckle (*Musculus gastrocnemius*) homogenised using a disperser with 13 mm extension arm diameter at 24'000 rpm for 30 s (Ultra-Turrax® T25, IKA) to an emulsion with 20 % linoleic acid (Figure 11). Phenol samples were then added in triplicates in three different concentrations; 50, 100 and 200 ppm concentration in relation to the total weight of the emulsion, into one emulsion each. One triplicate of emulsions with no added phenols was kept as the blank sample, i.e. to know how much the meat model oxidises as such. All emulsions (without and with antioxidants in different concentrations) were heated until the inner temperature reached 72°C, as would an industrial processed meat product. Then, after cooling, the emulsions were stored in a refrigerator at 4°C for one day, one week and two weeks, before lipid oxidation was measured using thiobarbituric reactive substances (TBARS) and the inhibitory capacity of the phenols were calculated using the blank samples.

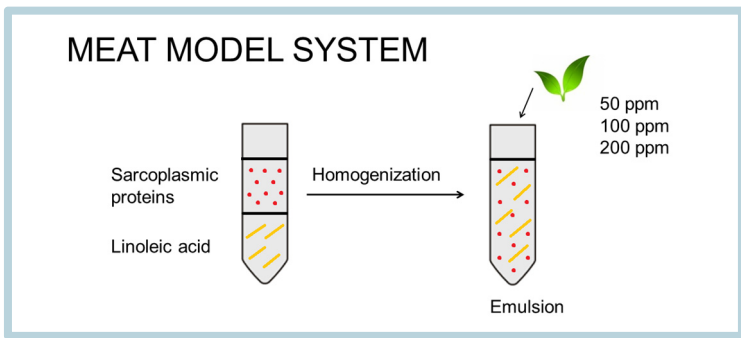


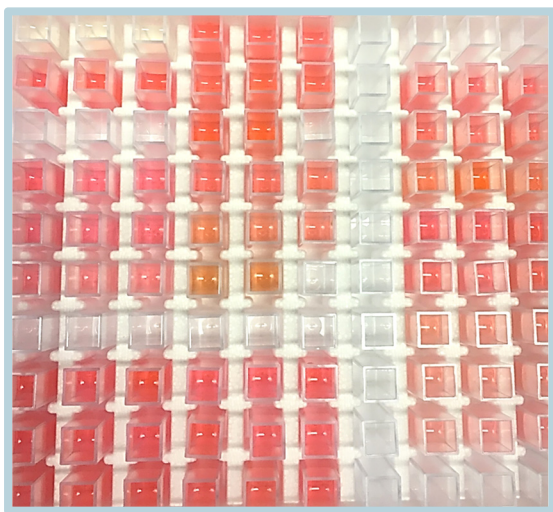
Figure 11.
Schematic picture of the meat model system where sarcoplasmic proteins were homogenised with linoleic acid together with given antioxidants of differing concentrations.

4.2.2 TBARS in meat model system

Malondialdehyde (MDA) is a secondary lipid oxidation product created upon the breakdown of PUFA and is typically measured when studying lipid oxidation in foods. MDA binds to two equivalents of thiobarbituric acid (TBA) resulting in a pink/magenta chromogen which is measured at an absorbance of approximately 534 nm. All emulsions were tested using TBARS based on the protocol of Buege and Aust [65]. The assay was performed on all emulsions after one day, one week and two weeks of storage, to study the development of lipid oxidation, and the inhibition thereof, over time. The results were given in µM MDA per gram emulsion, but was later transposed into a percentage lipid oxidation compared to the blank sample with no antioxidants.

Modification of original TBARS protocol

The protocol of Buege and Aust [65] was modified slightly, due to that the end product of the emulsions in our study yielded products of yellow/orange colouration, rather than pink/magenta. A calibration curve of the TBA working solution was made, lowering the amount TBA in every step, which gradually increased the yellow colouration. Thus, we argued that too little TBA was available for binding to the MDA adequately, creating different TBA-aldehyde complexes of different colours. Hence, we instead doubled the concentration of the TBA compared to the original protocol, which resulted in emulsion products of more pink/magenta colouration as desired. In this way, we were convinced that measurements were carried out on the correct type of molecule (Picture 1).



Picture 1.
Example of TBARS experimental setup on the meat model system where a somewhat orange colouration can still be noticed in some samples, but the majority of samples are of pink colouration.

4.3 The most oxidised meatball (paper III)

The next step of the project was to test our antioxidants in real meat products. But in order to do that, a representable, commonly consumed, meat product needed to be found. The Swedish meatball was chosen as the target meat product. However, now new questions arose regarding which type of meatball would be subjected to these antioxidants. Typically, Swedish meatballs are made of beef and/or pork, have fat contents ranging between approximately 10 to 20 %, have salt contents ranging between approximately 2 to 4 %, and they are either industrially deep-fried or pan-fried at home. Moreover, storage times differ, so naturally, there were some different types of meatballs to choose from. Hence, all of these combinations of parameters were tested, except for the mixture between beef and pork. The two extreme values of both salt and fat contents were chosen to better distinguish parameters between groups from each other. A schematic picture of the experimental setup can be seen in Figure 12 where the combinations of parameters for the pan-fried meatballs can be seen. A similar figure can be imagined for the deep-fried set of samples. Each meatball type was prepared in triplicates resulting in 144 meatball samples in total, counting both deep fried and pan fried samples.

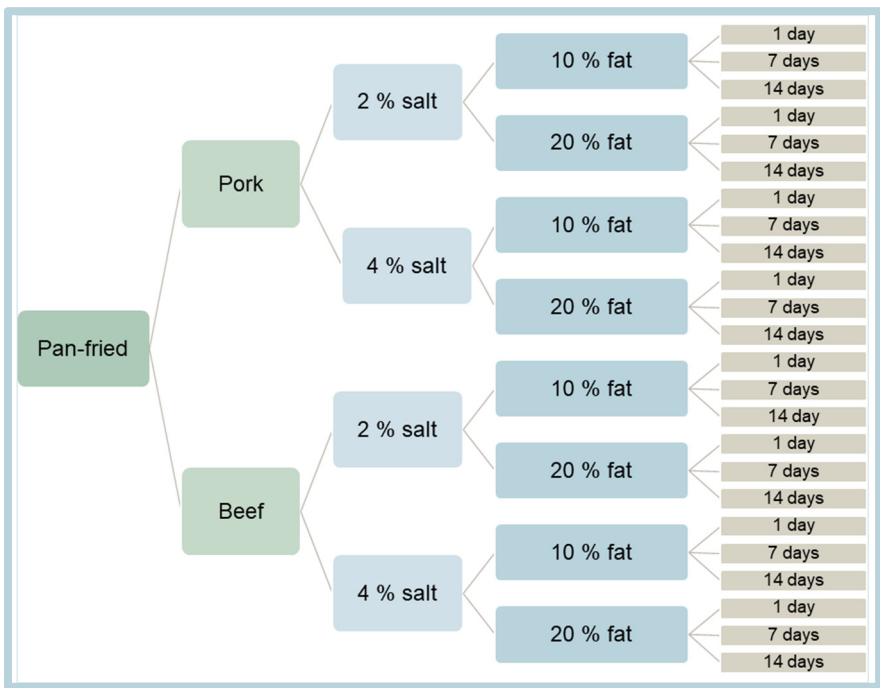


Figure 12.
Schematic picture of the experimental setup of meatball preparations with different combinations of parameters modified from Granheimer [66].

4.3.1 TBARS in meatballs

After one day, one week and two weeks respectively, TBARS were measured using the protocol of Buege and Aust [65], only now the sample preparation differed slightly. Meatball pieces of approximately 6 grams each were crushed using a mortar and a pestle before they mere mixed into a slurry with distilled water. Next, trichloroacetic acid (TCA) was added to precipitate proteins, before 96 % ethanol was added to the mixture to solubilise fats. The mix was then filtered using Munktell filter papers grade 1F in order to obtain clear filtrates which were then used for the TBARS assay. The absorbance was again measured at 534 nm and the results were reported as µM MDA per gram meatball. The type of meatball that oxidised most was then to be used as the model meat product for further studies to incorporate antioxidants.

4.4 The most oxidised meatball with plant phenols (paper III)

The most oxidised type of meatball showed to be deep-fried, made of pork, containing 2 % salt, 20 % fat and had been stored for 2 weeks. This combination of parameters was then used in the trials to follow.

4.4.1 The production of meatballs with plant phenols

Eleven different types of plant phenols were added to the meatball type found to oxidise the most (Table 3). The phenols were added in both 100 and 200 ppm concentration respectively in relation to the total weight of the meatball end product. Hence, 90 g of minced meat was mixed by hand with 9 mL plant phenol water solution until all the water had been absorbed into the mince. The meatballs were then hand rolled and weighed before they were deep-fried. They were then let to rest until they cooled down prior to being refrigerated for 14 days after which the lipid oxidation was measured using the TBARS assay. Meatballs with no added phenols were also manufactured where only water was added to the mince instead. These samples were then used as sample blanks for evaluation of lipid oxidation inhibition.

Table 3.
Plant samples with latin names, countries of origin, extraction methods and total phenols content in gallic acid equivalents (mg GAE mL^{-1}) incorporated into the model meatball for trial of inhibition of lipid oxidation.

Plant samples	Latin name	Country of origin	Extraction method	Total phenols (mg GAE mL^{-1} extract)
Sea buckthorn leaves	*Hippophae rhamnoides* L.	Finland	Pressurised hot water	7.0
Bilberry leaves	*Vaccinium myrtillus* L.	Finland	Pressurised hot water	11.6
Sea buckthorn leaves and sprouts	*Hippophae rhamnoides* L.	Latvia	80 % ethanol	13.2
Sea buckthorn leaves and sprouts	*Hippophae rhamnoides* L.	Latvia	Water	9.2
Summer savory leaves	*Satureja hortensis* L.	Denmark	Non-extracted	12.0
Sea buckthorn leaves	*Hippophae rhamnoides* L	Sweden	50 % ethanol	8.8
Olive polyphenols	*Olea europaea* L.	Sweden	50 % ethanol	3.8
Onion skin	*Allium cepa* L.	Sweden	50 % ethanol	3.0
Beetroot leaves	*Beta vulgaris* subsp. *vulgaris*	Sweden	50 % ethanol	1.0
Lyophilised rhubarb root	*Rheum rhabarbarum* L.	Estonia	20 % ethanol	18.1
Lyophilised black currantleaves	*Ribes nigrum* L.	Estonia	20 % ethanol	10.1

4.4.2 TBARS in meatballs with added antioxidants

The protocol was carried out as explained previously during the evaluation of parameters for lipid oxidation in meatballs. Results were reported as µM MDA per gram meatball, and later transposed as a percentage of the oxidation compared to the blank meatballs with no added antioxidants.

4.5 Animal trial (paper IV)

The last step of the project was to study possible health effects in mice consuming the previously found oxidised meatballs with the five most lipid oxidation inhibiting antioxidants. To do this, mice were fed a diet consisting of 20 % meatballs (the equivalent amount of a human average meat consumer) during approximately four months. The health effects of this meatball diet alone would not be clear enough to analyse during this trial period, why a low grade chronic inflammation was induced cyclically during the trial. After the trial, a number of parameters were studied to compare health effects between trial groups consuming the meatballs without and with antioxidants.

4.5.1 Meatball production

The meatball type used for the *in vivo* trial was the pork meatball that was deep-fried, contained 20 % fat and 2 % salt that had been stored for one week. Note that one week of storage was chosen instead of two weeks due to that one week more closely resembles real life storage conditions. The best performing phenols (Table 4) from the previous studies were added to this meatball type and were manufactured using a meatball machine at Atria AB, Malmö, Sweden. All meatballs were frozen (-18 °C) after production and thawed in a refrigerator one week before they were fed to the mice. A total of 1618 meatballs were produced for the trial including blank samples, i.e. meatballs with no added phenols for the meatball (MB) control group.

Table 4.
Plant samples with latin names, countries of origin, extraction methods and Gallic Acid Equivalents (mg GAE mL^{-1}) incorporated in meatballs for *in vivo* trial.

Plant samples	Latin name	Country of origin	Extraction method	Total phenols (mg GAE mL^{-1} extract)
Sea buckthorn leaves and sprouts	*Hippophae rhamnoides* L.	Latvia	Water	9.2
Summer savory leaves	*Satureja hortensis* L.	Denmark	Non-extracted	12.0
Olive polyphenols	*Olea europaea* L.	Sweden	50 % ethanol	3.8
Onion skin	*Allium cepa* L.	Sweden	50 % ethanol	3.0
Lyophilised black currant leaves	*Ribes nigrum* L.	Estonia	20 % ethanol	10.1

4.5.2 Animals

Eighty wild-type C57BL6/J mice (Janvier Labs, Le Genest-Saint-Isle, France) were randomly assigned to eight trial groups with ten animals per group. Each trial group was divided into two cages for ethical reasons. The mice were eight weeks old at arrival and weighed between 17 – 20 g. They were kept at 50 % humidity in a 12:12-h light-dark cycle and were allowed to acclimatise to these conditions for seven days prior to the start of the trial. The Ethics Committee for Animal Studies at Lund University approved the animal experiment (permit number 5.8.18-12636/2017).

4.5.3 *In vivo* trial

Dextran sulfate sodium (DSS)

In order to induce a chronic inflammation, dextran sulfate sodium (DSS) was administrated *ad libitum* in the drinking water in four cycles, with successively increased concentration in cycles (1st cycle 1.5 %, 2nd and 3rd cycle 2 % and 4th cycle

2.5 % (w/v)). Each cycle consisted of seven days DSS administration with ten days of regular water in between. The use of DSS for inducing a chronic inflammation in mouse models is common [67-69] but precautions regarding mouse strain and molecular weight of the DSS used should be taken. C57BL6/J mice are more sensitive to DSS than other mouse strains, and the molecular weight should be between 36 to 50 kD [67] why we chose one of 40 kD molecular weight (TdB Consultancy, Uppsala, Sweden). The mice were monitored daily and a disease activity index (DAI) [70] was measured during DSS-cycles to ensure the low grade inflammation did not increase to an acute one. The DAI included weight change, faecal consistency and a hemoccult testing for monitoring of presence of blood in stool. A faecal index (FI) was also calculated where the weight parameter was excluded and an average score of hemoccult (1/0), stool consistency (hard (score 0), semisoft (score 1), soft (score 3) and liquid (score 4)) and ocular presence of blood in stool (1/0) was evaluated.

Trial groups

The 80 mice were divided into eight trial groups, of which a negative control (NC group), a DSS control (DSS group) and a positive control with both DSS and meatball diet (MB group) were control groups, and five groups which all were administered DSS and were given a meatball diet containing one plant phenol each (Table 5). The NC group was fed standardised pelleted Lactamin R36 feed (Lantmännen, Sweden) and water.

Table 5.
Animal trial groups (n = 10) with group and antioxidant extract, abbreviation and diet description.

Group	Abbreviation	Treatment
Negative control	NC	Regular feed and water
Dextran sulfate sodium (DSS) control	DSS	Regular feed with cyclic DSS treatment
Positive control; DSS + meatball	MB	20 % meatball diet with cyclic DSS treatment
DSS + meatball with Olive polyphenols	OPP	20 % meatball diet + OPP with cyclic DSS treatment
DSS + meatball with Onion skin	OS	20 % meatball diet + OS with cyclic DSS treatment
DSS + meatball with Sea buckthorn	SBT	20 % meatball diet + SBT with cyclic DSS treatment
DSS + meatball with Summer savory	SS	20 % meatball diet + SS with cyclic DSS treatment
DSS + meatball with Black currant	BLC	20 % meatball diet + BLC with cyclic DSS treatment

All other trial groups were fed pulverised Lactamin R36 (Lantmännen, Sweden) mixed with water where mice with meatball diets were given a lower amount of feed replaced with 20 % meatball (w/w) and 28 % water (w/w) (Table 6). The feed was prepared daily by mixing the pulverised feed with water, as well as by mixing in the assigned meatball type for each trial group. The total amount of feed was prepared in excessive amounts compared to the number of animals per cage so they could be fed *ad libitum*.

The diet was regarded as balanced between trial groups in terms of protein and fat content (Table 6). The DSS water was also administered in excessive amounts and the consumption was monitored daily during DSS cycles where bottles were changed every second day. Water was given *ad libitum* between cycles.

Table 6.
Feed ingredient table for trial groups presented as g/100g (%). NFE = nitrogen-free extract. Antioxidants are presented as dry weight equivalents in the meatballs.

group	Lactamin R36 (%)	Water (%)	Meatball (%)	Protein (%)	Fat (%)	NFE (%)	Dietary fiber (%)	Antioxidants (%)
NC	Pelleted *ad lib.*	< 12	-	18	4	56	4	-
DSS	72	28	-	13	3	40	3	-
MB	52	28	20	12	5	29	2	-
OPP	52	28	20	12	5	29	2	0.53
SBT	52	28	20	12	5	29	2	0.01
SS	52	28	20	12	5	29	2	0.06
OS	52	28	20	12	5	29	2	0.66
BLC	52	28	20	12	5	29	2	0.02

4.5.4 End of trial

At the end of the trial, mice were anaesthetised by a subcutaneous injection of a mixture of Ketalar® and Domitor® (Apoteket, Sweden) before the mesenteric lymph nodes (MLN) were harvested and arterial blood was extracted. The mice were then sacrificed by a cardiac injection of a lethal dose of pentobarbital (Apoteket, Sweden). The spleen was removed and weighed, before the small intestine was removed and Peyer's patches (PP) were harvested. Small intestine biopsies and faecal contents were saved for further analyses. Next, the colon was removed where the length was measured before biopsies and faecal contents were saved for further analyses. The spleen weight was recorded and ocular evaluations were made on colonic lesions and dysplasias [71].

