Probiotics and berry-associated polyphenols: catabolism and antioxidative effects

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Food Hygiene
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Cover: bilberry, chokeberry, rosehips, *Lactobacillus* and chemical structure of anthocyanin

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Abstract

Oxidative stress can cause damage to DNA, proteins and lipids and is associated with inflammation and various human diseases as cancer, atherosclerosis, and autoimmune diseases. Polyphenol-rich diet, such as fruits and berries, may act as antioxidants and prevent oxidative stress and, thereby, associated diseases. Administration of lactic acid bacteria (LAB) can affect the microflora in the gastrointestinal (GI) tract and may increase the capability of the bacterial flora to digest polyphenols. Some strains of *Lactobacillus* may break down phenolic acids and hydrolyzable tannins into phenolic metabolites that are more easily absorbed in the body and may enhance antioxidative effects. The aim of this thesis was to clarify the protective effects of polyphenol-rich fruits and berries alone or in combination with different strains of LAB on oxidative stress in mice. Furthermore, transformation of polyphenols in a bilberry beverage by LAB was examined.

Supplementation with rosehips of the rose species *Rosa pimpinellifolia* or an LAB mixture decreased lipid peroxidation and oxidative stress in colon of mice after ischemia-reperfusion (I/R) injury. Adding an LAB supplement to the rosehips increased the concentrations of phenolic compounds, antioxidative capacity and total phenolic content in cecum. Rosehips of *R. piminellifolia* are a rich source of cyanidin-3-O-glucoside and this compound and its degradation product, protocatechuic acid, were detected in the cecum content.

Administration of bilberry, either alone or together with *Lactobacillus plantarum* HEAL19, decreased lipid peroxidation and oxidative stress in colon of mice after I/R injury. A chokeberry-supplement showed no antioxidative effect. Bilberry was found to have a more complex anthocyanin profile than chokeberry. Higher concentrations and a more varied composition of anthocyanins were seen in colon than in cecum. More phenolic metabolites were found in the intestines of bilberry-fed mice than in the chokeberry-fed ones. Chokeberry or bilberry alone decreased the number of LAB on the colonic mucosa but addition of *L. plantarum* HEAL19 prevented this reduction.

In a more extensive ischemia-reperfusion injury, diet supplemented with bilberry, but without addition of different LAB strains, reduced lipid peroxidation and protected the small intestine against oxidative stress. The highest concentration and recovery of anthocyanins was seen in the ileal content followed by that of

colon and finally cecum. Anthocyanin arabinosides, and especially malvidin-3-O-arabinoside, were accumulated in the colon content. Glucosides and galactosides of malvidin, peonidin and petunidin seemed to be digested by the microflora in the cecum. Supplementation of bilberry to the diet influenced the composition of cecum microflora.

Anthocyanins in bilberry beverages inoculated with different LAB strains, alone or in combination with wine yeast, decreased during 3 weeks incubation at 30°C. Arabinosides of malvidin and petunidin showed the greatest decrease. Addition of yeast improved the stability of the anthocyanins. In contrast to anthocyanins, quercetin, quercetin-3-glucoside and detected phenolic acids were relatively stable. Antioxidative capacity and total phenolic content decreased in all samples.

In conclusion, dietary supplementation of rosehips from *Rosa pimpinellifolia* or bilberry suppressed oxidative stress in colonic tissue of mice. Protective effects may be due to the high anthocyanin content, presence of phenolic metabolites and changed microflora. Addition of LAB improved status of the colonic but not the ileal tissue.

List of Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals.

Paper I
Jakesevic, M., Håkansson, Å., Adawi D., Jeppsson, B., Rumpunen, K., Ekholm, A., Ahrné, S. and Molin, G. (2009). Antioxidative protection of dietary rosehips and polyphenol active lactobacilli in mice subjected to intestinal oxidative stress by ischemia-reperfusion. *Microbial Ecology in Health and Disease* 21:193–202.

Paper II
Jakesevic, M., Aaby, K., Borge, G-I., Jeppsson, B., Ahrné, S. and Molin, G. (2011). Antioxidative protection of dietary bilberry, chokeberry and *Lactobacillus plantarum* HEAL19 in mice subjected to intestinal oxidative stress by ischemia-reperfusion. *BMC Complementary and Alternative Medicine*, 11: (8)
http://www.biomedcentral.com/1472-6882/11/8

Paper III
Jakesevic, M., Aaby, K., Borge, G-I., Rumpunen, K., Ahrné, S. and Molin, G. (2010). Phenolics in bilberry (*Vaccinium myrtillus*) beverage incubated with lactic acid bacteria and yeast (in manuscript).

Paper IV
Jakesevic, M., Xu, J., Aaby, K., Jeppsson, B., Ahrné, S. and Molin, G. (2010) Effects of bilberry (*Vaccinium myrtillus*) in combination with lactic acid bacteria on intestinal oxidative stress induced by ischemia-reperfusion in mouse (in manuscript).

The author´s contribution to the papers

Paper I — The author, Maja Jakešević (MJ), coordinated and performed the experimental work together with Åsa Håkansson and Diya Adawi. MJ performed the analysis regarding malondialdehyde, analysis of intestinal microflora by randomly amplified polymorphic DNA and viable count, analyses of total phenolic content and antioxidative capacity. MJ performed the analysis of polyphenols together with Anders Ekholm. MJ evaluated the results and wrote the paper.

Paper II — The author, Maja Jakešević, coordinated and performed the experimental work together with Åsa Håkansson, and performed the analysis of malondialdehyde and viable count. Analyses of anthocyanins and phenolic metabolites were performed by MJ in collaboration with Kjersti Aaby. MJ evaluated the results and wrote the paper.

Paper III — The author, Maja Jakešević, coordinated and performed the experimental work, performed the analyses of pH and alcohol content, analyses regarding total phenolic content and antioxidative capacity. MJ performed analysis of polyphenols in collaboration with Kjersti Aaby and Grethe Iren Borge. MJ evaluated the results and wrote the manuscript.

Paper IV — The author, Maja Jakešević, coordinated and performed the experimental work together with Siv Ahrné, performed the analysis regarding malondialdehyde, myeloperoxidase, and histology samples. Analysis of anthocyanins was performed by MJ together with Kjersti Aaby. MJ evaluated the results and wrote the manuscript.