4.5.5 Myeloperoxidase activity in colon and small intestine

Myeloperoxidase (MPO) is an enzyme produced by neutrophils during immune system activation. MPO is released during degranulation with the final aim to act as a bactericidal [72]. In our study, MPO was a measure of inflammatory response in the mice which was studied using ELISA from both colon and small intestine biopsies. The assay used was conducted according to the protocol of Osman, Adawi [73] where the results were reported as MPO units per gram tissue.

4.5.6 Malondialdehyde presence in colon and small intestine

The presence of the lipid oxidation end product malondialdehyde (MDA) was studied in both small and large intestine using a standardised lipid peroxidation kit (MAK085, Sigma-Aldrich, Saint Louis, Missouri, USA). The assay was performed following the manufacturer's protocol and results were reported as nmole MDA per µL tissue extract.

4.5.7 T-RFLP

Terminal restriction fragment length polymorphism (T-RFLP) is a fast and sensitive molecular biology method used to assess profiles of microbial communities [74]. In this project, this was done by extracting DNA from colon and small intestine contents using an EZ1 DNA tissue kit (Quiagen, Aarhus, Denmark). Thereafter, amplification of the 16S rRNA gene was done using PCR with universal primers FAM-ENV1 (5´-AGA GTT TGA TII TGG CTC AG-3´), fluorescently labelled with FAM dye at the 5´end and ENV2 (5´-CGG ITA CCT TGT TAC GAC TT-3´), in order to obtain a wide range of labelled 16S rRNA genes. These PCR products were then purified and digested with a 4-base cutter restriction endonuclease MspI, obtaining Terminal restriction fragments (T-RFs), now fluorescently labelled. Together with a DNA size standard, also fluorescently labelled, these T-RFs were then separated by gel electrophoresis at the DNA lab in Malmö (SUS, Malmö) equipped with a laser detector in order to visualize the different T-RFs. The electrophoresis allows for accurate sizing of T-RFs since it is sensitive within ± 1 nucleotide [74]. Since the base pair length of a T-RF corresponds to one genetic variant, this enables a visualisation, typically depicted in an electropherogram, where each peak represents a different variant. From this, a diversity index may be calculated, which could be compared to a finger print of the intestinal microflora of the mouse. Additionally, a table with the size of the T-RFs in base pairs and the area and peak height of each peak is provided from the results.

4.5.8 Fluorescence-activated cell-sorting (FACS)

In order to identify the immunological response of the different trial groups, leucocytes from MLN and PP were studied. The lymphoid tissues were collected and added to Hank's balanced salt solution (HBSS) before they were digested using an enzyme mix of Collagenase P, Dispase II and DNAse I from bovine pancreas (Sigma-Aldrich, Saint Louis, Missouri, USA). Samples were then added to foetal bovine serum (FBS) before they were centrifuged at 1400 rpm at 4°C for 5 minutes. Pellets were re-suspended in HBSS containing 10 % FBS. The suspension was filtered through 40 µM filters and re-suspended in HBSS. The cells were then incubated at 4°C for 15 minutes in the dark with four labels (FITC, APC, PE and PerCP Cy5.5) in three antibody panels;

Panel 1, macrophages and dendritic cells) F4/80, CD11c, TLR-2, and TLR-4

Panel 2, T-helper cells and Cytotoxic T-cells) CD4, CD8, CCR9, and CD69

Panel 3, activated regulatory T-cells) CD4, CD25, CD69, and FoxP3.

Cells were then washed and re-suspended in FACS buffer. Cells of panel 3 were treated with fixation and permeabilisation buffer before being incubated with FoxP3 in 4°C for 45 minutes in the dark. The cells were then washed and re-suspended in FACS buffer before all samples were run using Cytoflex (Beckman Coulter, Mountain view, CA, USA). Unstained cells and fluorescence minus one (FMO) were used as negative controls. Analysis was done using CytExpert software (Beckman Coulter Inc. version 2.0.0.153) with assessment of 50 000 events per sample.

4.5.9 Multiplex serum cytokine/chemokine profiling

Cytokines, of which chemokines are a family of small chemotactic cytokines, are peptides produced by a number of cells including immunological cells. They play a role in regulating the response from the immune system. Blood samples from our trial groups were allowed to clot for 2 h in room temperature (25 °C) before they were centrifuged at 2000 g for 10 minutes. The serum was then collected and stored at -80 °C until analysed. The expression of serum cytokines and chemokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, TNF-α) was determined using V-PLEX Pro-inflammatory Panel 1 kit and the Leptin expression was measured using a Leptin kit (Meso Scale Diagnostics (MSD), Rockville, MD, USA) following the manufacturers' protocols, using an MSD Sector S 600 plate reader. Results are given in pg/mL serum.

5 Statistical methods

For the entire thesis work, technical replicates in either triplicates or duplicates have been analysed. If the Coefficient of Variation (CV %) between these replicates exceeded 10 %, the analysis of that sample was re-run.

Paper I) Analysis of variance (ANOVA) was used to find differences between species and cultivars in regard to their antioxidant activity and contents. Tukey's post-hoc test was used to identify specific samples of interest that differed. A Pearson correlation analysis was conducted in order to identify possible correlations between antioxidant capacity and content among cultivars and species. Moreover, a principal component analysis (PCA) was made for identification of the phenols responsible for the antioxidant capacities. All statistical analyses for paper I were conducted using IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, N.Y., USA).

Paper II) A repeated measures general linear model (GLM) was conducted on data sets one to three and on data set four respectively. Logarithmic values were used to achieve normal distribution of samples. The Tukey post-hoc test was used for finding differences among samples. Estimated marginal means (EMM) were extracted for visualisation of overall effects of parameters. A Pearson correlation was made using the data from paper I to see if antioxidant capacity data correlated with inhibition of oxidation in the meat model system. All statistical analyses for paper II were conducted using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, N.Y., USA).

Paper III) A GLM was conducted on BoxCox transformed values for normal distribution purposes and post-hoc tests were conducted using the Scheffe method. Analyses were performed on both SPSS Statistics 25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp) and R for Windows GUI front-end version 3.5.3 (R version 3.5.2 (2018-12-20) -- "Eggshell Igloo" Copyright © 2018 The R Foundation for Statistical Computing Platform: x86_64-w64-mingw32/x64 (64-bit).

Paper IV) Univariate general linear model (UGLM) analyses were performed on normally distributed data, i.e. Shannon diversity index, MPO, MDA, faecal index, amount DSS consumed per group, colon length, and cytokines/chemokines (where KC/GRO and IL-6 were logarithmically transformed to ensure normality) together with Tukey post-hoc tests to identify the differing group(s). Kruskal-Wallis non-

parametric tests were performed on medians when normality was not achieved i.e in FACS analyses, T-RFLP peak analyses, spleen weight, colonic dysplastic zones, and colonic lesions together with Bonferroni-adjusted pairwise comparisons. A PCA was executed on FACS, MPO, and T-RFLP diversity index and peak area data to analyse if parameters correlated. All statistical analyses for paper IV were conducted using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp).

6 Main results and discussion

6.1 Screening of plant materials and extracts (Paper I)

6.1.1 Initial screening of plant material cultivars for total phenols content

Fifteen cultivars of sea buckthorn (SBT), black currant (BC) and red currant (RC) leaves respectively, as well as the olive polyphenol wastewater powder (OPP), were screened for their total phenol content using the FC reagent. Results showed there were large differences between species and cultivars and that SBT overall had the highest total phenols content (Figure 13) (Paper I).

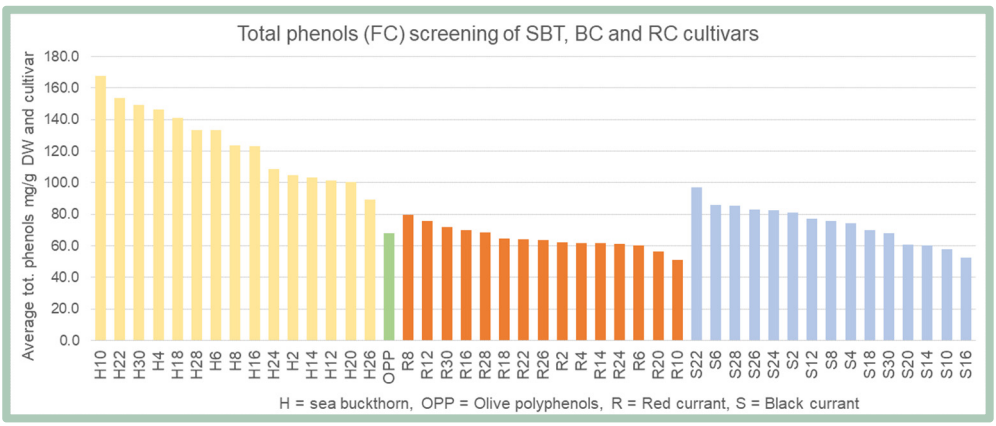


Figure 13.
Total phenols content in leaves (mg/g DW) of various sea buckthorn, black currant and red currant cultivars, as well as in olive polyphenol powder (OPP, shown in green). DW = Dry weight.

6.1.2 Screening of antioxidant capacity and total phenols content

The six best performing samples from SBT leaves, and the five best performing samples (with the highest FC values) from BC and RC leaves respectively, as well as all samples from carrot (C) and beetroot (BR) leaves, onion peel (OP), onion skin (OS) and olive polyphenols (OPP) were evaluated for their total phenols content using FC. FC results were reported as mg GAE per mg dry weight (DW) of the plant materials. The

antioxidant capacity was measured using FRAP, and ABTS where results were reported as mmol Fe^{2+} per 100 gram DW and mmol Trolox antioxidant equivalent capacity (TEAC) per 100 gram DW respectively. SBT cultivars showed to have the highest average phenol contents and the highest average antioxidant capacities, whereas the BR leaves showed to have the lowest antioxidant capacities (Figure 14). There was a strong correlation (Pearson) between the three antioxidant capacity analyses FC-FRAP 0.956, FC-ABTS 0.974, FRAP-ABTS 0.994 ($p < 0.001$) which was expected since it is shown in Figure 14 that values between the three antioxidant capacities correspond to each other in the different species. For instance, SBT had overall high values in all three capacities whereas BR had overall lower values. The interesting sample is OPP where the three capacities differ. FRAP is considerably higher than in ABTS. This implies that the OPP sample is a strong ferric reducer, rather than a radical scavenger. In order to analyse which phenolic compounds were responsible for which antioxidant capacity, all cultivars from each species was analysed using HPLC-MS. Thereafter, a principal component analysis was carried out on antioxidant capacity data together with data on phenol compounds.

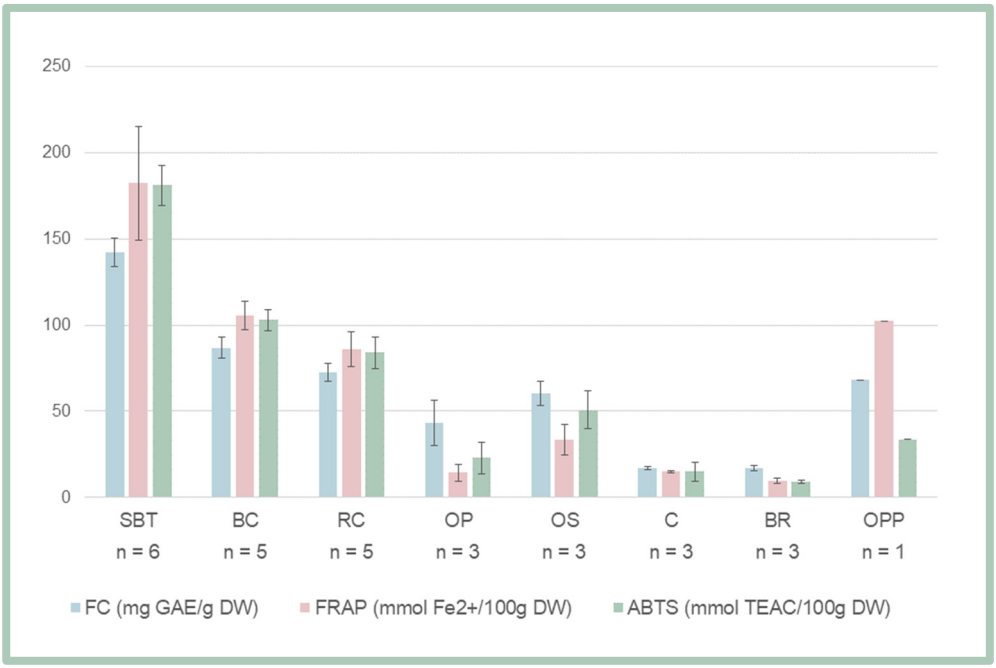


Figure 14.
Average total phenols content (FC), ferric reducing ability of plasma (FRAP) and radical scavenging capacity (ABTS) of cultivars where SBT = sea buckthorn, BC = black currant, RC = red currant, OP = onion peel, OS = onion skin, C = carrot, BR = beetroot, OPP = olive polyphenols, and DW = dry weight. The error bars show the standard deviation between cultivars (number of cultivars shown under cultivar abbreviation).

6.1.3 HPLC-MS analysis of phenolic compounds

Samples from each species was analysed using a specific chromatography method (Paper I). A principal component analysis (PCA) was carried out in order to study which phenolic compounds had the highest impact on antioxidant content and capacity. A total of 15 phenolic compounds were identified in the SBT samples (Figure 15) of which hydrolysable tannins Ia, Ib, and III were the most prevalent (Table 7). Tannins Ia and Ib were closely associated to FC and ABTS whereas tannin II was associated to FRAP. This could be of importance in further research when phenolic samples are to be chosen for specific purposes. In the BC samples, 19 phenolic compounds could be identified (Figure 16) of which quercetin-3-*O*-glucoside and quercetin-3-*O*-malonyl-glucoside A and B were the most prevalent (Table 8). Quercetin-3-*O*-glucoside and kaempferol-3-O-gluoside B were closely associated to ABTS, whilst kaempferol-3-*O*-rutinoside, chlorogenic acid and rutin where slightly associated to FC. In the RC samples 21 phenolic compounds were identified (Figure 17) of which rutin and quercetin-3-*O*-malonyl-glucoside were the most prevalent (Table 9). Isorhamnetin-3-*O*-glucoside A, isorhamnetin-3-*O*-rutinoside and quercetin were associated with FC, FRAP, and ABTS. This would imply that the choice of usage for this type of phenolic samples plays a minor role since all antioxidant capacities are related to the same phenolic compounds. In the onion peel and skin samples 12 and 11 compounds were identified respectively. A chromatogram of an onion peel sample represents both types of samples and is shown in Figure 18. The most prevalent compound in both onion peel and skin was quercetion-4-*O*-glucoside (Table 10), however differences were found in levels of quercetin which was considerably higher in the skin, which is in accordance to what Mizuno, Tsuchida [75] previously found, as well as in ferulic acid, found in the peel only (Paper I). Kaempferol and vanillic acid were associated with FC, FRAP, and ABTS. However, quercetin-4-*O*-glucoside, found to be the most prevalent compound, was not associated with any of the antioxidant variables. This reinforces the theory that the chemical structure of the antioxidant is more important than the amount. Seven compounds were identified in the carrot leaf samples (Figure 19) where chlorogenic acid and kaempferol-malonyl-glucoside were the most prevalent phenols (Table 11). Kaempferol-malonyl-glucoside and quercetin-3-*O*-malonyl glucoside A were closely related to FRAP, whereas rutin, cynarin, neo-chlorogenic acid and caffeic acid were found to be closely related to ABTS. As in the case of SBT, this could be important knowledge in choosing the right phenol extract for a certain purpose. In the beetroot samples, 5 main compounds were found (Figure 20) where xylosylvitexin was the most prevalent (Table 12). Xylosylvitexin was found to be closely associated to ABTS whereas rutin was associated with FC. The olive polyphenol powder is a wastewater-extracted powder of which hydroxytyrosol is the main hydrophilic phenolic compound which was in accordance to what we found in our sample. Hydroxytyrosol was more associated with FRAP than with ABTS, which can also be seen in Figure 14 where the FRAP value is more than twice as high as the ABTS value.

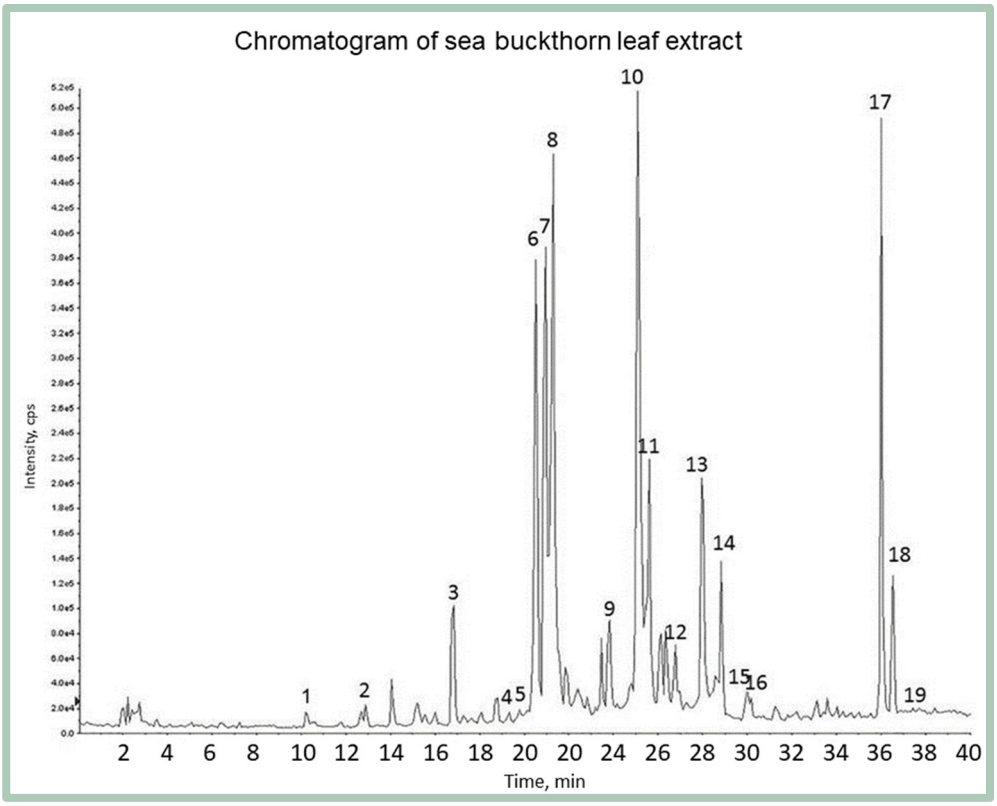


Figure 15.
Representative sea buckthorn (SBT) chromatogram where peak 1) kaempferol-3-*O*-glucoside, 2) gallic acid, 3) unknown peak 783.1, 4) procyanidin monomer glucoside, 5) epigallocatechin, 6) SBT tannin II, 7) SBT tannin Ia, 8) SBT tannin Ib, 9) unknown peak 609.5, 10) SBT tannin III, 11) unknown peak 623.2, 12) rutin, 13) quercetin-3-*O*-galactoside, 14) isorhamnetin-3-*O*-rutinoside, 15) kaempferol, 16) isorhamnetin-3-*O*-glucoside, 19) quercetin. Peaks 17 and 18 were unknown peaks in this particular chromatogram.

Table 7.
The five major phenolic compounds in sea buckthorn (SBT) leaves, average of six cultivars (biological replicate n = 1, technical replicate n = 3). CV = coefficient of variation, T_R = retention time.

Phenolic compound	*m/z* $[M - H]^-$	Content (µg/g DW)	CV %	Detection limit (µg/g DW)	t_R (min)	Peak number
SBT tannin Ia	935.5	4874.2	9.9	31.6	21.0	6
SBT tannin II	953.5	5368.1	5.6	27.1	20.9	7
SBT tannin Ib	935.5	6118.0	8.9	31.6	21.6	8
SBT tannin III	935.5	6794.4	6.5	31.6	25.4	10
Isorhamnetin-3-*O*-rutinoside	623.5	1885.2	8.7	27.8	28.9	14

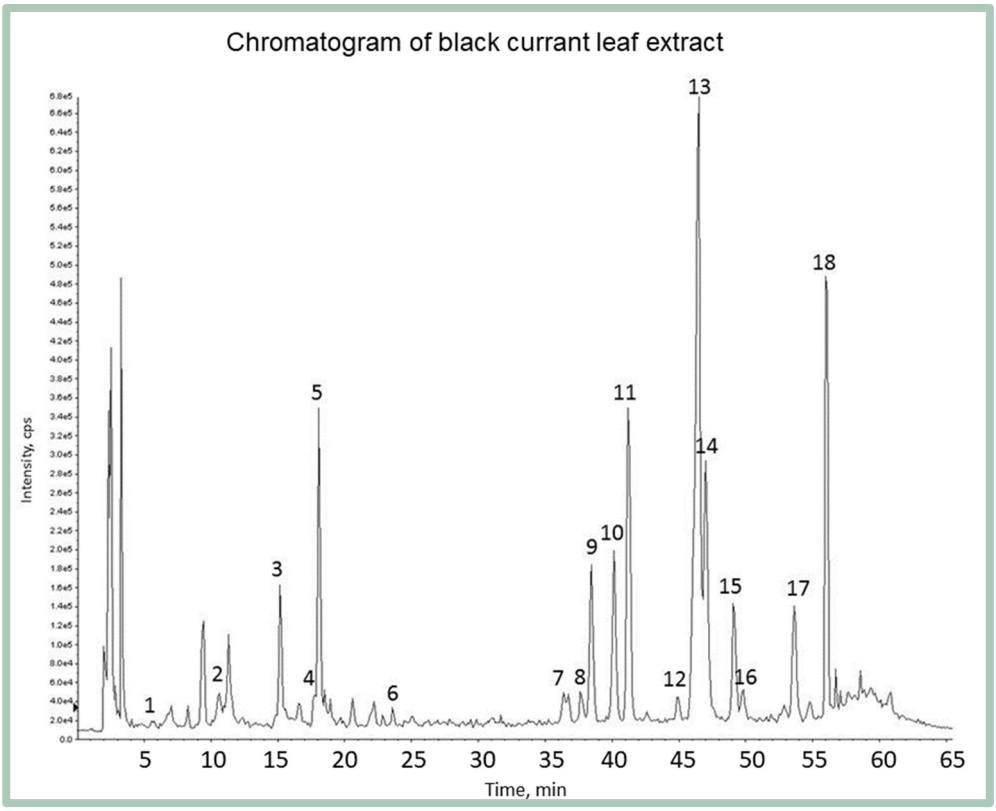


Figure 16.
Representative black currant (BC) chromatogram where peak 1) gallic acid, 2) neo-chlorogenic acid, 3) epigallocatechin, 4) catechin, 5) chlorogenic acid, 6) epicatechin, 7) myricitin-malonyl-glucoside A, 8) myricitin-malonyl-glucoside B, 9) rutin, 10) quercetin-3-*O*-galactoside, 11) quercetin-3-*O*-glucoside, 12) kaempferol-3-*O*-rutinoside, 13) quercetin-3-*O*-malonyl-glucoside A, 14) quercetin-3-*O*-malonyl-glucoside B, 15) kaempferol-3-*O*-glucoside, 16) isorhamnetin-3-*O*-glucoside, 17) kaempferol-malonyl-glucoside A, 18) kaempferol-malonyl-glucoside B.

Table 8.
The five major phenolic compounds in black currant (BC) leaves, average of five cultivars (biological replicate n = 1, technical replicate n = 3). CV = coefficient of variation, T_R = retention time.

Phenolic compound	*m/z* $[M - H]^-$	Content (µg/g DW)	CV %	Detection limit (µg/g DW)	t_R (min)	Peak number
Chlorogenic acid	353.3	1481.2	46.1	18.8	18.0	5
Rutin	609.4	1816.4	34.0	22.8	38.2	9
Quercetin-3-*O*-glucoside	463.1	1705.1	23.4	7.2	41.0	11
Quercetin-3-*O*-malonyl-glucoside A	549.2	4458.9	16.8	7.7	46.4	13
Quercetin-3-*O*-malonyl-glucoside B	549.2	2553.0	59.6	7.7	47.0	14

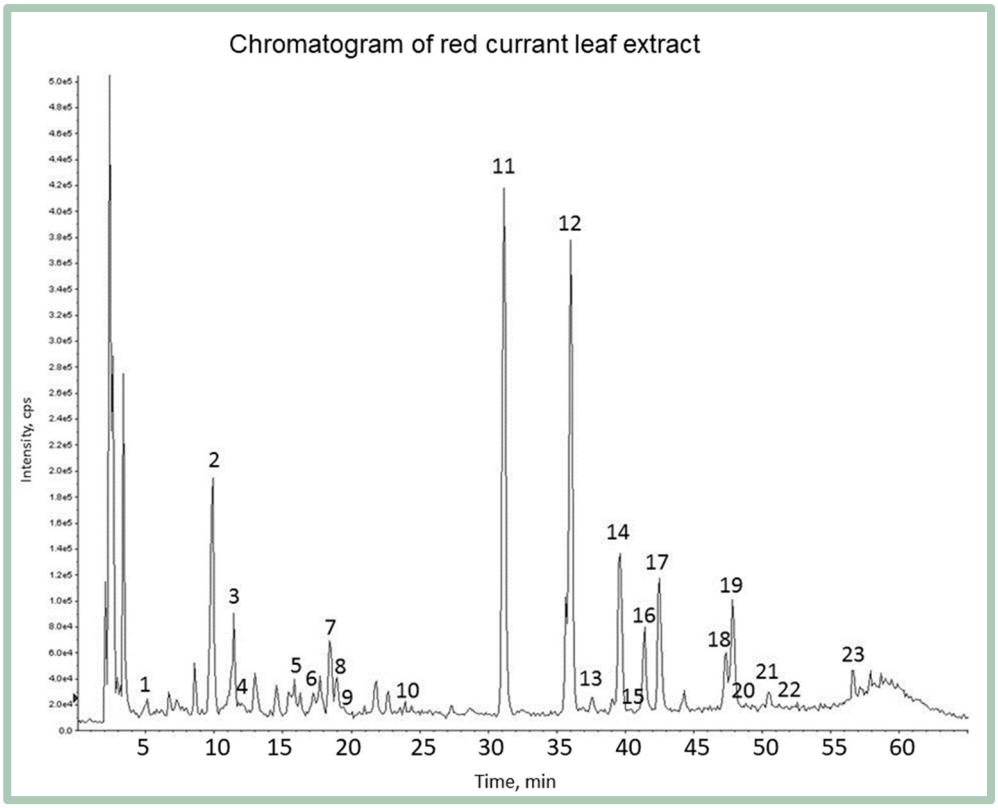


Figure 17.
Representative red currant (RC) chromatogram where peak 1) gallic acid, 2) unknown peak 299.0, 3) unknown peak 447.3, 4) neo-chlorogenic acid, 5) epigallocatechin, 6) caffeoyl hexose, 7) catechin, 8) unknown peak 319.0, 9) chlorogenic acid, 10) red currant tannin I, 11) red currant tannin Ib, 12) red currant tannin II, 13) myricitin-malonyl-glucoside, 14) rutin, 15) quercetin-3-O-galactoside, 16) unknown peak 592.8, 17) quercetin-3-O-glucoside, 18) kaempferol-3-O-rutinoside, 19) quercetin-3-O-malonyl-glucoside, 20) isorhamnetin-3-O-rutinoside, 21) kaempferol-3-O-glucoside, 22) isorhamnetin-3-O-glucoside, 23) kaempferol-malonyl-glucoside.

Table 9.
The five major phenolic compounds in red currant (RC) leaves, average of five cultivars (biological replicate n = 1, technical replicate n = 3). CV = coefficient of variation, T_R = retention time.

Phenolic compound	*m/z* $[M - H]^-$	Content (µg/g DW)	CV %	Detection limit (µg/g DW)	t_R (min)	Peak number
RC tannin Ib	755.3	2146.0	118.9	7.1	30.8	11
RC tannin II	739.4	1917.9	119.9	2.7	36.0	12
Rutin	609.4	4900.7	43.6	17.8	38.2	14
Quercetin-3-O-glucoside	463.1	1618.4	46.4	5.0	41.0	17
Quercetin-3-O-malonyl-glucoside	549.2	2665.5	59.2	27.1	46.4	19

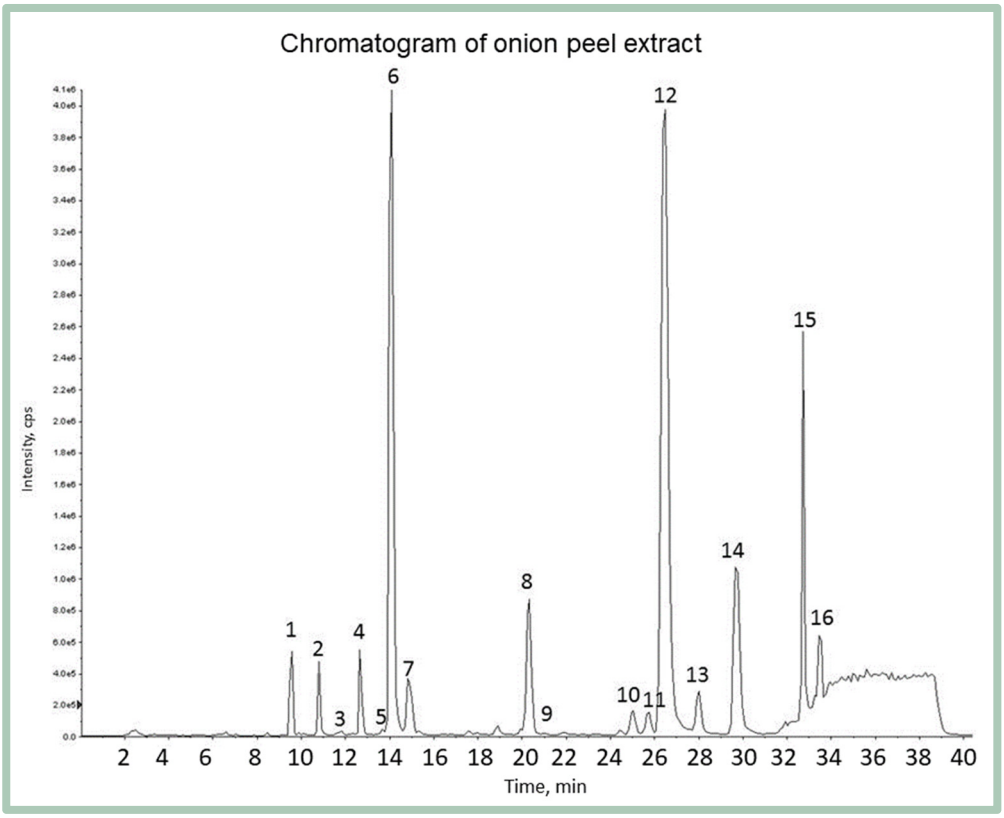


Figure 18.
Representative onion (OP) chromatogram where peak 1) quercetin-3,7,4-triglucoside, 2) unknown peak 625.3, 3) p-hydroxybenzoic acid, 4) quercetin-7,4-diglucoside, 5) vanillic acid, 6) quercetin-3,4-diglucoside, 7) unknown peak 639.2, 8) quercetin-3-*O*-glucoside, 9) ferulic acid, 10) kaempferol-3-*O*-glucoside, 11) isorhamnetin-3-*O*-glucoside, 12) quercetin-4-*O*-glucoside, 13) unknown peak 447.2, 14) unknown peak 477.2, 15) quercetin, 16) kaempferol.

Table 10.
The five major phenolic compounds in onion peel (OP) and skin (OS), average of three cultivars (biological replicate n = 1, technical replicate n = 3). CV = coefficient of variation, T_R = retention time.

Phenolic compound	*m/z* $[M-H]^-$	Peel content (µg/g DW)	CV %	Skin content (µg/g DW)	CV %	Detection limit (µg/g DW)	t_R (min)	Peak number
Quercetin-7,4-diglucoside	625.3	416.2	15.6	366.4	23.0	2.2	12.7	4
Quercetin-3,4-diglucoside	625.2	952.8	14.5	444.7	57.4	0.5	14.3	6
Quercetin-3-*O*-glucoside	463.1	729.5	19.8	301.8	79.2	24.5	20.1	8
Quercetin-4-*O*-glucoside	463.2	1936.6	10.2	1767.6	35.0	7.1	26.4	12
Quercetin	301.1	623.8	33.2	1779.8	18.3	3.5	32.7	15

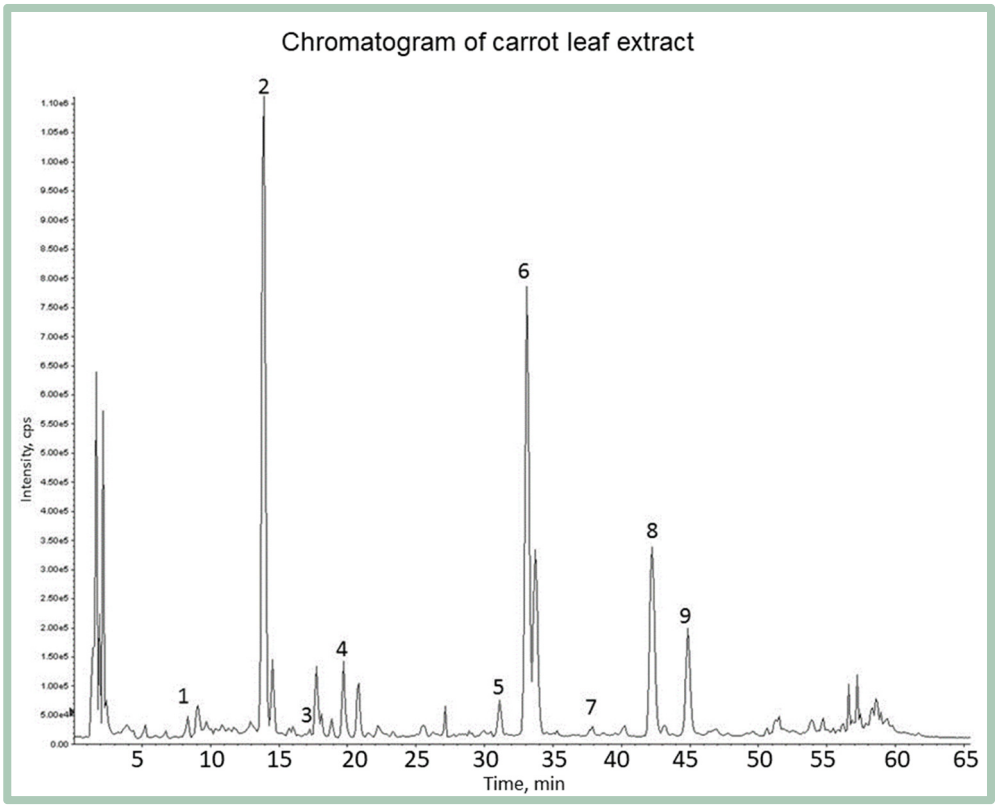


Figure 19.
Representative carrot (C) leaf chromatogram where peak 1) neo-chlorogenic acid, 2) chlorogenic acid, 3) caffeic acid, 4) unknown peak 593.3, 5) rutin, 6) peak 447.3, 7) quercetin-malonyl-glucoside, 8) disregarded peak, 9) kaempferol-malonyl-glucoside.