Abbreviations

I/R	Ischemia-reperfusion
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
MDA	Malondialdehyde
MPO	Myeloperoxidase
RAPD	Randomly Amplified Polymorphic DNA
T-RFLP	Terminal Restriction Fragment Length Polymorphism
H&E	Hematoxylin and Eosin
FRAP	Ferric Reducing Activity Power
TP	Total phenolic content
PCR	Polymerase Chain Reaction
CFU	Colony-forming units
DAD	Diode array absorbance detector
ESI	Electrospray ionization
HPLC	High-Performance Liquid-Chromatography
MS	Mass Spectroscopy
UV-vis	Ultraviolet-visible light
GI	Gastrointestinal
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
GRx	Glutathione reductase
GSH	Glutathione

XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
SMA	Superior Mesenteric Artery
SCFA	Short-chain fatty acids
LAB	Lactic acid bacteria
TBARS	Thiobarbituric acid-reacting substances
TFA	Trifluoroacetic acids
ICAM-1	Intercellular adhesion molecule
VCAM-1	Vascular cellular adhesion molecule
PCA	Principal component analysis

Contents

Abstract 3
List of papers 5
The author´s contribution to the papers 6
Abbreviations 7
Contents 9
Introduction 11
Background 13
Oxidation and antioxidants 13
Intestinal ischemia-reperfusion 15
Polyphenols 17
Flavonoids and phenolic acids 19
Absorption and metabolism 22
Berries 24
Chokeberry 24
Bilberry 25
Rosehip 26
Intestinal microflora 27
Development of microflora 28
Functions of the microflora 29
Probiotics 30
Aims 33
Methodology 34
Experimental design 34
Intestinal ischemia-reperfusion (I/R) procedure 38

Analyses 38
Extraction of phenolic comppounds 38
Analysis of phenolic compounds 39
Ferric reducing activity power (FRAP) 40
Total phenolic content (TP) 40
Malondialdehyde (MDA) 41
Myeloperoxidase (MPO) 41
Histopathology 41
Randomly amplified polymorphic DNA (RAPD) 42
Terminal restriction fragment length polymorphism (T-RFLP) 42
Statistics 42
Results and discussion 44
Polyphenols 44
Anthocyanins 44
Phenolic compounds other than anthocyanins 49
Antioxidative capacity and total phenolic content 52
Inflammatory markers 53
Ischemia-reperfusion 53
Malondialdehyde (MDA) 54
Myeloperoxidase (MPO) 60
Histopathology 61
Bacteriology 61
Lactobacilli and Enterobacteriaceae 61
The composition of the bacterial microflora 62
Conclusions 64
Populärvetenskaplig sammanfattning 66
Acknowledgements 69
References 71

Introduction

Oxidative stress occurs when oxygen free radicals are overproduced in the cells. It can cause damage to DNA, proteins and lipids and is associated with inflammation and various human diseases such as cancer, type-2 diabetes and cardiovascular diseases. Furthermore, oxidative stress and inflammation increase intestinal permeability, allowing translocation of pathogenic bacteria and endotoxins to extraintestinal sites which may lead to systemic inflammation, sepsis and eventually multiple-organ failure. Antioxidant therapy has been suggested to prevent and attenuate oxidative stress and thereby associated diseases. Polyphenols are secondary metabolites in plants. They are proposed to act as antioxidative and anti-inflammatory agents. Fruits and berries are important dietary sources of phenolic compounds. Antimicrobial properties of polyphenols may influence the composition and function of the indigenous intestinal microflora by inhibiting certain bacterial groups (those sensitive to the antimicrobial effect of polyphenols) and promote others (those more resistant to poyphenols). Hopefully the sensitive ones are also those with negative effects on the health and the resistant ones are bacterial groups with health-promoting effects. The bacterial flora of the gut can influence the nutritional status and the health of the host via modulation of metabolic functions. Polyphenols that are not absorbed in the small intestine can be degraded by colonic microflora to simpler and more easily absorbable bioactive compounds with potential physiological effects.

Dietary supplements probiotics (microorganisms with health-promoting properties after ingestion) may, at least theoretically, change the intestinal microflora and increase the number of polyphenol-degrading groups in the intestines, at least if a probiotic bacterium capable of degrading polyphenols has been chosen. Furthermore, probiotics may mitigate the inflammation by promoting the normalization of intestinal microflora and exclusion of pathogens, decreasing intestinal permeability, improving the intestine´s immunological barrier functions and alleviating the intestinal inflammatory response.

A polyphenol-rich diet and supplementation with probiotics may improve the intestinal status and possibly attenuate oxidative stress and suppress inflammation.

The work described in this thesis is devoted to the study of antioxidative and anti-inflammatory effects of the diet when supplemented with polyphenol-rich fruits and berries and potential probiotic strains in an oxidative stress model in mice. All

bacterial supplements involved lactic acid bacteria, mostly *Lactobacillus* strains with tannase activity. The influence of potential probiotic strains on transformation of polyphenols is also investigated.

Background

Oxidation and antioxidants

A free radical is any atom or molecule that contains one or more unpaired electrons, which makes it highly reactive with other atoms and molecules (Halliwell, 1994a). Oxidation is a free radical chain reaction comprising initiation, propagation and termination. Reactive oxygen species (ROS) include not only oxygen-centered free radicals such as superoxide (O_2^-), peroxyl (ROO^-), alkoxyl (RO^-) and hydroxyl (OH^-) but also some nonradical derivates, e.g. hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Nitric oxide (NO^-) is one of the more important free radicals known as reactive nitrogen species (RNS) (Pietta, 2000; Shahidi *et al*, 2010; Halliwell, 1994b). The chemical reactivity of free radicals varies greatly, with OH^- being the most reactive one.

In biological systems, ROS are involved in energy production, synthesis of biologically important compounds, regulation of cell growth and intracellular signaling (Pietta, 2000). They are also an important part of immune defense since phagocytes (neutrophils, monocytes, macrophages, eosinophils) produce large amounts of O_2^- and H_2O_2 to kill bacteria and fungi and to inactivate virus (Halliwell, 1997; Pietta, 2000). Some physiopathological situations such as cigarette smoke, air pollutants, UV radiation, high polyunsaturated fatty acid diet, drugs, inflammation and ischemia-reperfusion lead to overproduction of ROS, known as oxidative stress (Halliwell, 1997; Pietta, 2000). Oxidative stress can cause damage to DNA, proteins in tissues and lipids in cell membranes and is associated with different human diseases such as cancer, atherosclerosis, chronic inflammation, neurodegenerative diseases (Parkinson´s Disease and Alzheimer´s Disease), rheumatoid arthritis and tissue injury (Halliwell, 1997; Halliwell, 1994b; Pietta, 2000).

As a protection against ROS, the human body has evolved an antioxidant system in the form of endogenous antioxidative enzymes, e.g. superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GRx) and nonenzymatic antioxidant defenses, e.g. glutathione (GSH), α-tocopherol,

ascorbic acid, iron-binding proteins transferrin and ferritin, histidine-peptides, urate and plasma-protein thiols (Halliwell, 1994a; Pietta, 2000; Shahidi *et al*, 2010). SOD enzymes convert O_2^- to H_2O_2. Generated H_2O_2 is then removed by GPx enzymes that contain selenium at their active site. Superoxide and hydrogen peroxide are not so reactive chemically but if brought into contact with iron and/or copper ions they generate the highly reactive and harmful OH^- that attack and damage almost all molecules in living cells (Halliwell, 1994a; Halliwell, 1997; Pietta, 2000). The human body does not synthesize an overwhelming excess in its antioxidant defense system but seems to aim at a balance between production of ROS and levels of antioxidant protection.

Since the body´s endogenous antioxidant defenses are not always sufficient to prevent oxidative stress, dietary antioxidants can be needed for diminishing the cumulative effects of oxidative damage. Antioxidants are substances that, when present at low concentrations compared to that of an oxidizable substrate, markedly delay or prevent its oxidation (Shahidi *et al*, 2010). In addition to providing vitamins C, E, A and carotenoids, plant polyphenols are important antioxidants derived from the diet (Pulido R, 2000; Pietta P-G, 2000). Different mechanisms by which antioxidants may exert their inhibitory effects against oxidation include free radical scavenging, chelation of metal ions, inactivation of peroxides and other ROS and inhibition of pro-oxidative enzymes (Pulido *et al*, 2000; Shahidi *et al*, 2010). Based on their mode of action, antioxidants are broadly divided into primary and secondary antioxidants. Most polyphenols are primary antioxidants, so they break the chain reaction of oxidation and neutralize free radicals by donating a hydrogen atom. The resulting antioxidant radicals are stabilized by delocalization of the unpaired electron around the phenol ring to form stable resonance hybrids. These radicals have low reactivity and generally do not initiate creation of new radicals (Shahidi *et al*, 2010).

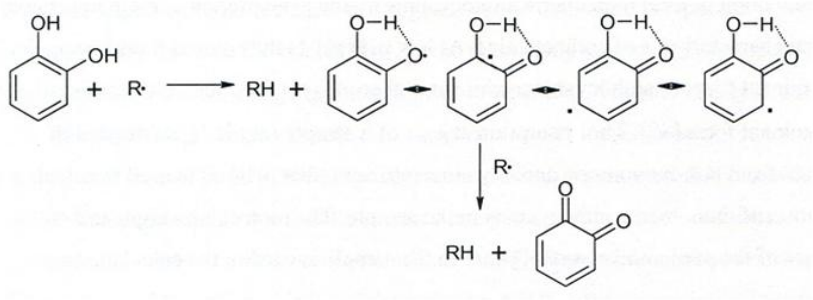


Figure 1. Scavenging of free radicals (R•) by a phenol (catechol) (Shahidi *et al*, 1992)

Intestinal ischemia-reperfusion

Ischemia-reperfusion (I/R) injury occurs when the blood supply returns (reperfusion) to a tissue that temporarily has been deprived of blood supply (ischemia) and triggers an intense inflammatory response caused by oxidative stress (Arumugam *et al*, 2004; Ozkan *et al*, 2009; Thomson *et al*, 1998). Lack of oxygen during the ischemic phase causes multiple cellular and metabolic changes such as acidosis due to anaerobic metabolism and increased production of lactic acid, depletion of energy-rich adenosine 5´-triphosphate (ATP), altered ion distribution favoring intracellular increase of calcium, sodium and water causing cell swelling, leakage and eventually cellular death (Collard & Gelman, 2001; Grace, 1994; Mallick *et al*, 2004). Furthermore, during ischemia, cellular ATP is degraded to hypoxanthine. Xanthine dehydrogenase (XDH) that normally oxidizes hypoxanthine to xanthine, is converted to xanthine oxidase (XO) during the ischemic period. This leads to accumulation of XO and hypoxanthine in ischemic tissue (Cerqueira *et al*, 2005; Grace, 1994; Mallick *et al*, 2004).

During reperfusion, oxygenated blood returns to the tissue and initiates a reaction that causes greater damage than the ischemic phase. Reintroduced oxygen reacts with hypoxanthine and XO to produce a burst of ROS such as O_2^- and H_2O_2. Furthermore, the most reactive and harmful OH^- is produced. The most damaging effect of ROS is lipid peroxidation of the polyunsaturated fatty acids and phospholipids in cell membranes causing alternations in their structure and permeability (Kellog, 1975; Slater, 1984; Cerqueira *et al*, 2005).

Besides participating in lipid peroxidation, the ROS are involved in attraction and activation of leukocytes at the sites of injury (Santen, 2008; Riaz, 2002; Cerqueira *et al*, 2005). Leukocyte recruitment is a multiple-step process that includes bordering the vessel wall, rolling along endothelium, firm adherence and finally transmigration through endothelium (Kubes & Kerfoot 2001; Mölne & Wold, 2007). Adhesion molecules P- and E-selectins, expressed on the endothelial surface, are up-regulated and weakly interact with L-selectin expressed by leukocytes resulting in rolling of leukocytes along the endothelium (Mölne & Wold, 2007; Granger, 1988). As the rolling step proceeds, the expression and binding activity of β_2-integrins (LFA-1 and MAC-1) on leukocytes is increased, resulting in firm adhesion to ICAM-1 (intercellular adhesion molecule) and VCAM-1 (vascular cellular adhesion molecule) expressed on endothelial cells. The firm adhesion is followed by leukocyte migration out of the circulation to the site of injury (Mölne & Wold, 2007; Panes *et al*, 1999). After migration through endothelium, the active leukocytes release toxic ROS, proteases and elastases causing local damage and destruction (Panes *et al*, 1999).

I/R is encountered in strangulated bowel, hemorrhagic shock and organ transplantation, and is associated with high morbidity and mortality in surgical and trauma patients (Mallick *et al*, 2004; Collard & Gelman, 2001). Among the internal organs, intestinal mucosa is highly sensitive to ischemia-reperfusion injury (Mallick *et al*, 2004; Ozer *et al*, 2005; Ozkan *et al*, 2009). Colonic mucosa is less sensitive to I/R than that of the small intestine, but once damaged it recovers more slowly (Stallion *et al*, 2005; Robinson *et al*, 1981; Leung *et al*, 1992). The superior mesenteric artery (SMA) has been shown to maintain intestinal perfusion and mucosal integrity in rodents (Leung *et al*, 1992). Occlusion of SMA results in mucosal damage of small intestine, cecum and colon. Loss of mucosal barrier-function due to the I/R leads to increased translocation of enteric bacteria and local production of cytokines. If sufficiently severe, intestinal I/R injury can lead to multiple organ failure and death (Leung *et al*, 1992; Stallion *et al*, 2005; Riaz, 2002). The clinical setting that occurs in critically ill patients has been mimicked by using a murine model, in which occlusion of SMA results in intestinal I/R injury.

Polyphenols

Polyphenols are produced as secondary metabolites of plants, and are involved in plant growth and reproduction and provide protection against ultraviolet radiation, oxidative stress and pathogens (Ross & Kasum, 2002). Phenolic compounds are regular constituents of human diet and are found in fruits, beverages such as tea, coffee, wine and fruit juices, chocolate and, to lesser extent, in vegetables, cereals and legume seeds (Scalbert *et al*, 2002). The astringency and bitterness of foods and beverages depend on the content of polyphenolic compounds which are partially responsible for the sensory and nutritional qualities of plant foods (Bravo, 1998). Polyphenols possess antioxidative, metal-chelating and free radical-scavenging abilities, and they may prevent various diseases associated with oxidative stress, such as cardiovascular diseases, cancer, chronic inflammation, atherosclerosis and type-2 diabetes (Ross & Kasum, 2002; Scalbert *et al*, 2000). Chemically, phenolics can be defined as molecules possessing an aromatic ring bearing one or more hydroxyl groups. More than 8,000 phenolic structures are currently known (Ross & Kasum, 2002; Bravo, 1998). According to the nature of their carbon skeleton polyphenols can be classified into four major groups: phenolic acids, flavonoids, stilbenes and lignans (Scalbert *et al*, 2000).