Table 11.
The five major phenolic compounds in carrot (C) leaves, average of three cultivars (biological replicate n = 1, technical replicate n = 3). CV = coefficient of variation, T_R = retention time.

Phenolic compound	*m/z* [M – H]$^-$	Content (µg/g DW)	CV %	Detection limit (µg/g DW)	t_R (min)	Peak number
Neo-chlorogenic acid	353.3	100.0	28.5	24.2	8.4	1
Chlorogenic acid	353.3	6322.2	5.1	6.6	13.9	2
Peak 447.3	447.3	2874.6	7.4	1.4	33.1	6
Quercetin-malonyl-glucoside	549.2	23.1	5.0	7.2	37.9	7
Kaempferol-malonyl-glucoside	533.2	1387.9	17.3	10.9	44.7	9

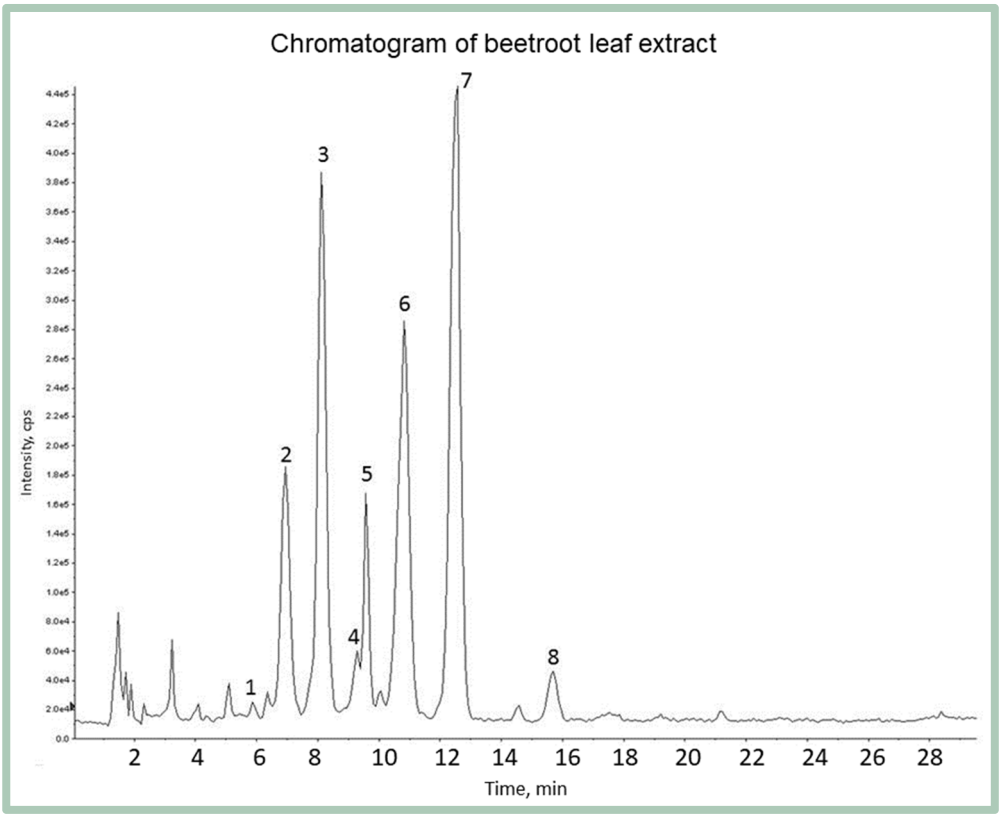


Figure 20.
Representative beetroot (BR) chromatogram where peak 1) kaempferol-malonyl-glucoside, 2) unidentified peak 593.3, 3) xylosylvitexin, 4) rutin, 5) glucopyranosyl-glucopyranosyl-rhamnetin, 6) glucopyranosyl-xylosyl-rhamnetin, 7) unidentified peak 605.2, 8) unidentified peak 577.2.

Table 12.
The five major phenolic compounds in beetroot (BR) leaves, average of three cultivars (biological replicate n = 1, technical replicate n = 3). CV = coefficient of variation, T_R = retention time.

Phenolic compound	*m/z* $[M - H]^-$	Content (µg/g DW)	CV %	Detection limit (µg/g DW)	t_R (min)	Peak number
2,4,5-trihydroxybenzaldehyde	153.0	33.7	26.2	5.1	5.8	1
Xylosylvitexin	563.0	2596.1	73.6	7.6	8.2	3
Rutin	609.5	18.9	84.1	2.6	8.9	4
Glucopyranosyl-glucopyranosyl-rhamnetin	639.0	485.3	44.7	2.6	10.8	5
Glucopyranosyl-xylosyl-rhamnetin	609.3	856.8	50.7	3.6	9.6	6

6.1.4 General discussion for paper I

The SBT samples had the highest antioxidant capacities compared to samples from other species. This may imply that a lower quantity of the sample is needed to obtain the same antioxidant activity than the other samples. However, the chemical structure of the phenolic compounds showed to have greater impact on antioxidant capacities rather than the amount of the different compounds. Moreover, these samples were extracted with 50 % ethanol to obtain a broader range of phenolic compounds (both hydro- and lipophilic). This extraction solvent evidently showed to be the most successful for the SBT samples whereas different solvents might have been more beneficial for others. For instance, hydroxytyrosol which is strictly hydrophilic, might have been extracted in a more efficient way using water only.

The CV % between cultivars of the same species, e.g. BC cultivars (Table 8), RC cultivars (Table 9), and BR cultivars (Table 12) show that there is a difference in phenolic composition in the leaves between cultivars. In the RC case, a possible explanation for this might be that two of the cultivars had white berries, as opposed to the other cultivars with red berries. These two cultivars showed to group together in the PCA (Paper I) which may imply that the phenolic compounds differ, not only in the berries, but in the leaves as well.

These analytical methods are commonly used for analysis of various antioxidants [53]. With this screening, we saw that not only the species of antioxidant chosen is of great importance, but also the cultivar in terms of antioxidant properties, which is in accordance to what Morgenstern, Ekholm [61] previously found. Moreover, recognising the chemical composition and antioxidant properties of these compounds, as well as that of the matrix they will be added to, is crucial for obtaining the best effect. Since one of the aims in this thesis work was to find antioxidants inhibiting lipid oxidation in processed meat products, another screening method needed to be used for testing of antioxidant in a relevant matrix.

6.2 The meat model system (Paper II)

The analysis of TBARS was conducted in four sets of emulsion samples due to the large amount of antioxidant powders and extracts that were to be studied. Extracts and powders were added in three concentrations, 50, 100 and 200 ppm based on their phenols content (GAE per mL extract (see Paper I)). The level of lipid oxidation was measured after one day, one week and two weeks where the amount MDA was evaluated spectrophotometrically. All sets had their respective blank emulsion (with no added antioxidant) from which a percentage of oxidation was calculated (μM MDA per gram emulsion with antioxidants / μM MDA per gram blank emulsion * 100). The overall trend for most samples was that lipid oxidation was inhibited more with higher

concentration and more so over time. The repeated measures GLM analysis showed that the fixed factors; plant material and concentration, had significant effects on the repeated dependent variable TBAR ($p < 0.001$) where plant material had the largest impact of the factors. In set one, SBT, OS, and BR samples showed to be model examples of the trend where lipid oxidation was inhibited more with higher concentration and over time (Figure 21). The lipid oxidation was reduced to 31.2 %, 18.1 % and 16.6 % for SBT, OS and BR respectively at 200 ppm GAE after 2 weeks of storage.

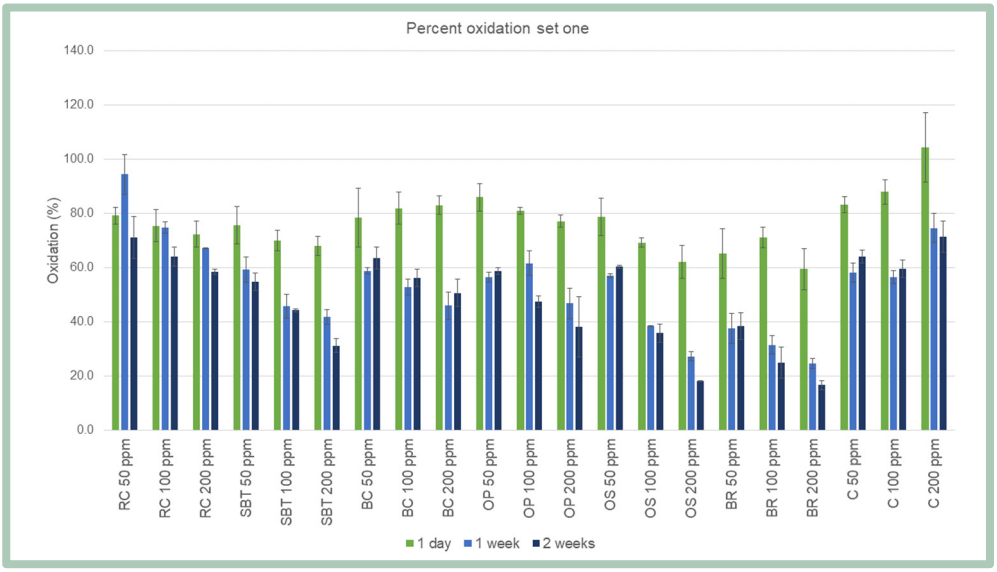


Figure 21.
Percent (%) lipid oxidation in first set of meat model system samples (replicates, n = 3). RC = red currant leaves, SBT = sea buckthorn leaves, BC = black currant leaves, OP = onion peel, OS = onion skin, BR = beetroot leaves, C = carrot leaves.

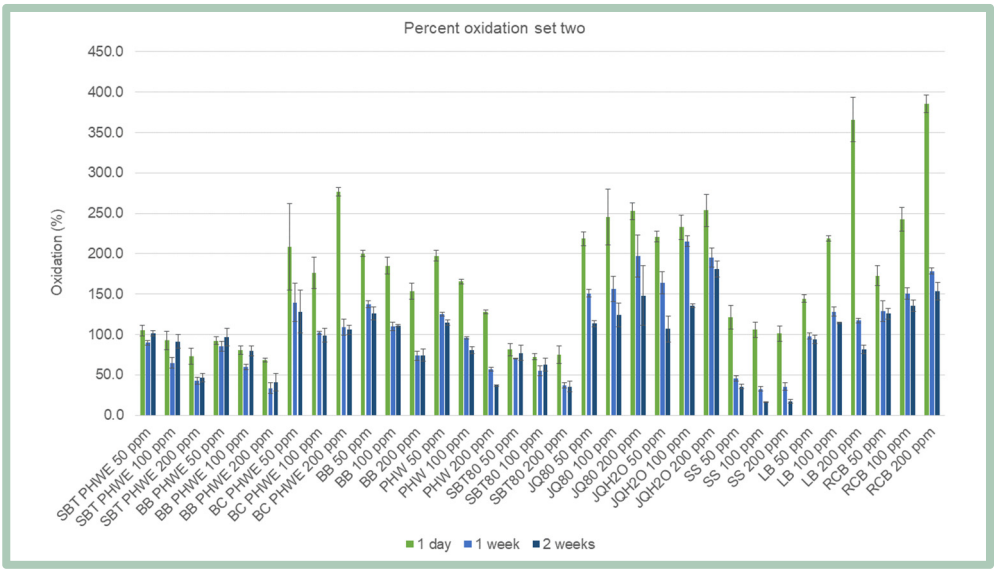


Figure 22.
Percent (%) lipid oxidation in second set of meat model system samples (replicates, n = 3). SBT PHWE = sea buckthorn leaves pressurized hot water extracted, BB PHWE = bilberry leaves pressurized hot water extracted, BC PHWE = black currant juice press residues pressurized hot water extracted, BB = bilberry leaves, PHW = pine heartwood, SBT80 = sea buckthorn 89 % ethanol, JQ80 = japanese quince 80 % ethanol, JQH2O = japanese quince water extracted, SS = summer savory, LB = lingonberry, RCB = red currant berries.

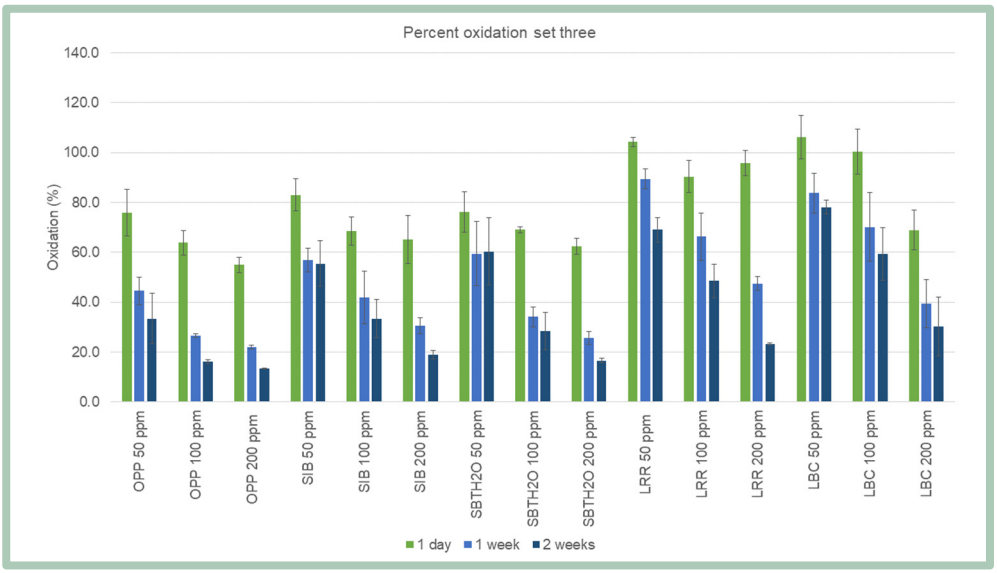


Figure 23.
Percent (%) lipid oxidation in third set of meat model system samples (replicates, n = 3). OPP = olive polyphenols, SIB = spruce inner bark, SBT H2O = sea buckthorn water extracted, LRR = lyophilised rhubarb root, LBC = lyophilised black currant leaves.

In set two, the summer savory (SS) powder and the pine heartwood (PHW) sample inhibited lipid oxidation more with higher concentration and with longer storage time (Figure 22) but only after one and two weeks of storage. SS inhibited lipid oxidation to 17.2 % and PHW to 35.4 % at 200 ppm after two weeks of storage. Pressurised hot water extracted sea buckthorn (SBT PHWE) and bilberry leaves (BB PHWE) showed to be the most efficient after one week of storage, and similar tendencies could be seen in sea buckthorn extracted with 80 % ethanol (SBT80) at 50 and 100 ppm. In this set of samples, many phenols showed to have a pro-oxidant effect instead of antioxidant. Black currant extracted with pressurised hot water (BC PHWE), the bilberry sample (BB), the Japanese quince samples (JQ80 and JQH2O), the lingonberry and red currant powders all showed great pro-oxidant effects already after one day of storage. It is widely known that some antioxidants may act as pro-oxidants, depending on their concentration, mode of action, and the presence of transitional metal ions [58, 59]. BB did however inhibit lipid oxidation at 200 ppm after one and two weeks of storage.

In set three, the inhibition trend was similar to samples in set one, where samples inhibited lipid oxidation more efficiently with higher concentration and with longer storage time (Figure 23). The olive polyphenol (OPP) sample was the most efficient in inhibiting lipid oxidation was to 13.5 % in relation to the blank emulsion. The spruce inner bark (SIB), sea buckthorn extracted with water (SBTH2O) and the lyophilised rhubarb root (LRR) efficiently inhibited oxidation to 19.0 %, 16.6 %, and 23.2 % respectively. Set three consisted of Estonian samples together with re-run samples that previously had a coefficient of variation (CV) % between technical replicates that exceeded 10 %.

The Tukey post-hoc test for sets one to three showed that OPP had the statistically strongest inhibiting capacity of lipid oxidation of all samples ($p < 0.001$) except when compared to the BR sample ($p = 0.151$). BR statistically differed from all samples except for OPP (see above) and SBT H2O ($p = 0.289$). All concentrations lowered the level of oxidation statistically ($p < 0.001$), where 200 ppm < 100 ppm < 50 ppm.

The fourth set consisted of samples that were tested at lower concentrations. This was done due to their expected proneness to act as pro-oxidants. This hypothesis relied on similarities in antioxidant capacities between samples in set four and pro-oxidant samples from set two. The ten-fold difference in concentration was chosen arbitrarily, and would in retrospect been calculated from the GAE of samples to better suit the meat model system. The spray dried rhubarb juice (SDRJ) was the most effective sample, inhibiting lipid oxidation to 68.3 % at 5 ppm after 2 weeks Figure 24). The post-hoc test showed that SDRJ significantly differed from all other samples ($p < 0.001$). Significant differences in concentrations were found between 5 ppm and 20 ppm ($p < 0.001$) and between 10 ppm and 20 ppm ($p < 0.001$) but not between 5 ppm and 10 ppm ($p = 0.992$).

The Pearson correlation analysis showed there was no correlation between the previously found antioxidant capacities and the lipid oxidation inhibiting capacity. It is possible that the samples differed too much in composition and lipid oxidation inhibition capacity for them to produce unanimous results correlating with FC, ABTS or FRAP (Paper III).