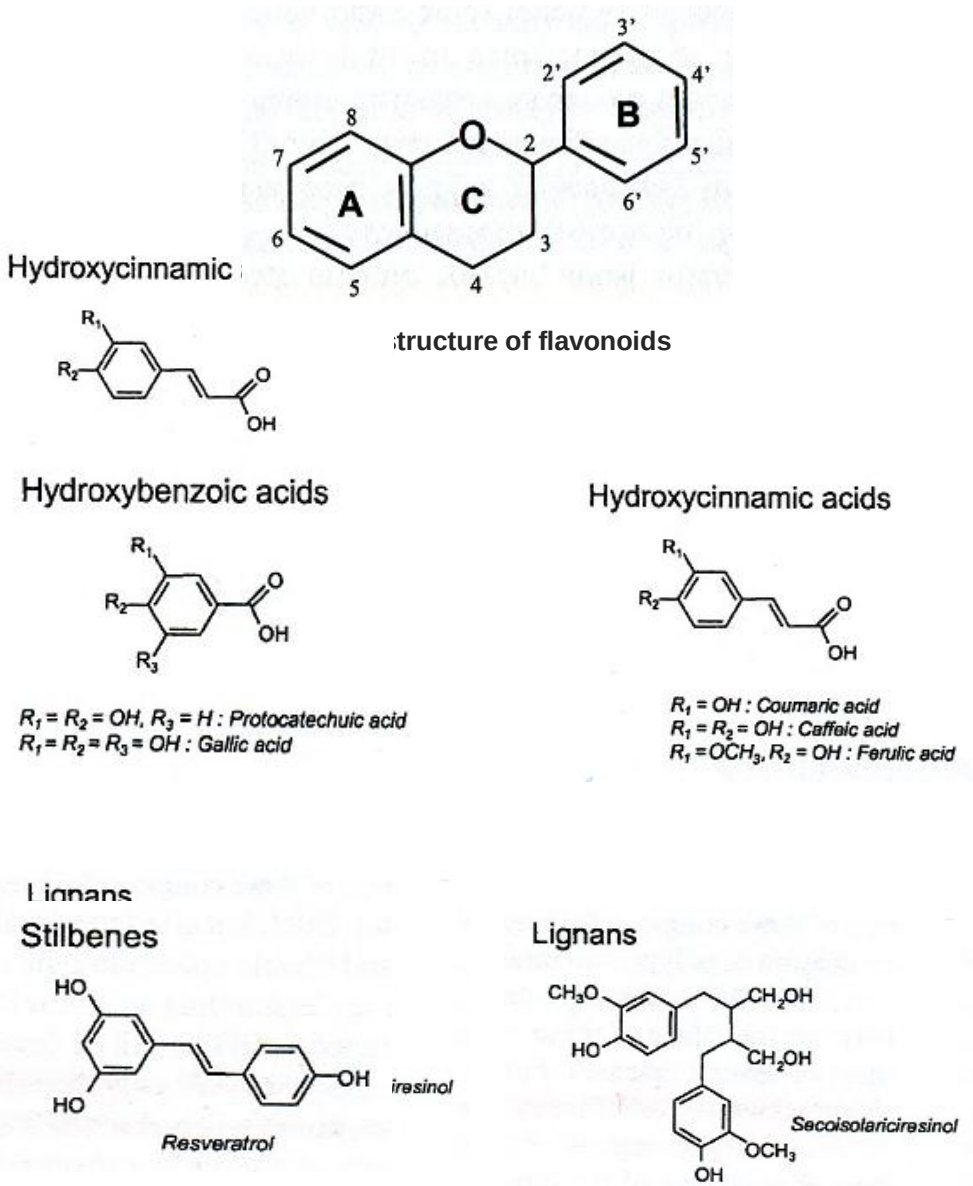


Figure 2. Chemical structures of polyphenols (Manach *et al*, 2004)

Flavonoids and phenolic acids

Flavonoids are the most abundant polyphenols in the diet. All flavonoid phenolics share a basic skeleton consisting of two aromatic rings (A and B) linked through three carbons that form an oxygenated heterocycle (C ring) (Bravo, 1998; Manach *et al*, 2004). According to the oxidation degree of the C ring, flavonoids can be divided into several classes: flavones, flavonols, isoflavones, anthocyanins, flavanols, flavanones and proanthocyanidins (Scalbert *et al*, 2000). Flavonoids occasionally occur in plants as aglycones, but are most commonly found attached to sugars (glycosides) (Ross & Kasum, 2002; Bravo, 1998).

Flavonols are the most ubiquitous flavonoids in foods with quercetin and kaempferol as the main representatives (Manach *et al*, 2004). These compounds are present in glycosylated forms. Glucose and rhamnose are the most common sugar residues, although other sugars such as galactose, xylose and arabinose are also found (Bravo, 1998). Blueberries are rich source of flavonols.

Catechin and epicatechin are the main flavanols in fruits. In contrast to the other flavonoid groups, flavanols are not glycosylated in food (Manach *et al*, 2004).

Proanthocyanidins, also known as condensed tannins, are found in large amounts in fruits, berries, nuts, cocoa and wine (Rasmussen *et al*, 2005). In proanthocyanidins, flavan-3-ol (catechin or epicatechin) units are linked mainly through a C4→C8 bond, but also through a C4→C6 bond, to form dimers, oligomers and high-molecular-weight polymers. These linkages are both called B-type linkages. An additional ether bond between C2→C7, resulting in double linked catechin units is called A-type linkage (Gu *et al*, 2004). B-type proanthocyanidins are the most common, but A-type linkages were also found in some foods (Rasmussen *et al*, 2005). Proanthocyanidins consisting exclusively of epicatechin units are called procyanidins (Gu *et al*, 2004). Through the formation of the complexes with salivary proteins, proanthocyanidins are responsible for the astringent character of some fruits and beverages. When fruit becomes ripe, this astringency often disappears (Santos-Buelga *et al*, 2000).

As a major sub-group of flavonoids, anthocyanins are water-soluble plant pigments responsible for pink, red, blue and purple colors of plants. Most anthocyanins occur as glycosides of their respective anthocyanidin (aglycone), with the sugar moiety mainly bound to the 3-position on C-ring or the 5, 7-position on the A-ring (Prior & Wu, 2006). The most common sugars are glucose,

galactose, arabinose, rhamnose and xylose while most common aglycones are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (Bravo, 1998; Prior & Wu, 2006). In addition to glycosylation, esterification with organic acids and phenolic acids also occurs. Anthocyanins are reactive compounds and readily degrade in the presence of oxygen, various enzymes, light and at high temperatures (Kalt *et al*, 2000). Variations in pH also affect the anthocyanin stability. The basic anthocyanidin structure, red flavylium cation, is the predominant molecular form at low pH (<2). Increase in pH leads to the loss of a proton and generates the blue quinonoidal structure. At the same time, a much slower hydration of the flavylium cation occurs, yielding small portions of the colorless chalcone forms (McGhie *et al*, 2007; Rivas-Gonzalo, 2003).

Phenolic acids can be classified as hydroxybenzoic acids and hydroxycinammic acids. They are present in fruits, vegetables, beverages and cereals. The hydroxycinammic acids are more commonly found in foods than hydroxybenzoic acids and consist mainly of p-coumaric, caffeic, ferulic and sinapic acids. Hydroxycinnamic acids occur in foods as simple esters with quinic acid or glucose. Chlorogenic acid, the most abundant hydroxycinnamic acid, is composed of caffeic and quinic acids. Hydroxybenzoic acids are generally present in foods in the form of glucosides and are often the components of complex structures like lignins and hudrolyzable tannins. Gallic acid, protocatechuic acid, vanillic acid and syringic acid are the most common hydroxybenzoic acids (Manach *et al*, 2004; Mattila *et al*, 2006). Hydroxycinnamic acids are more effective antioxidants than hydroxybenzoic acids. The presence of the –CH=CH-COOH group in hydroxycinammic acids ensures greater H-donating ability and radical stabilization by chemical resonance than the –COOH group in hydroxybenzoic acids (Rice-Evans *et al*, 1996).

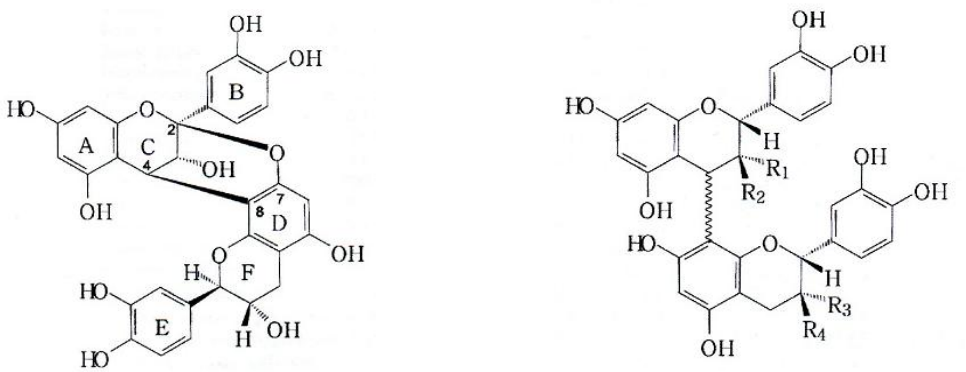


Figure 3. Procyanidin dimer, A-type linkage and B-type linkage (Rasmussen *et al*, 2005).

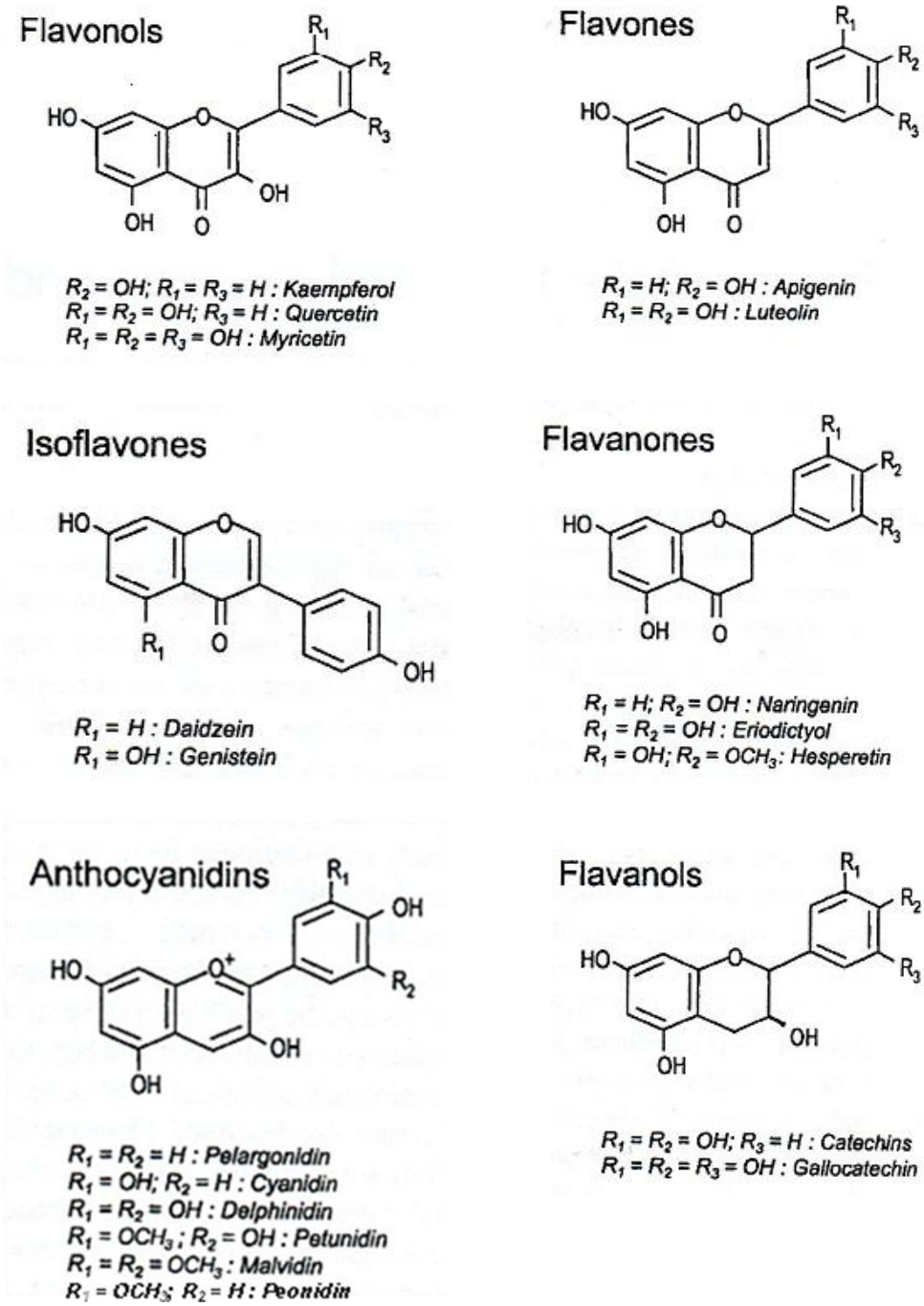


Figure 4. Chemical structures of flavonoids (Manach *et al*, 2004).

Absorption and metabolism

The absorption and metabolism of polyphenols are determined by their chemical structure, which includes the degree of glycosylation, acylation and polymerization, their basic structure, conjugation with other phenolics, molecular size and solubility. Aglycones and phenolic acids can be directly absorbed through the small intestinal mucosa (Lafay *et al*, 2006). Most polyphenols exist in food in the form of esters, glycosides or polymers that cannot be absorbed in their native form. These compounds probably resist acid hydrolysis in the stomach and arrive intact in the small intestine where they are hydrolyzed by intestinal enzymes to their aglycones. Free aglycones are conjugated by methylation, sulfation or glucuronidation, first in the small intestine and later in the liver (Scalbert *et al*, 2000). This is a metabolic detoxication process that facilitates biliary and urinary elimination of xenobiotics by increasing their hydrophilicity (Manach *et al*, 2004). Once they are absorbed through the gut barrier, polyphenols are able to penetrate tissues where they are metabolized. Polyphenols that are not absorbed in the small intestine and re-excreted in the bile reach the colon where they are metabolized by the microflora (Scalbert *et al*, 2002). Glycosides are hydrolyzed by the microflora and the resulting aglycones are further metabolized into various low molecular aromatic acids such as phenylvaleric, phenylpropionic, phenylacetic and benzoic acids that can be absorbed (Rechner *et al*, 2004; Lee *et al*, 2006; Gonthier *et al*, 2003a).

Proanthocyanidins differ from other polyphenols because of their polymeric nature and high molecular weight which limit their absorption. Only dimers and trimers are absorbed in the small intestine and the large non-absorbed fraction of polymers is degraded by colonic microflora to phenylvaleric, phenylpropionic, phenylacetic and benzoic acids (Déprez *et al*, 2000; Gonthier *et al*, 2003b; Rios *et al*, 2003).