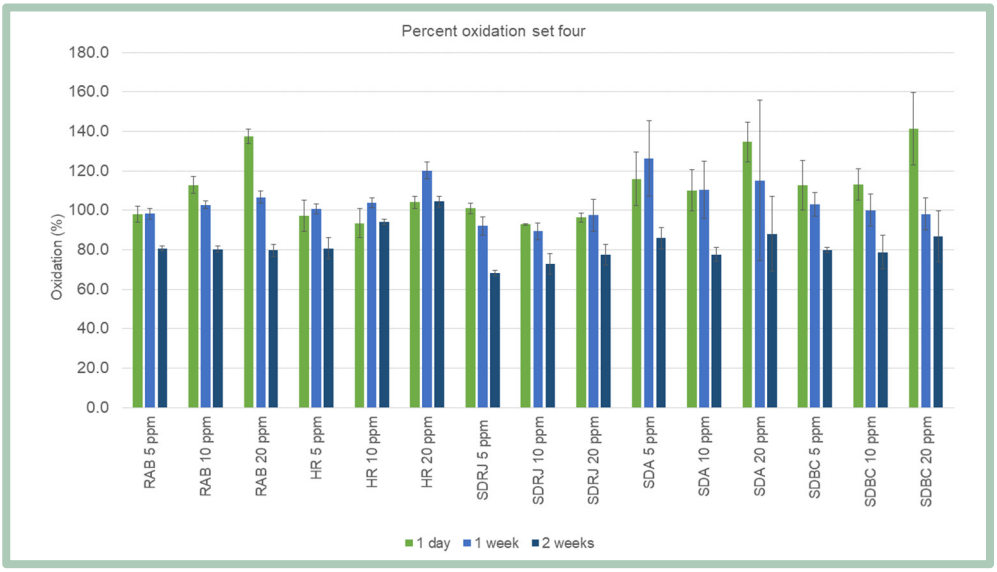


Figure 24.
Percent (%) lipid oxidation in fourth set of meat model system samples (replicates, n = 3). RAB = ramson bulb, HR = horseradish, SDRJ = spray dried rhubarb juice, SDA = spray dried aronia juice, SDBC = spray dried black currant juice press residues.

6.2.1 General discussion for paper II

The meat model system showed to be an efficient matrix for analysing the main ingredients in a processed meat product in a standardised manner, as previously found [76, 77]. Together with an accurate heat treatment, this model system was a good option for relevant screening of lipid oxidation inhibiting capacity of antioxidant powders and extracts. Most antioxidant samples inhibited lipid oxidation better with higher concentration and with longer storage time when compared to the emulsions without added antioxidants. This was expected, since a higher concentration of antioxidants typically results in a stronger lipid oxidation inhibiting capacity. However, this study also showed that some antioxidants were more efficient at 100 ppm than at 200 ppm, which again points towards that the reaction speed depends on the chemical composition of the antioxidants [60] rather than relying solely on concentration. Some antioxidants showed to be pro-oxidant which, as previously mentioned, could be expected [58, 59]. The results from this study highlights the importance of finding the

right antioxidant for the right matrix, and the importance of studying the compounds thoroughly prior to further usage. The next step in this thesis work consisted in testing the best performing antioxidants in an actual processed meat product using the newly acquired knowledge from the meat model system study.

6.3 Most oxidised meatball with plant phenols (Paper III)

6.3.1 The most oxidised meatball

All meatball samples of different ingredient combinations were evaluated for their TBARS substances (MDA per gram meatball) in triplicates after one day, one week and two weeks of storage (Figure 25). The GLM-analysis showed that meat type, storage time, cooking type, and salt content all had significant effects on the lipid oxidation ($p < 0.001$) as had the fat content ($p < 0.01$). Nearly all interactions had significant effects on lipid oxidation *(*$p < 0.05$) except for interactions between; Salt × Cooking, Meat × Salt × Storage, Meat × Salt × Fat × Cooking, and Meat × Salt × Fat × Storage, although interactions showed the effect sizes were minor (shown by partial Eta squared in Paper III). The factors affecting lipid oxidation the most were meat type and storage time ($p < 0.001$) where pork oxidised more than beef, and where oxidation increased with longer storage times for both meat types (Paper III). The combination of parameters that oxidised the most contained pork meat, 20 % fat and 2 % salt, was deep fried and stored for 2 weeks (Figure 25).

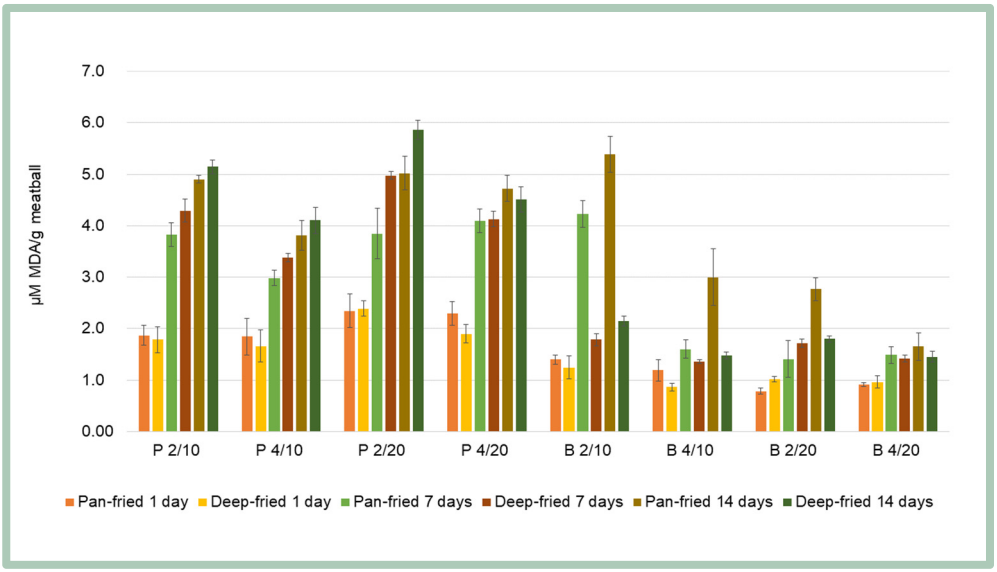


Figure 25.
Lipid oxidation in model meatballs with differing parameters shown in µM malondialdehyde (MDA) per gram meatball where P = pork and B = beef meat. The numbers 2 and 4 correspond to the salt content in % and the numbers 10 and 20 correspond to the fat content in %. The standard deviation is shown by the error bars (n = 3).

Initially, the hypothesis was that beef meatballs would be more prone to oxidise, due to the higher amount of heme iron in beef compared to pork meat [78], which did not show to be the case. Moreover, since salt (NaCl) is known to act as pro-oxidants in meat and meat products [6], it was expected that the meat samples with the higher salt concentration (4 %) would be more prone to oxidise, which did not show to be the case either. A possible explanation for this might be that salt contents above 3 % previously have shown little to no pro-oxidant effect [11]. Instead, the crucial factor in this study is hypothesised to be the fat content, where beef contains less PUFA than pork in both muscular and adipose tissue [12]. Another interesting finding was that the beef meatball with 2 % salt and 10 % fat oxidised considerably more after one and two weeks of storage than the other beef meatball samples. This is discussed thoroughly in Paper III where the hypothesis was that the frying fat had been taken up by these meatballs. The frying fat was a commercially available rapeseed oil (Zeta, Sweden) with 7.5 % saturated fatty acids, 62.5 % mono-unsaturated fatty acids, and 30 % poly-unsaturated fatty acids. This was particularly interesting since these particular meatballs showed to have lost the most fat among all meatball samples [66]. Hence, if they lost their own fat and instead took up the frying fat, this would have changed the fatty acid composition to one more prone to oxidise, which would be in accordance to what has previously been shown [79]. The meatball type that oxidised the most was used as the highly oxidised model meatball for the following study of inclusion of antioxidants.

6.3.2 The most oxidised meatball with plant phenols

All meatball samples without and with antioxidants were evaluated for their TBAR substances (MDA per gram meatball) in triplicates after 14 days of storage. The results were calculated as a percentage of oxidation compared to samples without added antioxidants (µM MDA per gram meatball with plant material / µM MDA per gram meatball without antioxidant * 100) (Figure 26). The antioxidant species, concentrations, and the interaction of both significantly affected the level of lipid oxidation compared to the meatball without added antioxidants ($p < 0.001$), where species had the largest effect size (shown by partial Eta squared in Paper III). Antioxidant species showed to have the largest effect size on the level of lipid oxidation. The SS powder at 200 ppm and 100 ppm, SBTH2O sample at 100 ppm, and OPP sample at 200 ppm were the most efficient antioxidants, lowering lipid oxidation to 13.8 %, 21. 8 %, 22.9 % and 26.1 % respectively compared to the meatball with no added antioxidants. SS statistically differed from all other samples ($p < 0.001$) and has previously shown to effectively inhibit lipid oxidation in pork meatballs [80]. There were no significant correlations (Pearson) between the total phenols content and the lipid oxidation inhibition capacity of the antioxidants (Paper III).

6.3.3 General discussion for paper III

The meatball model was based on the most common ingredients in industrially produced meatballs in Sweden. However, no breadcrumbs, onions, eggs, nor spices were added, since the aim was to isolate the most important ingredients included in the lipid oxidation process. Moreover, in a typical Swedish recipe, beef and pork is mixed in the mince, but to get a clear picture of the effects, and because of the already large sample size, this sample type was disregarded.

Natural plant phenols such as olive leaf extracts [76] and oregano and sage oils [81] have previously shown to be effective antioxidants in meat products and meat model systems. In this study, antioxidants were more efficient at 200 ppm than at 100 ppm, except for SBT which was statistically more efficient at 100 ppm ($p < 0.05$). SBTH2O also showed tendencies to be more efficient at 100 ppm than at 200 ppm. Again this could be attributed to the previously mentioned difference in reaction speed due to chemical composition, rather than the concentration [60]. The five best performing species, SS, OS, OPP, SBTH2O, and LBC were chosen for evaluation *in vivo*. Even though SBT80 was more efficient at 200 ppm than OS and LBC, the focus was put on species differences, rather than extraction methods for the same species.

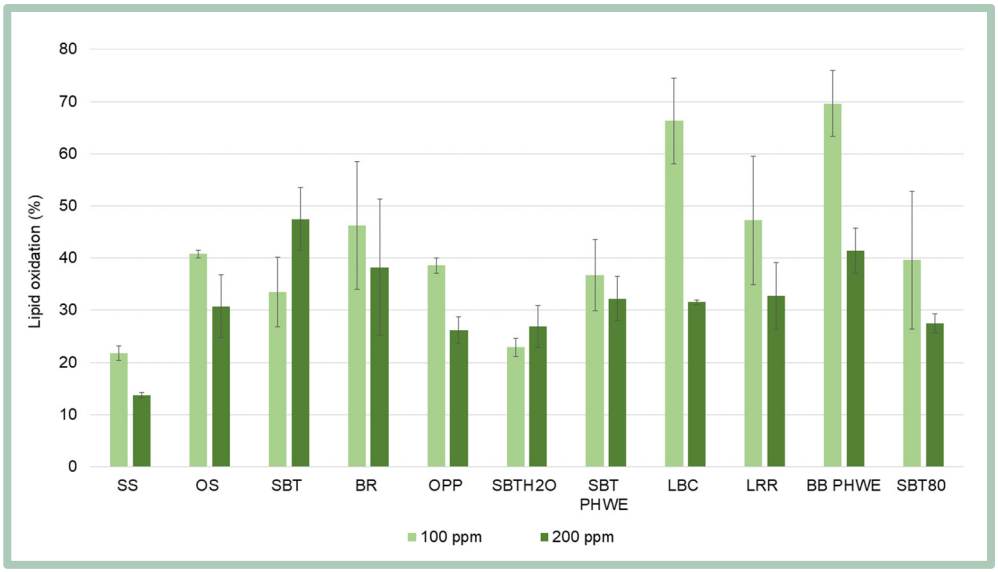


Figure 26.
Lipid oxidation in the meatball type most prone to oxidise (pork with 2 % NaCl, 20 % fat, deep-fried) with different antioxidants shown as a percentage of oxidation compared to the meatball without added antioxidants at two concentrations. SS = summer savory, OS = onion skin, SBT = sea buckthorn leaves, BR = beetroot leaves, OPP = olive polyphenols, SBTH2O = water extracted sea buckthorn leaves and sprouts, SBT PHWE = sea buckthorn leaves and sprouts – pressurised hot water extraction, LBC = lyophilised black currant leaves, LRR = lyophilised rhubarb root, BB PHWE = bilberry leaves – pressurised hot water extraction, and SBT80 = ethanol (80 %) extracted sea buckthorn leaves and sprouts. The standard deviation is shown by the error bars (n = 3).

6.4 Animal trial results (Paper IV)

6.4.1 DSS consumption and faecal index

When studying the results from the various analyses of the animal trial, large variations were found within trial groups. Hence, results were divided per cage to see if there were any differences, which showed to be the case. The total volume of DSS consumed was calculated for each cycle, group and cage respectively. A significant difference in consumption was found over cycles ($p = 0.025$), where the highest consumption was in cycle 1 and the lowest consumption was in cycle 2, but not between cages ($p = 0.202$) nor groups ($p = 0.327$). However, the main effect plots (estimated marginal means) showed there were differences between cages, which is worth keeping in mind when evaluating results between groups and cages (Figure 27).

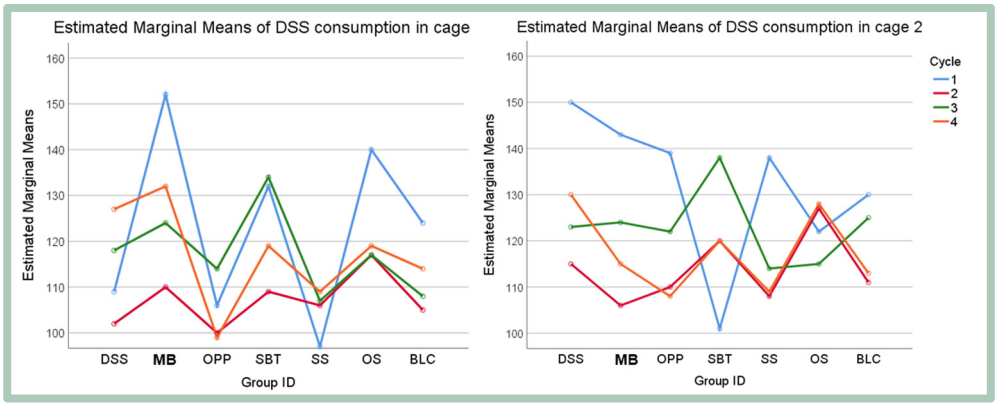


Figure 27.
DSS consumtion across groups divided by cage 1 and cage 2.

The disease activity index (DAI) was analysed and showed to differ according to weight changes (data not shown), which fluctuated depending on the amount DSS consumed. This was noticed either as a result of the mice not liking the taste of the DSS, hence not drinking, and therefore losing weight during the cycle, or due to the ingestion of DSS, leading to diarrhoea and therefore losing weight for that matter. It was thus more valuable to analyse the faecal index (FI) independently of the weight changes. The FI did not differ significantly among groups ($p = 0.240$), cages ($p = 0.511$) or cycles (Figure 28) except for in cycle 2 where OS had a significantly higher FI than all other groups ($p < 0.05$) except for OPP ($p = 0.115$) and SS ($p = 0.207$) (Table 13).

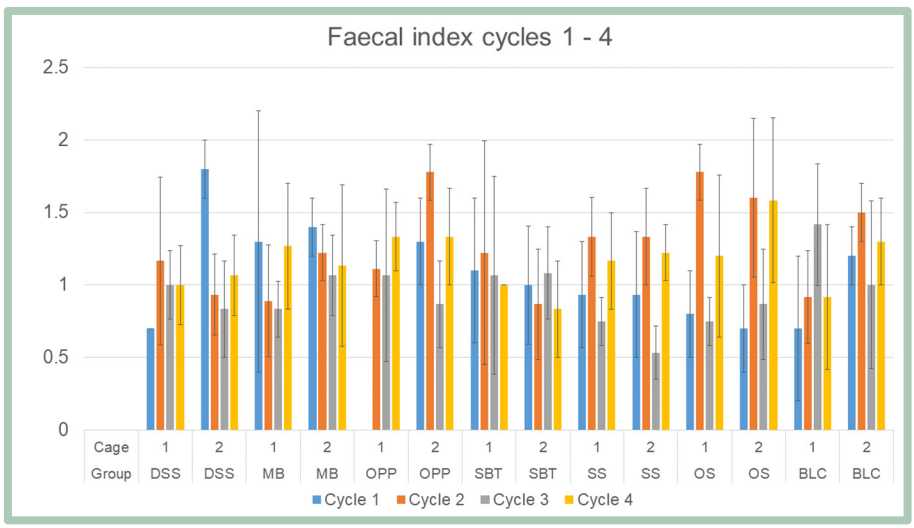


Figure 28.
Faecal index across cycles divided by groups and cages. The error bars show the standard deviation.

Table 13.
Faecal index (FI) across cycles where SD = standard deviation.