The absorption of anthocyanins also differs from the other flavonoids because they are mainly absorbed as intact glycosides from the upper part of the small intestine but also from the stomach (McGhie *et al*, 2007; Matuschek *et al*, 2006; Passamonti *et al*, 2003). Since absorption of anthocyanins is very low most of the anthocyanins are transformed to phenolic acids by microbial populations in the colon (Fleschhut *et al*, 2005; Aura *et al*, 2005; Keppler & Humpf, 2005).

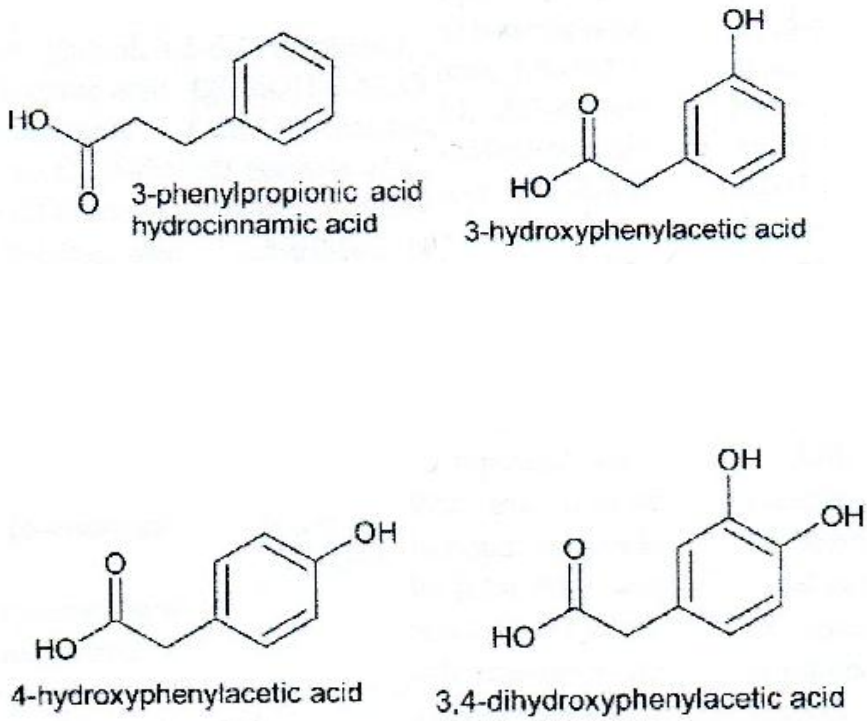


Figure 5. Some of the metabolites from microbial degradation of polyphenols in large intestine (Jenner *et al*, 2005).

Berries

Berry fruits are rich sources of phenolic compounds that many people believe possess health-promoting properties. The most important group of phenolics in berries is the flavonoids, which consist of flavonols, anthocyanidins, proanthocyanidins and flavan-3-ols. The predominant phenolic acids in berries are hydroxycinnamic and hydroxybenzoic acid derivates. The berry composition is affected by genetic differences, cultivar, fertilization, fruit maturation, harvest time, weather and location (Howard & Hager, 2007).

Chokeberry

Shrubs of the *Aronia* genus have been traditionally used as a food resource and in herbal medicine, first by Native Americans, and later by Russians and Eastern Europeans. *Aronia* is still commonly used in the food industry as a natural food colorant and for production of syrup, fruit juice, fruit wine and jams (Kulling *et al*, 2008). The genus *Aronia* (*Rosaceae* family) includes two species - *Aronia melanocarpa*, known as black chokeberry, and *Aronia arbutifolia*, known as red chokeberry. Apart from the black and red chokeberries there is also an intermediate hybrid, *Aronia prunifolia*, a purple chokeberry (Kokotkiewicz *et al*, 2010). Polyphenols, especially anthocyanins and proanthocyanidins, make up the main group of biologically active components in the chokeberry fruits. As a major class of polyphenols, polymeric procyanidins represent 66% of polyphenols in chokeberry and are responsible for the astringent taste of the berry. Procyanidins are oligomeric and polymeric B-type linked epicatechins, with >10-mers dominating in *Aronia* (Wu *et al*, 2004). Anthocyanins are the second largest group in chokeberry and represent 25% of total polyphenols. Four anthocyanins are responsible for the dark red color of the fruits: 3-O-galactoside, 3-O-glucoside, 3-O-arabinoside and 3-O-xyloside of cyanidin, with cyaniding-3-O-galactoside being the main one. Phenolic acids, of which chlorogenic and neochlorogenic acids are dominant ones, represent 7.5% of total polyphenols in chokeberry (Oszmiański *et al*, 2005). The content of flavonols is low compared to these other groups and constitutes only 1.3% of total polyphenols. The main flavonols detected in the chokeberry are quercetin-3-rutinoside, quercetin-3-galactoside and quercetin-3-glucoside (Oszmiański *et al*, 2005; Slimestad *et al*, 2005). After crowberry, chokeberry showed the highest content of total phenolics (40 mg GAE/g) among the berries (Kähkönen *et al*, 1999).

Aronia has been claimed to alleviate oxidative stress, exhibits anti-inflammatory, antimicrobial and antiviral activities due to the high content of polyphenols, and has antioxidative properties. It has been reported that chokeberry juice and chokeberry extract reduced lipid peroxidation (measured by MDA or TBARS), decreased tissue damage and improved antioxidative status in different organs exposed to oxidative stress induced by chemical treatment such as carbon tetrachloride, indomethacin and N-nitrosodiethylamine (Valcheva-Kuzmanova *et al*, 2004; Valcheva-Kuzmanova *et al*, 2005; Kujawska *et al*, 2010), high-fructose diet in combination with low-dose streptozotocin injection (Jurgoński *et al*, 2008) or exercise (Pilaczynska-Szczesniak *et al*, 2005). Administration of chokeberry extract showed a dose-dependent anti-inflammatory effect on endotoxin-induced uveitis in rats (Ohgami *et al*, 2005). *Aronia* juice exhibited a bacteriostatic activity *in vitro* against *Staphylococcus aureus* and *E. coli* and an antiviral activity against type A influenza virus (Valcheva-Kuzmanova *et al*, 2006).

Bilberry

Blueberries belong to the *Vaccinium* genus and *Ericaceae* family. The main species are the North American highbush *Vaccinium carymbosum*, lowbush *Vaccinium angustifolium* and the native European *Vaccinium myrtillus*, also called bilberry. Bilberry is one of the most important wild berries in Northern Europe and has a long tradition in folk medicine (Riihinen *et al*, 2008). Anthocyanins are the main polyphenols in bilberry and comprise around 90% of total phenolics. They are present in both peel and pulp and their composition is rather complicated, with cyanidin- (Cy), delphinidin- (Dp), petunidin- (Pt), peonidin- (Pn) and malvidin- (Mv) glycosides (glucoside, galactoside and arabinoside). Flavonols are the second largest group of polyphenols in the bilberry fruit and are dominated by quercetin-3-galactoside, quercetin-3-glucoside and quercetin-3-rhamnoside (Määttä-Riihinen *et al*, 2004). Myricetin-3-galactoside and myticetin-3-glucoside are the most abundant flavonols in the peel (Riihinen *et al*, 2008; Määttä-Riihinen *et al*, 2004). Chlorogenic acid is the dominant phenolic acid and is responsible for the high content of hydroxycinnamic acids in bilberry (Määttä-Riihinen *et al*, 2004). Syringic, caffeic, p-coumaric, vanillic and protocatechuic acids are also detected in relatively high amounts while gallic, ferulic and sinapic acids are present in smaller amounts (Matilla *et al*, 2006). The major flavan-3-ol in bilberry is epicatechin. Proanthocyanidins composed of rare A-type linked procyanidin units are characteristic of the berries in the Ericaceae family (Määttä-Riihinen et al 2004). *V. myrtillus* showed one of the highest contents of total phenolics (around 30 mg GAE/g) among the berries (Kähkönen *et al*, 1999).