		Cycle 1			Cycle 2			Cycle 3			Cycle 4		
Group	Cage	Average	SD	N	Average	SD	N	Average	SD	N	Average	SD	N
DSS	1	0.7	NA	1	1.2	0.6	4	1.0	0.2	5	1.0	0.3	4
DSS	2	1.8	0.2	2	0.9	0.3	5	0.8	0.3	4	1.1	0.3	5
MB	1	1.3	0.9	2	0.9	0.4	3	0.8	0.2	4	1.3	0.4	5
MB	2	1.4	0.2	3	1.2	0.2	3	1.1	0.3	5	1.1	0.6	5
OPP	1	0	NA	1	1.1	0.2	3	1.1	0.6	5	1.3	0.2	5
OPP	2	1.3	0.3	4	1.8	0.2	3	0.9	0.3	5	1.3	0.3	3
SBT	1	1.1	0.5	3	1.2	0.8	3	1.1	0.7	5	1.0	0.0	2
SBT	2	1.0	0.4	5	0.9	0.4	5	1.1	0.3	4	0.8	0.3	4
SS	1	0.9	0.4	5	1.3	0.3	4	0.8	0.2	4	1.2	0.3	4
SS	2	0.9	0.4	5	1.3	0.3	3	0.5	0.2	5	1.2	0.2	3
OS	1	0.8	0.3	4	1.8	0.2	3	0.8	0.2	4	1.2	0.6	5
OS	2	0.7	0.3	5	1.6	0.5	5	0.9	0.4	5	1.6	0.6	4
BLC	1	0.7	0.5	4	0.9	0.3	4	1.4	0.4	4	0.9	0.5	4
BLC	2	1.2	0.2	2	1.5	0.2	4	1.0	0.6	5	1.3	0.3	4

6.4.2 MDA

Malondialdehyde was not detected in neither biopsies from colon nor small intestine (data not shown). This might be a result of too little exposure to secondary lipid peroxidation products due to that the amount of meatballs present in the feed was 20 %, and/or due to that the trial period was too short for these products to penetrate to the intestinal tissues. Our screening hence showed that the onset of potential disease could not be seen in MDA levels from intestinal biopsies.

6.4.3 MPO

MPO was found in higher concentrations in colon biopsies than in small intestine biopsies (Figure 29), where no significant differences were found between groups ($p = 0.548$). The GLM-analysis showed there were significant differences between groups in colon ($p = 0.040$) where the Tukey post-hoc test showed that all other groups had higher values than the control group ($p < 0.05$) and where the SBT group had a higher value than the OPP group ($p = 0.022$). Large differences within groups can be seen in e.g. the DSS and SBT groups (more specifically for cage 1 in each group) where high

MPO values could not be righteously claimed to be outliers and were hence kept in the data set. This result also shows the difficulty of *in vivo* trials due to individual variations.

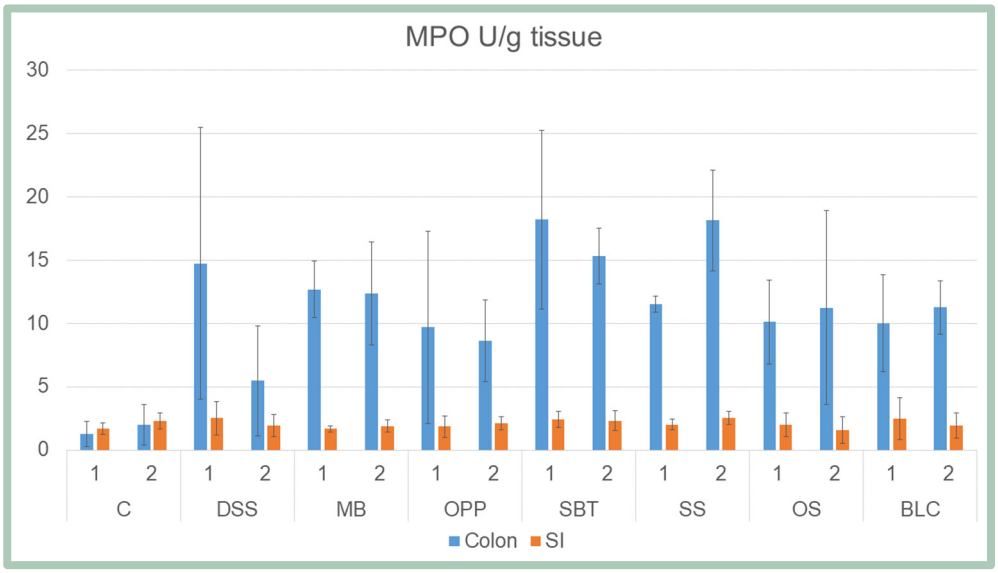


Figure 29.
Myeloperoxidase (MPO) levels in small intestine (SI) and colon divided by groups and cages. The standard deviation is shown by the error bars.

6.4.4 Physical properties

A significant difference was found in colon length (cm) between the C group and the groups ingesting DSS ($p < 0.05$) (Table 6, Paper IV), as well as a difference in colonic lesions (Figure 30A) between groups DSS and C ($p = 0.026$) and MB and C ($p = 0.029$). However, no significant differences were revealed between the trial groups with plant phenols either between groups or when compared to groups C, MB or DSS (Paper IV). No significant difference was found among groups for spleen weights ($p = 0.368$) (Supplementary Table 7, Paper IV) nor epithelial dysplastic zones ($p = 0.093$) (Figure 30B).

6.4.5 Cytokines/Chemokines

IL-12p70 and IL-2 were not detected in the analyses and there were too few detected samples per trial group of IL-4 to be analysed statistically. Levels of IFN-γ , IL-1β , IL-5, IL-6, IL-10, KC/GRO, TNF-α and Leptin were detected, but showed to not statistically differ between trial groups (Supplementary Table 6, Paper IV). Our

screening showed that the onset of potential disease could not be seen as changes in cytokine/chemokine concentrations in peripheral blood.

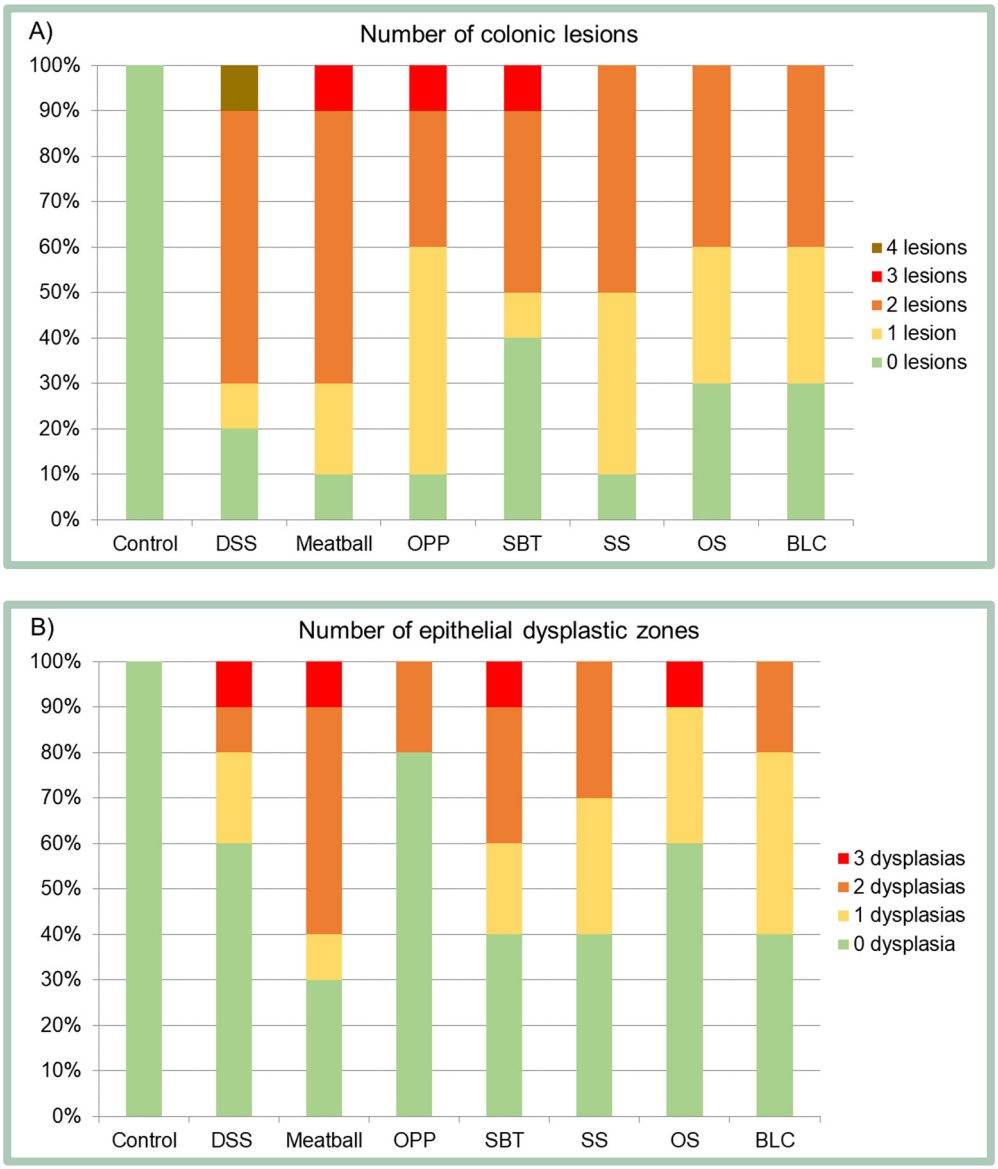


Figure 30.
Number of colonic lesions (A) and number of epithelial dysplastic zones (B) across groups (n = 10), where DSS = dextran sulfate sodium, OPP = olive polyphenol, SBT = sea buckthorn, SS = summer savory, OS = onion skin, and BLC = black currant. The percentage represents the number of mice where 100 % = 10 mice.

6.4.6 Shannon Diversity index

There were significant differences in the Shannon DI between groups of both colon ($p = 0.016$) and small intestine ($p = 0.001$) (Table 14) where SS had significantly higher DI than all other groups ($p < 0.001$) in the colon and both SS and OS had higher DI than all other groups ($p < 0.001$) in small intestine where OS and SS did not differ significantly. Significant differences were found between C and DSS, and C and MB, but not between DSS and MB groups in small intestine ($p < 0.05$). A high diversity index (DI) is commonly regarded as being positive for intestinal health [42] and it has been shown that microbial dysbiosis may have a cancer-promoting effect [46]. It is however important to note that a DI may be high but it does not reveal the composition in regards to bacterial species [44], i.e. the microbiota may be diverse with either potential pathogenic or health-associated bacteria, showing the same result in the DI.

Table 14.
Shannon Diversity Index (DI) in colon and small intestine (SI) where SD = standard deviation, and a different letter signifies a difference in p-value of 0.05.

		DI Colon				DI SI			
Group	*Cage no*	*Average*	*SD*	*N*	*P < 0.05*	*Average*	*SD*	*N*	*P < 0.05*
MB	1	2.14	0.36	5		0.90	0.40	5	
	2	2.33	0.22	5		0.89	0.45	5	
	Total	*2.23*	*0.30*	*10*	*b*	*0.89*	*0.40*	*10*	*a*
C	1	2.63	0.23	5		1.76	0.53	5	
	2	1.90	0.17	5		1.09	0.49	5	
	Total	*2.27*	*0.43*	*10*	*b*	*1.43*	*0.60*	*10*	*b*
DSS	1	2.32	0.22	5		0.95	0.60	5	
	2	1.86	0.37	5		0.64	0.19	5	
	Total	*2.09*	*0.37*	*10*	*b*	*0.79*	*0.45*	*10*	*a*
OPP	1	2.28	0.34	5		0.76	0.33	5	
	2	2.39	0.20	5		1.04	0.20	5	
	Total	*2.33*	*0.27*	*10*	*b*	*0.90*	*0.30*	*10*	*a*
SBT	1	2.41	0.27	5		1.21	0.15	4	
	2	2.19	0.39	5		1.19	0.18	5	
	Total	*2.30*	*0.34*	*10*	*b*	*1.20*	*0.16*	*9*	*ab*
SS	1	3.25	0.21	5		2.11	0.31	5	
	2	3.21	0.17	5		2.13	0.16	5	
	Total	*3.23*	*0.18*	*10*	*a*	*2.12*	*0.23*	*10*	*c*
OS	1	2.45	0.21	5		2.36	0.24	5	
	2	2.17	0.26	5		2.16	0.14	5	
	Total	*2.31*	*0.27*	*10*	*b*	*2.26*	*0.21*	*10*	*c*
BLC	1	2.21	0.27	5		1.17	0.22	5	
	2	2.01	0.23	5		1.38	0.32	5	
	Total	*2.11*	*0.26*	*10*	*b*	*1.27*	*0.28*	*10*	*ab*

6.4.7 Terminal-Restriction Fragment analysis

In the terminal-restriction fragments (T-RFs) analysis of colon, T-RFs 85, 92 and 177 showed to be present in the C group only, whereas T-RF 547 was present in the MB group only (Figure 31A). T-RFs 88 and 304 were only present in the groups consuming DSS. The microflora profiles in the colon differed more than those of the small intestine (Figure 31A), although some patterns could be recognized. For instance, the DSS group and the SS group had resembling T-RF profiles except for T-RF 495 which was found in all meatball-consuming groups except for OS. When looking at groups with antioxidant preparations, OPP and SS both had T-RFs 546 and 547, which were not present in the other groups.

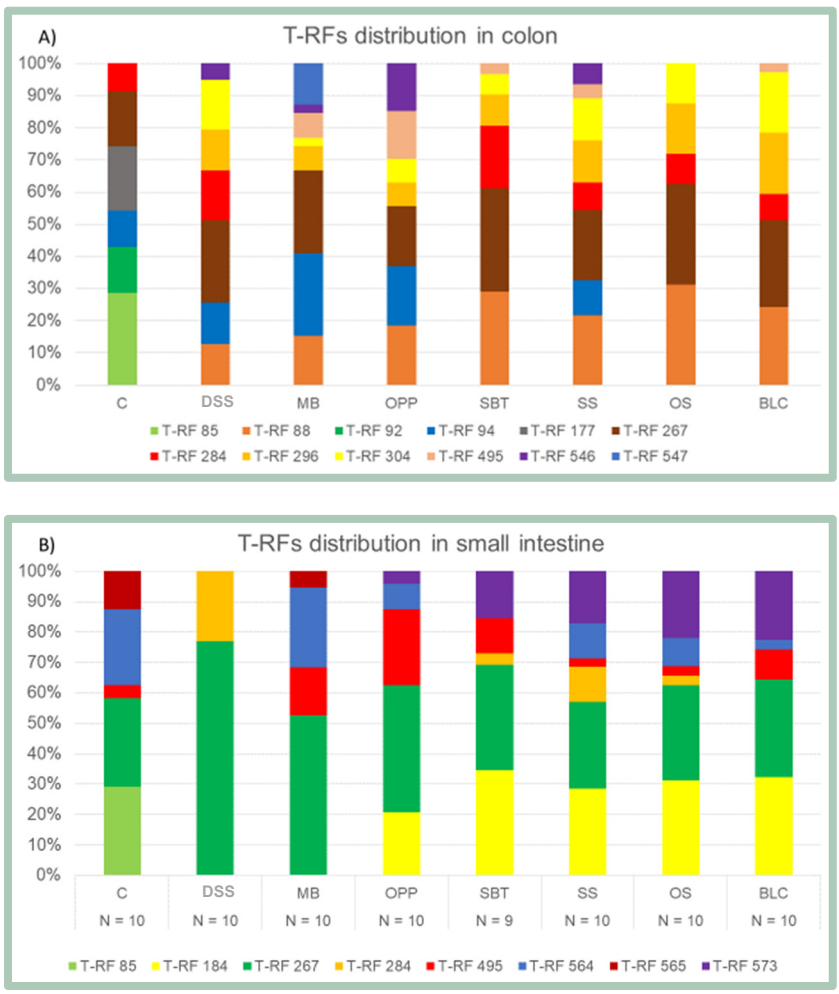


Figure 31.
T-RFs distribution in colon (A) and small intestine (B) respectively. The relative abundance of T-RFs is given in %.

In the small intestine, the DSS group consisted of two main T-RFs, namely 267 and 284 (Figure 31B) and T-RF 184 was only found in groups consuming meatballs with antioxidant preparations. These results point towards that although the DI does not necessarily differ significantly, the bacteria constituting the diversity might. The T-RF analysis also showed that the antioxidant preparations seemed to standardise the microflora in the small intestine (Figure 31B) since all groups consuming meatballs with antioxidant preparations showed similar bacterial profiles, except for T-RF 284 which was only present in SBT, SS and OS.

6.4.8 FACS

In MLN panel 1, SBT cage 1 and 2 as well as MB cage 2 had a significantly higher percentage of gated F4/80+ cells expressing TLR4+ than the control group cages ($p < 0.05$). No other antioxidant groups differed from the C group which could point towards a counteracting effect of the other plant phenols on immunological response in comparison to the MB group. For the F4/80+TLR2+TLR4+ cells, cage 1 from the control group had a significantly higher value than MB cage 2 ($p = 0.020$), SBT cage 1 and 2 ($p < 0.01$), and SS cage 2 ($p = 0.039$). Moreover, the control group cage 2 differed significantly from SBT cage 1 and 2 ($p < 0.05$). BLC, OPP, OS and SS did not differ significantly from the C group which, as previously mentioned, could be a positive effect of the plant materials. For CD11c+TLR2+ cells, C cage 2 had significantly lower values than the SBT group ($p < 0.05$), and OS cage 2 ($p < 0.01$) (Supplementary Table 1, Paper IV) where instead the other groups (including the MB group) showed results not differing from the healthy control group.

In panel 2, significantly lower values were found between C cage 1 - BLC cage 1 ($p = 0.22$), as well as SBT cage 2 ($p = 0.013$) for CD8+CCR9+ cells. MB cage 1 had significantly lower results than SBT cage 2 among CD4+CCR9+ cells ($p = 0.047$), similarly to group C having lower values than SBT ($p < 0.01$), and C cage 1 - OS cage 2 ($p = 0.048$). A higher percentage of gated cells were also identified between MB cage 1 and group OS in activated T_H-cells (CD4+CD69+) ($p < 0.05$), as well as between C cage 2 and OS cage 2 ($p = 0.025$) (Supplementary Tables 1 – 2, Paper IV).