High polyphenol content has been responsible for antioxidative, anti-inflammatory and antimicrobial activities of bilberries. *V. myrtillus* has been used in different studies to prevent oxidative stress injury in liver, kidney, skin and eyes (Bao *et al*, 2008a; Bao *et al*, 2008b; Svobodová *et al*, 2008; Yao *et al*, 2010). Bilberry prevented oxidative stress by increasing the levels of endogenous antioxidative enzymes (SOD, GPx), by improving the antioxidative status, and by decreasing lipid peroxidation (measured by MDA) and ROS production. Oral administration of *V. myrtillus* decreased the number of adhering leucocytes to venular vessels, preserved the capillary perfusion and reduced microvascular permeability during ischemia-reperfusion in hamster cheek pouch microcirculation (Bertuglia *et al*, 1995).

Supplementation with bilberry was suggested to modulate the inflammation process and prevent atherosclerosis and cardiovascular disease by decreasing the plasmatic total cholesterol, reducing the release of pro-inflammatory mediators in the liver and through reduction of several NF-κB regulated inflammatory mediators in plasma (Mauray *et al*, 2010; Karlsen *et al*, 2010). *Salmonella enterica* sv. Typhimurium, *Salmonella enterica* sv. Infantis and *Staphylococcus aureus* were inhibited by bilberry extract (Puupponen-Pimiä *et al*, 2005a). Bacteriostatic effects due to anti-adhesion have been observed in *Vaccinium* berries. Procyanidins with A-type linkages in *Vaccinium* berries have been associated with inhibition of *Escherichia coli* and *Helicobacter pylori* adhesion in uroepithelium and gastric epithelial cells, respectively (Puupponen-Pimiä *et al*, 2005b).

Rosehip

Rosa canina and *Rosa pimpinellifolia* are fruits, or rosehips, of *Rosa* genus in the *Rosaceae* family. *R. pimpinellifolia* has the black fruits while fruits of *R. canina* are red-orange. Both species show strong resistance to harsh environmental conditions. Rosehips are used in many European countries in food products such as tea, jam, marmalade, soup and for medical purposes (Demir *et al*, 2001). Rosehips are a rich source of minerals (K and P), vitamins C and E, carotenoids (lycopene), folate and phenolic compounds (Demir *et al*, 2001; Böhm *et al*, 2003; Strålsjö *et al*, 2003; Hvattum, 2002). Analysis of phenolics in rosehips revealed the presence of 15 individual proanthocyanidin aglycones and 19 proanthocyanidin glycosides as the major phenolics (Salminen *et al*, 2005). Several glycosides of quercetin and quercetin aglycone are identified flavonols in rosehips. The major flavan-3-ol is catechin, which is also the building unit for proanthocyanidins (Hvattum, 2002). Cyanidin-3-glucoside is the only anthocyanin present in the rosehips (Hvattum, 2002).

Rosehips are believed to possess antioxidative and anti-inflammatory properties. Administration of rosehips, especially together with probiotics, reduced lipid peroxidation (measured by MDA) in cecum and colon and decreased the count of pro-inflammatory *Enterobacteriaceae* in cecum of mice subjected to oxidative stress injury caused by intestinal I/R (Håkansson *et al*, 2006; Jakesevic *et al*, 2009). *R. canina* extract composed of proanthocyanidins and flavonoids was able to scavenge ROS released by activated neutrofils as inflammatory response (Daels-Rakotoarison *et al*, 2002). An extract made from dry powder of *R. canina* inhibited chemotaxis and generation of ROS by polymorphonuclear leucocytes (*in vitro* and *in vivo*) and showed anti-inflammatory effects comparable to the non-steroid anti-inflammatory drugs ibuprofen and acetylsalicylic acid (aspirin) in arthritis (Kharazmi & Winther, 1999; Winther *et al*, 1999).

Intestinal microflora

The human gastrointestinal tract is inhabited by a complex and dynamic population of different microbial species that exist in a complex equilibrium (Collado *et al*, 2009). The GI tract of an adult is estimated to harbor about 10^{14} viable bacteria which is 10 times more than the total number of eukaryotic cells in the human body (Holzapfel *et al*, 1998). This complex microbial community differs in composition and population levels in specific regions along the GI tract (Berg, 1996). Due to the low pH and relatively short transit times, the stomach contains approximately 10^3 bacteria/mL and is dominated by Gram-positive species such as streptococci and lactobacilli, and by *Helicobacter pylori* and yeasts (Holzapfel *et al*, 1998; Berg, 1996; Dunne, 2001). In the distal part of small intestine (ileum) the number of microbes (10^8 bacteria/mL) as well as the diversity increases. In addition to streptococci and lactobacilli, high concentrations of bifidobacteria, *Bacteroides*, *Fusobacteria* and *Enterobacteriaceae* are found in the ileum (Holzapfel *et al*, 1998). Slow intestinal motility and hence slow transit time and the very low oxidation-reduction potentials make the large intestine (colon) the primary site of microbial colonization with 10^{10}-10^{11} bacteria/g intestinal content (Holzapfel *et al*, 1998; Berg, 1996; Dunne, 2001). Strict anaerobes such as *Bacteroides*, *Eubacterium*, *Bifidobacterium* and *Peptostreptococcus* dominate the colonic microflora and are 100 to 1,000-fold more numerous than facultative anaerobes constituting *Enterobacteriaceae*, streptococci and lactobacilli (Berg, 1996; Holzapfel *et al*, 1998).

Development of microflora

The fetus is sterile in utero and is subjected to microbial contamination first during the delivery process. The type of birth delivery has a significant effect on the development of the intestinal microflora. With vaginal or cesarean delivery newborn infants are contaminated by the mother´s fecal matter and vaginal flora and/or the surrounding environment such as equipment, air, nursing staff and other infants (Mackie *et al*, 1999; Dunne, 2001). *Escherichia coli* and streptococci are most commonly isolated immediately after birth (Mackie *et al*, 1999; Fanaro *et al*, 2003; Holzapfel *et al*, 1998).

After the initial colonization, the composition of the microflora is greatly influenced by the diet of the infant. In the infants fed solely with human breast milk, the microflora is dominated by bifidobacteria, whereas similar numbers of bifidobacteria and *Bacteroides* are found in formula-fed infants (Harmsen *et al*, 2000). As the minor components of the microflora, streptococci and lactobacilli are found in breast-fed infants whereas formula-fed infants possess more staphylococci and clostridia and are generally colonized by more diverse microflora (Harmsen *et al*, 2000; Stark & Lee, 1982). On the introduction of solid food and weaning, the microbial differences between breast-fed and formula-fed infants disappear. After the second year of life the infant flora become more complex and resembles that of adults (Mackie *et al*, 1999; Stark & Lee, 1982). Every individual has its own dominant microbial composition that is unique and stable over time during adulthood (Collado *et al*, 2009; Guarner & Malagelada, 2003).