In panel 3, significantly higher values in MB cage 2 were detected for T_{regs} (CD4+FoxP3+) compared to the C group ($p < 0.01$), in SBT cage 2 compared to OS cage 1 ($p = 0.014$), as well as between SBT and C groups ($p < 0.05$). There was also a significantly higher percentage of T_{regs} expressing activation marker (CD4+Foxp3+CD69+) ($p = 0.002$) in the C group when compared with SBT cage 2. All other groups showed no significant differences, hence no other immunological reaction could be detected for this phenotype.

In PP, the percentage of F4/80+TLR2+TLR4+ cells was significantly lower in the MB group compared to C cage 2 ($p < 0.05$) as between SS cage 1 and C cage 2 ($p = 0.044$).

No significant differences were shown between groups for CD11c+ expressing cells. In panel 2, CD8+CCR9+ cells expressing homing to the gut was significantly higher in SBT cage 2 than in SS cage 2 (p = 0.01) as was DSS cage 1 when compared to SS cage 2 (p = 0.023), no further differences were found between groups. For CD4+CCR9+CD69+ cells there was a significantly lower percentage gated cells in SS cage 1 than C cage 2 (p = 0.013) as well as in SS cage 1 and BLC cage 2 (p = 0.020). Moreover, a lower percentage of CD4+CD69+ cells was found in C cage 2 than in the DSS group (p < 0.05). In panel 3, the population of regulatory T-cells (CD4+FoxP3+), was significantly higher in SS cage 1 than in C cage 1 (p = 0.041) (Supplementary Tables 4 – 5, Paper IV).

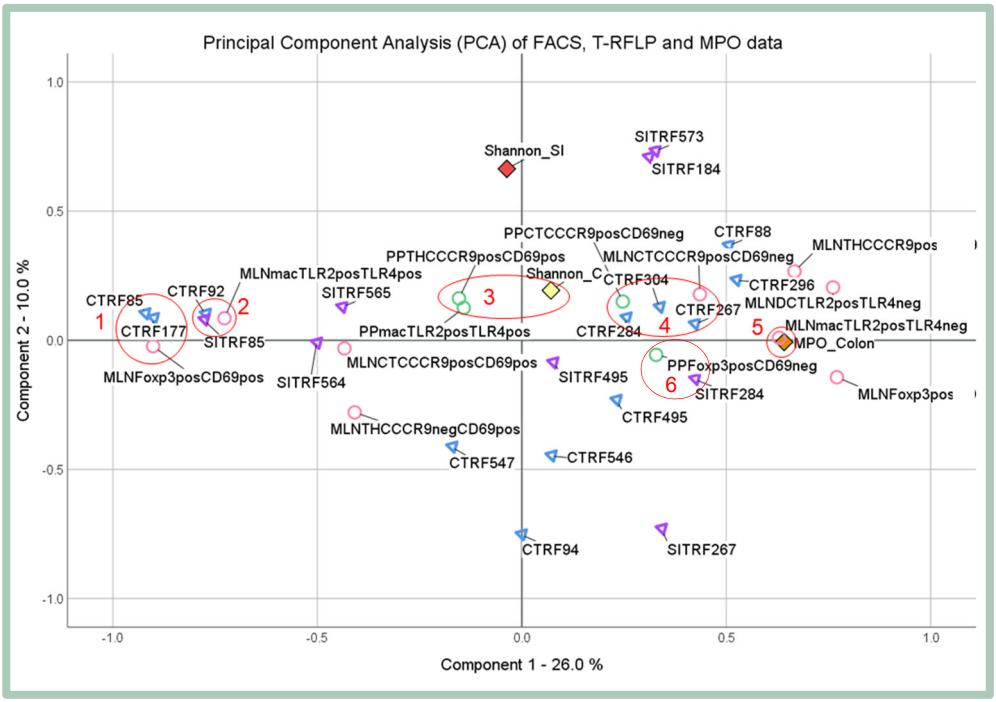


Figure 32.

Principal Component Analysis (PCA) of Shannon diversity index (DI) in colon (C♦) and small intestine (SI♦), MPO in colon (C♦), FACS data from Peyer's patches (PP○) and mesenteric lymph nodes (MLN○) as well as T-RFs from colon (C, ▲) and small intestine (SI▲). CD4_Foxp3 = regulatory T-cells, CD8_CCR9_CD69 = activated cytotoxic T-cells expressing homing to the gut, CD4_CD69 = activated T-helper cells, CD8_CCR9 = cytotoxic T-cells expressing homing to the gut, F480_TLR2 = macrophages expressing TLR2+, CD4_CCR9 = T-helper cells expressing homing to the gut, CD11c_TLR2 = marker for dendritic cells expressing TLR2+. T-RFs are shown as C for colon, SI for small intestine, followed by TRF and base pair length. The numbers 1 – 6 signifies areas of interest discussed in section 6.4.9.

6.4.9 Principal component analysis

A PCA was carried out to study if the analyses where significant results were found in the FACS analysis correlated with MPO, DI data, and T-RF analysis (KMO: 0.634, Bartlett's test: $p < 0.001$). T-RFs 85 and 177, which were only present in the control group, correlated positively with activated regulatory T-cells from MLN (Figure 32, circle 1). This is interesting because T_{regs} often are upregulated in patients with cancer, but are also known to regulate homeostasis and control potential inflammation induced by commensal bacteria in colon [82]. T-RF 92 from colon and 85 from small intestine, both only occurring in the control group, correlated with TLR2+TLR4+ macrophages from MLN (Figure 32, circle 2) which is interesting since the pattern recognition receptors TLR2 and TLR4 are associated with an increased CRC risk [46]. Activated T-helper cells expressing homing to the gut (CCR9+CD69+) and TLR2+TLR4+ macrophages from PP correlated with the DI in colon (Figure 32, circle 3), which may point towards that a high DI in this case might not be advantageous due to the association to an increased cancer risk with TLR2+TLR4+ cells, as previously mentioned. In circle 4, Figure 32, T-RFs 284 (found in all but MB and OPP groups), 304 (found in all but C group), and 267 (found in all groups) from colon grouped together with CD8+CCR9+ from MLN and PP respectively. MPO levels in colon correlated with TLR2+ macrophages from MLN (Figure 32, circle 5) and T-RF 284 in small intestine, present in DSS, SBT, SS and OS groups, grouped with T_{regs} in PP (Figure 32, circle 6). These findings might be clues to first immunological responses to inflammation upon consumption of processed meat but results are difficult to interpret due to the early stage of possible disease progression.

6.4.10 General discussion for paper IV

To induce a chronic inflammation in mice by only adding 20 % processed meat to the mouse feed would be far too time consuming and difficult considering the short life span of a mouse. Inducing a low-grade chronic inflammation using dextran sodium sulfate (DSS) has been used widely to investigate pathophysiological mechanisms in chronic inflammations [67] and facilitates studying the possible implication(s) meat consumption might have on health.

There were, as previously mentioned, no significant differences in DSS consumption between groups or cages, however seeing the DSS consumption in Figure 27 points towards that the consumption might inflict physiological differences in further analyses. Additionally, it cannot be excluded that high levels of DSS consumption may be a result of dripping from the drinking bottles, why a non-statistical difference in DSS-consumption instead could be regarded as a positive outcome (DSS-group cycle 1, cage 2 in Figure 27).

The animals were fed a standardised feed, naturally containing dietary fibre (Table 6) which is known to be protective against intestinal inflammation [50] due to the SCFA produced via fermentation [42]. It is possible that this may have had a positive impact on the intestinal health of the mice. Moreover, the meatballs used in this model were oxidised to a normally expected level from industrially produced products, since they contain regular ingredients in typically found proportions (Paper III). The main aim of this study was to screen initial effects of consuming processed meat products during a mild colonic inflammation. The risk of CRC increases with 19 % when consuming 50 g processed meat per day for humans [22]. This number was scaled down to fit the trial animals. However, individual differences in meat consumption could be expected since the animals were housed in groups of five, making it hard to map the exact consumption per mouse. The housing of five animals per cage was carried out for ethical reasons according to Swedish legislation, as was the presence of bedding material, the latter leading to difficulties in quantifying the exact amount of feed consumed per cage due to the hiding behaviour the animals have innately.

There were no differences in FI among groups which was a predictable result since the goal of the animal trial was to induce a low-grade chronic inflammation without crossing over to an acute colitis. Additionally, the number of animals differed greatly in FI among groups (Table 13) which may have added to the difficulty in analysing results statistically. The Shannon diversity index of colon was significantly higher ($p < 0.001$) in animals consuming meatballs enriched with plant phenols of summer savory (SS) (Table 14) as well as in the small intestine where SS and onion skin (OS) plant phenols showed to increase DI compared to other groups ($p < 0.001$). This change in microbial diversity at an early stage of inflammation and processed meat consumption is a promising finding which points towards that the aetiology of inflammation and/or CRC may be initialized by a response in the gut microbiota as previously found [36, 42]. Many differences in immunological responses analysed using FACS were detected although they are difficult to interpret at this early stage. Differing results for different phenotypes were found, which might imply that a pro-inflammatory response is initiated to protect against the induced chronic inflammation during meatball consumption. The addition of plant phenols, although at very low concentrations, seems to play a role either by acting as bioactive components physiologically [6], by acting as antimicrobial compounds [60], or by inhibiting the iron oxidation of fats in the meatball [83], thereby minimizing the ROS production and the health risks they come with.

7 Conclusions

7.1.1 Paper I

Many horticultural waste materials from crops are not harvested or are at harvest separated and left in the field, although they may be of value for different purposes. Handling these materials post-harvest could result in residues with high contents of bioactive compounds used upon refining. In Paper I, the aim was to screen a large number of plant materials from different crops with regards to their antioxidant capacities and their content of major phenolic compounds. The fact that atypical plant materials from food production could have high antioxidant properties is interesting but not new to the research field. However, the phenolic compounds that give rise to these antioxidant properties been identified in this study. A large variation was found among and within species, which emphasises the importance of proper choice of cultivar, its antioxidant capacity, as well as the content of specific phenolic compounds to increase its value for further use.

7.1.2 Paper II

The meat emulsion model system showed to be an effective matrix for mapping antioxidant capacities in different concentrations over time. However, the model needs further validation in future studies where the effects of phenol-rich plant material may be measured in a more optimised way. Overall, the plant materials and extracts successfully inhibited lipid oxidation with the highest efficacy at 200 ppm GAE and increasingly so over time where the summer savory powder, beetroot leaf sample and olive polyphenol samples inhibited oxidation down to 17.2 %, 16.6 % and 13.5 % respectively compared to the blank sample. Even at the low concentrations of 5, 10 and 20 ppm, samples showed lipid oxidation inhibiting capacities of importance.

7.1.3 Paper III

The meatball type that was found to be the most prone to oxidise was deep-fried, made of pork, contained 20 % fat and 2 % salt, and had been stored for 14 days. Hence, 11 plant materials and extracts were evaluated in this meatball type at two concentrations, 100 ppm and 200 ppm GAE, which were stored for 14 days. All samples showed to be

effective in inhibiting lipid oxidation in both tested concentrations. The summer savory powder was the most efficient in both concentrations, lowering lipid oxidation to 21.8 % (100 ppm) and 13.8 % (200 ppm), compared to meatballs with no added antioxidants. Thus, antioxidant rich plant materials and extracts could efficiently prevent lipid oxidation in meatballs highly prone to oxidise.

7.1.4 Paper IV

The screening in paper IV was conducted at an early stage of inflammation and together with meat consumption. Despite the short trial period, initial reactions could still be noticed in the diversity index of both colon and small intestine, as well as in T-RF composition between trial groups. Differences in immunological reactions could be seen between trial groups, where the SBT group differed significantly from other groups in a number of cases when analysing lymphocyte phenotypes, which is a step in the right direction for further studies. No differences were shown in MPO concentrations in small intestine, MDA, cytokine levels, or in some physical properties of the animals. The latter findings may indicate that the onset of potential disease does not begin with these physiological reactions, but might potentially be seen more clearly in later stages. The aetiology of colon inflammation and CRC is complex and multifactorial. Finding initial differences in immunological factors and gut microbiota could be of valuable interest for further studies during longer trial periods.

7.1.5 Overall conclusions of thesis work

Plant phenols derived from not commonly used sources showed to differ in content and antioxidant capacity between species and cultivars, showed to be effective antioxidants in both meat model system and meat product, and gave rise to significant differences in the composition of the intestinal microflora and immunological responses in mice. This thesis was built on four screenings in which both significant and non-significant results are expected, and many valuable results for further research were obtained.

8 Future perspectives

- **Paper I.** It would be interesting to evaluate other plant materials and extraction methods to optimise antioxidant potential for each specific plant derived preparation. Moreover, analysing plant phenol components prior to application in any matrix would be valuable to obtain the most successful combination of antioxidant versus matrix.
- **Paper II.** Similarly to the future perspectives of paper I, calculating the accurate concentration for each plant derived preparation (as opposed to three pre-determined concentrations as in this case), whilst being aware of the phenol composition, would be advantageous for proper application in any matrix. In the case of meat applications, the FRAP analysis would possibly be the antioxidant mode of action to rely on.
- **Paper III**. Other meat products of differing proportions of meat, fat, salt and cooking methods together with storage times would be interesting to study further. As well as testing plant phenols in already existing recipes which include other ingredients than the ones mentioned above to see if these play a role in the lipid oxidation reaction.
- **Paper IV**. A longer trial period would probably give more differences between trial groups. Moreover, in future research, it would be interesting to study the effect of combined plant phenols in both animal and human trials.

9 Acknowledgements

I would like to thank my supervisor **Åsa Håkansson** for her help and guidance throughout my PhD journey. Thank you for your kindness, for always caring and for all the chocolate and crackers I consumed in your office.

I would like to thank my co-supervisor **Kimmo Rumpunen** for his support and thoroughness throughout my article and thesis writing. Thank you for always being patient with me and for all our long statistical discussions.

I would like to thank my co-supervisor **Marilyn Rayner** (the 4th most cited food scientist in Sweden 2019) for her mental support during the last phase of my PhD journey. Thank you for your endless encouragements and for all the skunk skinning.

I would also like to thank **Eva Tornberg** for her supervision in the first part of my PhD studies. Thank you for teaching me to stand up for myself.

Many thanks to **Peter Eklöv** for all the help in administrative work, laughs and for all the jump scares. Thank you **Hans Bolinsson** and **Dan Johansson** for always helping me with machines and chemicals as well as all the good laughs at lunch, parties and coffee brakes. A special thanks to Hans for all the shoulder puns (1/10!).

I would like to thank all **seniors** at the department for interesting discussions, laughs, and support along the way.

Thank you **Anders Ekholm** for your chemical knowledge and for all the motorcycle rides.

I would like to thank **Jan-Eric Englund** and **Adam Flöhr** for their guidance through statistical jungles and for bravely trying to teach me how to run analyses in R.

Thank you **Åhus Grönt** at Horna gård, **Nyskördade morötter AB** and **Alléns organic farm** in Åhus for providing me with plant materials for antioxidant extraction.

I would also like to thank **Taxi Allians** in Kristianstad for the interesting morning rides to Balsgård.

Thank you to **Sofia Stüffe**, **Hasse Mathiason**, and **Alexandra Malmsjö** at Atria AB for your kind help and for providing me with meat for my studies.

I would like to thank all colleagues from Denmark, Finland, Latvia, and Estonia within the **SUSMEATPRO** collaboration for their help in manuscript revision and for achieving my results.

Many thanks to the colleagues at the Clinical Research Centre (CRC) in Malmö, **Anne-Marie Rohrstock** for her laboratory expertise and kindness, **Milladur Rahman** for his invaluable help during FACS analyses, and to all **PhD students** that happily gave a helping hand during my time in their lab.

A big thank you to the colleagues in the animal facility at CRC Malmö. Thank you **Gerry Jönsson** for your patience and help, **Henrik Åfors** for your guidance, and **Julia Eriksson** and **Lennart Olsson** for being such cheerful and helpful co-workers.

A special thanks to my wonderful master students, without whom this thesis would have been impossible to complete. Thank you **Katerina Tzima** and **Revekka Papaioannou** for your endless help and kindness and for teaching me how to do a real tzatziki. Thank you **Kajsa Granheimer** for your great work in both the meatball project and in the animal study. I am forever grateful for your help and I'm sorry I gave you nightmares. Thank you **Marine Rémy** for all your patience, your hard work during the meatball production for the animal study and for all the nice talks. Thank you **Tawanda Mandoga** and **Shandana Jois** for your endurance when helping me through the termination of the animal trial (by providing me with chocolate!). Thank you **Vandana Tannira** for helping me with the DNA-extractions and to **Yunjeong So** for your valuable help in the T-RFLP analyses.

A warm thank you to my fellow PhD students **Atma Sol**, **Catalina Fuentes**, **Cecy Curi**, **Daniel Osorio**, **Eda Demir**, **Grant Thamkaew**, **Klara Sjölin**, **Mukul Hossain**, **Nabilah Abdul**, **Paula Viola**, **Peyman Olad**, **Randi Phinney**, **Sander Perez**, **Shuai Bai**, **Vanesa Castro**, and **Yi Lu** for being fantastic colleagues, party animals, performers and friends during my PhD journey. Thank you to my three microbiologists and good friends who helped me immensely during my lab works despite risking to give birth, being deported, or succumb to an anaphylactic shock, **Bettz Uhlig**, **Pam Canaviri Paz** and **Elin Oscarsson** (respectively). A huge thank you to my silverfish-party partner **Ida-Marie Andersson** (Pulvrén) and crazy cat lady equal **Kajsa Nilsson**, as well as my dear friend and psychiatrist **Maria Rosenlöw** who helped me through rough times with support and encouragement. A big gracias to my dear friend **Juanito Salinas Andrade** for our long and emotional talks along the way.

Thank you **Shia Labeouf** for your motivational speech when my motivation for statistical analyses was low (youtube.com/watch?v=ZXsQAXx_ao0).

Thank you **Hoch Tirol** for your song "Konis Hupen" and for celebrating all disco Fridays with me (and all co-workers although unwillingly).

Thank you **Dr. Todd Grande** for all statistical help with SPSS via Youtube.

A special thank miou to my cats **Kini**, **Murmel** and **Louie**, for the oxytocin releases and warm love throughout my journey.

I would like to thank my aunt **Nettis Fridh** for always believing in me.

Thank you **Björken Bjurling** for being my friend and for supporting me for all these years.

Thank you **Trellebål Niskanen** for being my number one fan, listening to me endlessly, whilst providing me with huge amounts of coffee and wine.

Thank you **Kaky Levall**, for being there for me through hard times and forever encouraging me to pursue a career in your field of expertise.

Thank you to my very best friend and cousin **Palle Ibraim** for knowing me the best of all. Thank you for your love and caring that gets me through life.

Thank you to my one and only **Ronas Benjaminsson Nielsen** for keeping my feet on and off the ground, taking care of me, and for loving me no matter what.

Thank you to my brother and idol **Jaff Burri** for always having my back and supporting me through everything I do. Thank you for the love you and **Maika Beato** and my little **Alba Burri Beato** have for me.

The warmest of thank yous to my mother **Ewa Linde-Burri** and my father **Philippe Poupou Burri**, my pillars in life, for the strongest love and support of all.

Finally, this doctoral project was funded by the **Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning** (grant 222-2014-1923). So thank you for that!

10 References

1. Norat T, Bingham S, Ferrari P, Slimani N, Jenab M, Mazuir M, et al. Meat, fish and colorectal cancer risk: the European prospective investigation into cancer and nutrition (EPIC). Journal of the National Cancer Institute. 2005; [97]: 906-16.
2. Tomé D, Chan DSM, Lau R, Aune D, Vieira R, Greenwood DC, et al. Red and Processed Meat and Colorectal Cancer Incidence: Meta-Analysis of Prospective Studies. PLoS ONE. 2011; [6] (6): e20456.
3. Norat T, Scoccianti C, Boutron-Ruault MC, Anderson A, Berrino F, Cecchini M, et al. European Code against Cancer 4th Edition: Diet and cancer. Cancer Epidemiology. 2015; [39 Suppl 1]: S56-66.
4. Micha R, Khatibzadeh S, Shi P, Andrews KG, Engell RE, Mozaffarian D, et al. Global, regional and national consumption of major food groups in 1990 and 2010: a systematic analysis including 266 country-specific nutrition surveys worldwide. BMJ Open. 2015; [5] (9): e008705.
5. Kumar Y, Yadav DN, Ahmad T, Narsaiah K. Recent trends in the use of natural antioxidants for meat and meat products. Comprehensive Reviews in Food Science and Food Safety. 2015; [14]: 796-812.
6. Jiang J, Xiong YL. Natural antioxidants as food and feed additives to promote health benefits and quality of meat products: A review. Meat Science. 2016; [120]: 107-17.
7. Barbut S. The science of poultry and meat processing. Guelph, Ontario, Canada 2015.
8. Lawrie RA, Ledward DA. Lawrie's meat science. 7th ed. Camebridge, England: Woodhead publishing limited; 2006.
9. Faustman C, Sun Q, Mancini R, Suman SP. Myoglobin and lipid oxidation interactions: Mechanistic bases and control. Meat Science. 2010; [86]: 86-94.
10. Enser M, Hallett K, Hewitt B, Fursey GA, Wood JD. Fatty acid content and composition of english beef, lamb and pork at retail. Meat Science. 1996; [42] (4): 443-56.
11. Amaral AB, da Silva MV, Lannes SCdS. Lipid oxidation in meat: mechanisms and protective factors - a review. Food Science and Technology. 2018; [38] (1): 15.
12. Pereira FAL, Abreu GVK. Lipid Peroxidation in Meat and Meat Products. In: Catala A, Mansour MA, editors. Lipid peroxidation, Edited Volume. London, United Kingdom: IntechOpen; 2018.
13. Frankel EN. Lipid oxidation. Progress in Lipid Research. 1980; [19]: 1-22.

14. Binnie MA, Barlow K, Johnson V, Harrison C. Red meats: Time for a paradigm shift in dietary advice. Meat Science. 2014; [98]: 445-51.
15. Oostindjer M, Alexander J, Amdam GV, Andersen G, Bryan SN, Chen D, et al. The role of red and processed meat in colorectal cancer development: a perspective. Meat Science. 2014; [97]: 583-96.
16. Joosen AM, Kuhnle GG, Aspinall SM, Barrow TM, Lecommandeur E, Azqueta A, et al. Effect of processed and red meat on endogenous nitrosation and DNA damage. Carcinogenesis. 2009; [30]: 1402-7.
17. Ishikawa S, Tamaki S, Ohata M, Arihara K, Itoh M. Heme induces DNA damage and hyperproliferation of colonic epithelial cells via hydrogen peroxide produced by heme oxygenase: A possible mechanism of heme-induced colon cancer. Molecular Nutrition and Food Research. 2010; [54]: 1182-91.
18. Domingo JL, Nadal M. Carcinogenicity of consumption of red meat and processed meat: A review of scientific news since the IARC decision. Food and Chemical Toxicology. 2017; [105]: 256-61.
19. Kim E, Coelho D, Blachier F. Review of the association between meat consumption and risk of colorectal cancer. Nutrition Research. 2013; [33]: 983-94.
20. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians. 2018; [68] (6): 394-424.
21. Ferlay J, Steliarova-Foucher E, Lortet-Tiulent J, Rosso S, Coebergh JWW, Comber H, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. European Journal of Cancer. 2013; [49]: 1374-403.
22. IARC Monographs evaluate consumtion of red meat and processed meat [press release]. Lyon, France, 26 October 2015 2015.
23. Ferlay J, Colombet M, Soerjomataram I, Dyba T, Randi G, Bettio M, et al. Cancer incidence and mortality patterns in Europe: Estimates for 40 countries and 25 major cancers in 2018. European Journal of Cancer. 2018; [103]: 31.
24. Cascella M, Bimonte S, Barbieri A, Del Vecchio V, Caliendo D, Schiavone V, et al. Dissecting the mechanisms and molecules underlying the potential carcinogenicity of red and processed meat in colorectal cancer (CRC): an overview on the current state of knowledge. Infectious Agents and Cancer. 2018; [13]: 3.
25. Kanner J. Dietary advanced lipid oxidation endproducts are risk factors to human health. Molecular Nutrition and Food Research 2007; [51]: 1094-101.
26. Fodde R. The APC gene in colorectal cancer. European Journal of Cancer. 2002; [38]: 867-71.
27. Demeyer D, Honikel K, De Smet S. The world cancer research fund report 2007: A challenge for the meat processing industry. Meat Science. 2008; [80]: 953-9.

28. Hammerling U, Bergman Laurila J, Grafström R, Ilbäck N-G. Consumption of Red/Processed Meat and Colorectal Carcinoma: Possible Mechanisms Underlying the Significant Association. Critical Reviews in Food Science and Nutrition. 2016; [56] (4): 20.
29. Kamada N, Seo S-U, Chen GY, Núñez G. Role of the gut microbiota in immunity and inflammatory disease. Nature reviews: Immunology. 2013; [13] (5).
30. Håkansson Å, Molin G. Gut microbiota and inflammation. Nutrients. 2011; [3]: 637-82.
31. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. Science. 2012; [336] (6086): 1268-73.
32. Opal SM, DePalo VA. Anti-inflammatory cytokines. Impact of Basic Research on Tomorrow's Medicine. 2000; [117] (4): 1162-72.
33. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nature reviews: Immunology. 2014; [14] (3): 141-53.
34. Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nature reviews: Immunology. 2008; [8]: 411-20.
35. Janakiram NB, Chinthalapally VR. The Role of Inflammation in Colon Cancer Springer Basel Heidelberg New York Dordrecht London: Springer Basel 2014; 2014.
36. Louis P, Hold GL, Flint HJ. The gut microbiota, bacterial metabolites and colorectal cancer. Nature reviews microbiology. 2014; [Advanced online publication].
37. Feagins LA, Souza RF, Spechler SJ. Carcinogenesis in IBD: potential targets for the prevention of colorectal cancer. Nature Reviews Gastroenterology &Amp; Hepatology. 2009; [6]: 297.
38. Olén O, Askling J, Sachs MC, Frumento P, Neovius M, Smedby KE, et al. Increased mortality of patients with childhood-onset inflammatory bowel diseases, compared with the general population. Gastroenterology. 2019: 1-9.
39. Olén O, Askling J, Sachs MC, Neovius M, Smedby KE, Ekbom A, et al. Mortality in adult-onset and elderly-onset IBD: a nationwide register-based cohort study 1964-2014. Gut. 2019.
40. Atreya I, Neurath MF. Immune cells in colorectal cancer: prognostic relevance and therapeutic strategies. Expert Review of Anticancer Therapy. 2008; [8] (4): 561-72.
41. Murray PR, Rosenthal KS, Pfaller MA. Medical microbiology. 7th ed: Saunders, Elsevier Inc.; 2013.
42. Vivarelli S, Salemi R, Candido S, Falzone L, Santagati M, Stefani S, et al. Gut microbiota and cancer: from pathogenesis to therapy. Cancers. 2019; [11] (38): 1-26.
43. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. Nature Neuroscience. 2017; [20] (2): 145-55.

44. Jeffery IB, Lynch DB, O'Toole PW. Composition and temporal stability of the gut microbiota in older persons. International Society for Microbial Ecology. 2016; [10]: 170-82.
45. Azimi T, Javad Nasiri M, Salimi Chirani A, Pouriran R, Dabiri H. The role of bacteria in the inflammatory bowel disease development: a narrative review. Journal of Pathology, Microbiology and Immunology. 2018.
46. Sheflin AM, Whitney AK, Weir TL. Cancer-promoting effects of microbial dysbiosis. Current Oncology Reports. 2014; [16] (406): 1-9.
47. Samraj AN, Pearce OM, Laubli H, Crittenden AN, Bergfeld AK, Banda K, et al. A red meat-derived glycan promotes inflammation and cancer progression. Proc Natl Acad Sci U S A. 2015; [112] (2): 542-7.
48. Tomova A, Bukovsky I, Rembert E, Yonas W, Alwarith J, Barnard ND, et al. The effects of vegetarian and vegan diets on gut microbiota. Frontiers in Nutrition. 2019; [6]: 1-10.
49. WCRF. World Cancer Research Fund International/ American Institute for Cancer Research. Continous update project report: Diet, Nutrition, Physical activity, and colorectal cancer. American Institute For Cancer Research; 2017.
50. Vulcan A. Nutrition and colorectal cancer - The role of BMI, sex, biomarkers and dietary index [Doctoral dissertation]. Lund: Lund University; 2018.
51. Pogorzelska-Nowicka E, Atanasov AG, Horbanczuk J, Wierzbicka A. Bioactive Compounds in Functional Meat Products. Molecules. 2018; [23] (2).
52. Bunaciu AA, Aboul-Enein HY, Fleschin S. FTIR Spectrophotometric methods used for antioxidant activity assay in medicinal plants. Applied Spectroscopy Reviews. 2012; [47] (4): 245-55.
53. Dai J, Mumper RJ. Plant phenolics: Extraction, Analysis and their antioxidant and anticancer properties. Molecules. 2010; [15]: 7313-52.
54. Shahidi F, Wanasundara PK. Phenolic antioxidants. Critical Reviews in Food Science and Nutrition. 1992; [32] (1): 67-103.
55. Kala KH, Mehta R, Tandey R, Sen KK, Mandal V. Ten years of research on phenolics (2005-2015): A status report. Pacific Science Review A: Natural Science and Engineering. 2016; [18]: 1-4.
56. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Bological efects of essential oils - A review. Food and Chemical Toxicology. 2008; [46]: 446-75.
57. Bozinou E, Karageorgou I, Batra G, Dourtoglou VG, Lalas SI. Pulsed electric fields extraction and antioxidant activity determination of *Moringa oleifera* dr leaves: A comparative study with other extraction techniques. Beverages. 2019; [5] (8): 1-13.
58. Eghbaliferiz S, Iranshahi M. Prooxidant Activity of Polyphenols, Flavonoids, Anthocyanins and Carotenoids: Updated Review of Mechanisms and Catalyzing Metals. Phytotherapy Research. 2016; [30] (9): 1379-91.

59. Rahal A, Kumar A, Singh V, Yadav B, Tiwari R, Chakraborty S, et al. Oxidative stress, prooxidants, and antioxidants: the interplay. BioMed Research International. 2014; [2014]: 761264.
60. Radenkovs V, Püssa T, Juhnevica-Radenkova K, Anton D, Seglina D. Phytochemical characterization and antimicrobial evaluation of young leaf/shoot and press cake extracts from Hippophae rhamnoides L. Food Bioscience. 2018; [24]: 10.
61. Morgenstern A, Ekholm A, Scheewe P, Rumpunen K. Changes in content of major phenolic compounds during leaf development of sea buckthorn (Hippophaë rhamnoides L.). Agricultural and Food Science. 2014; [23]: 2017-219.
62. Singleton VL, Orthoffer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology. 1999; [299]: 152-78.
63. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine. 1999; [26] (9): 1231-7.
64. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidants power": The FRAP assay. Analytical Biochemistry. 1996; [239]: 70-6.
65. Buege AJ, Aust SD. Microsomal Lipid Peroxidation. Methods in Enzymology. 1978; [52]: 302-10.
66. Granheimer K. Different parameters affecting lipid oxidation in meatballs [Master Thesis]. Lund, Sweden: Lund University; 2017.
67. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. Nature Protocols. 2007; [2] (3): 541-6.
68. Bylund-Fellenius AC, Landström E, Axelsson LG, Midtvedt T. Experimental Colitis Induced by Dextran Sulphate in Normal and Germfree Mice. Microbial Ecology in Health and Disease. 1994; [7] (4): 207-15.
69. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran Sulfate Sodium (DSS)-induced Colitis in Mice. Current Protocols in Immunology. 2015; [104] (15.25): 1-16.
70. Murthy S, Murthy NS, Coppola D, Wood DL. The efficacy of BAY y 1015 in dextran sulfate model of mouse colitis. Inflammation Research. 1997; [46]: 224-33.
71. Håkansson Å, Bränning C, Molin G, Adawi D, Hagslätt M-L, Jeppsson B, et al. Blueberry Husks and Probiotics Attenuate Colorectal Inflammation and Oncogenesis, and Liver Injuries in Rats Exposed to Cycling DSS-Treatment. PLoS ONE. 2012; [7] (3): 1-14.
72. Klebanoff SJ. Myeloperoxidase. Proceedings of the Association of American Physicians. 1999; [111] (5): 383-9.
73. Osman N, Adawi D, Molin G, Ahrne S, Berggren A, Jeppsson B. Bifidobacterium infantis strains with and without a combination of Oligofructose and Inulin (OFI)

attenuate inflammation in DSS-induced colitis in rats. BMC Gastroenterology. 2006; [6] (31).

74. Karlsson C. The Gut Bacterial Flora - Focus on Early Life and Physiological Traits [Doctoral Dissertation]. Lund, Sweden: Lund University; 2011.
75. Mizuno M, Tsuchida H, Kozukue N, Mizuno S. Rapid quantitative analysis and distribution of free quercetin in vegetables and fruits. Nippon Shokuhin Kogyo Gakkaishi. 1992; [39] (1): 88-92.
76. Hayes JE, Stepanyan V, Allen P, O'Grady MN, O'Brien NM, Kerry JP. The effect of lutein, sesamol, ellagic acid and olive leaf extract on lipid oxidation and oxymyoglobin oxidation in bovine and porcine muscle model systems. Meat Science. 2009; [83] (2): 201-8.
77. Lee SK, Han JH, Decker EA. Antioxidant activity of phosvitin in phosphatidylcholine liposomes and meat model systems. Food Chemistry and Toxicology. 2002; [67] (1): 37-41.
78. Hedrick HB, Aberle ED, Forrest JC, Judge MD. Principles of Meat Science. 3rd ed 2013.
79. Haak L, Sioen I, Raes K, Camp J, Smet S. Effect of pan-frying in different culinary fats on the fatty acid profile of pork. Food Chemistry. 2007; [102] (3): 857-64.
80. Lindberg-Madsen H, Andersen L, Christiansen L, Brockhoff P, Bertelsen G. Antioxidative activity of summer savory (*Satureja hortensis* L.) and rosemary (*Rosmarinus officinalis* L.) in minced, cooked pork meat. Zeitschrift für Lebensmittel-Untersuchung und -Forschung. 1996; [203]: 333-8.
81. Fasseas MK, Mountzouris KC, Tarantilis PA, Polissiou M, Zervas G. Antioxidant activity in meat treated with oregano and sage essential oils. Food Chemistry. 2008; [106] (3): 1188-94.
82. Adeegbe DO, Nishikawa H. Natural and Induced T regulatory cells in cancer. Frontiers in Immunology. 2013; [4] (7): 1-14.
83. Burri CMS, Granheimer K, Rémy M, Ekholm A, Håkansson Å, Rumpunen K, et al. Lipid oxidation inhibition capacity of 11 plant materials and extracts evaluated in highly oxidised cooked meatballs. Foods. 2019; [8] (406): 1-1410.3390/foods8090406.

Printed by Media-Tryck, Lund 2019 NORDIC SWAN ECOLABEL 3041 0903

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LUND UNIVERSITY

ISBN 978-91-7895-300-4
Food Technology,
Engineering and Nutrition
Faculty of Engineering
Lund University