In comparison to humans, mice are dominated by *Lactobacillus* spp., *Streptococcus* spp., *E. coli* and flavobacteria during the first few days after birth (Inoue *et al*, 2005; Schaedler *et al*, 1965). Lactobacilli and streptococci are isolated from the stomach, small intestine and large intestine and remain at a high and approximately constant level throughout life. Strict anaerobic bacteria including *Bacteroidaceae*, eubacteria, clostridia and fusiform-shaped bacteria become established after weaning and are mainly isolated from the large intestine in high numbers (Inoue *et al*, 2005; Hirayama *et al*, 1995). Stabilized intestinal microflora of mice is established within 3 to 5 weeks after the birth (Hirayama *et al*, 1995).

Functions of the microflora

Since potentially pathogenic microorganisms can be members of normal, resident microflora, a balance among the bacterial groups present in the gut can be crucial for maintaining health. Microbial imbalance has been associated with enhanced risk of different diseases, such as antibiotic-associated diarrhea (Marteau *et al*, 2001), inflammatory bowel diseases (Marteau *et al*, 2001), allergy (Wang *et al*, 2008), obesity (Ley, 2010) and type-2 diabetes (Larsen *et al*, 2010). Aging, stress, diet and use of medicines are the major factors influencing changes in the composition of the gut microflora.

The major functions of the gut microflora include metabolic activities, protection against pathogens and interaction with immune system. The essential metabolic function of colonic microflora is to supply the colon with energy by fermenting non-digestible carbohydrates such as resistant starches, cellulose, hemicelluloses, pectins and gums (Guarner & Malagelada, 2003; Sekirov *et al*, 2010). The principal end products of carbohydrate fermentation are short-chain fatty acids (SCFA), especially acetate, propionate and butyrate. All three SCFA have trophic effects on the intestinal epithelium but butyrate seems to be the most effective. Butyrate provides intestinal epithelium with around 70% of all energy and regulates cell growth and differentiation. It is important for maintaining mucosal health in the colon. Acetate and propionate are taken up by the epithelium, appear in portal blood and eventually pass through the liver to peripheral tissues where they are metabolized by muscle. Acetate inhibits fatty acid oxidation, while propionate may lower cholesterol and may also have a role as modulator of glucose metabolism (Cummings *et al*, 1987a; Cummings & Englyst, 1987b; Salminen *et al*, 1998). Several members of intestinal microflora can also synthesize vitamin K and some vitamins B (Berg, 1996).

Gut microflora provides its host with a physical barrier to incoming pathogens by competitive exclusion, such as occupation of attachment sites, exhaustion of or competition for the same nutrient sources, and production of antimicrobial substances (bacteriocins) that inhibit the growth of pathogens. An additional mechanism involved in the barrier effects is the creation of physiologically restrictive environments in terms of pH, redox potential, hydrogen sulfide production or production of metabolites (ammonia, phenol compounds, amines etc) toxic to invading bacteria (Bourlioux *et al*, 2003; Sekirov *et al*, 2010; Collado *et al*, 2009).

The intestine is the largest immune organ of the body. Gut-associated lymphoid tissue (GALT) contains 80% of all antibody-producing cells (Ouwehand *et al*, 2002). Intestinal microflora seems to be crucial for the development of the host immune system by increasing the number of Peyer´s patches and immunoglobulin (Ig) A-producing cells. Microflora also plays an important role in the regulation of immune responses at local and systemic levels (Salminen *et al*, 1998; Isolauri *et al*, 2004).

Probiotics

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). A potential probiotic strain should be of human origin and non-pathogenic, tolerate low pH and bile in order to survive passage through the stomach and upper intestinal tract and be able to adhere to the epithelial cells, resist technological processes and withstand incorporation into a foodstuff at high cell counts, and remain viable throughout the shelf-life of the product (Dunne, 2001; Collado *et al*, 2009; Rolfe, 2000; Parvez *et al*, 2006). The most commonly used probiotics are strains of *Bifidobacterium* or lactic acid bacteria (LAB), especially of the genus *Lactobacillus*. A probiotic mixture may contain one or several different strains of microorganisms (Timmerman *et al*, 2004).

Beneficial effects of probiotic consumption include stabilizing of intestinal mucosal barrier function, normalizing of indigenous microflora, stimulating of the immune system, synthesizing and enhancing the bioavailability of nutrients and reducing risk of certain diseases (Collado *et al*, 2009; Rolfe, 2000).

Probiotics have been successfully used in prevention and treatment of rotavirus diarrhea, traveller´s diarrhea and antiobiotic-associated diarrhea (Isolauri *et al*, 1991; Oksanen *et al*, 1990; Black *et al*, 1989; Siitonen *et al*, 1990; Vanderhoof & Young, 2002; Marteau *et al*, 2001). Certain probiotic strains of *Lactobacillus* have shown an improvement in bacterial flora and maintained the remission of pouchitis (Gionchetti *et al*, 2000), reduction in abdobinal pain, bloating, flatulence and constipation in inflammatory bowel disease and irritable bowel syndrome (Nobaek *et al*, 2000; Niedzielin *et al*, 2001), down-regulation of inflammation (Parvez *et al*, 2006), atopic eczema (Isolauri *et al*, 2000) and food allergy (Kirjavainen *et al*, 1999), decrease in intestinal permeability and bacterial translocation (Mangell *et al*, 2006).

Several mechanisms of action have been proposed to explain those beneficial effects of probiotics, including: i) inhibition of pathogens by production of antimicrobial substances e.g. organic acids, hydrogen peroxide and bacteriocins, ii) competition with pathogens for nutrients and adhesion sites on the intestinal epithelial surfaces, iii) modulation of pH in the gut, and iv) stimulation of immunomodulatory cells (Collado *et al*, 2009; Parvez *et al*, 2006; Rolfe, 2000).

Lactobacillus plantarum possesses PAD (phenolic acid decarboxylase) enzymes that transform phenolic acids (p-coumaric, m-coumaric, caffeic and ferulic acid) into their vinyl derivates and phenylpropionic acids (Barthelmebs *et al*, 2000, Rodríguez *et al*, 2009). Furthermore, *L. plantarum* strains possess enzyme tannase (tannin acylhydrolase) and are able to degrade hydrolyzable tannins and tannic acid, thereby producing gallic acid and the antioxidant pyrogallol (Osawa *et al*, 2000). Hydrolyzable tannins are composed of esters of gallic acid (gallotannins) or ellagic acid (ellagitannins) with a sugar moiety, usually glucose, and may be hydrolysed into monomeric products.

Additionally, some strains of LAB possess antioxidative ability, scavenge reactive oxygen species and chelate metal ions providing protection against oxidative stress and lipid peroxidation (Lin & Yen, 1999a; Lin & Yen, 1999b; Kullisaar *et al*, 2002; Kaizu *et al*, 1993; Songisepp *et al*, 2005). Consumption of foods containing LAB may also contribute to the health effects associated with dietary antioxidants.

AIMS

The general aims of this thesis were to evaluate the protective effects of different polyphenol-rich berries and probiotics in oxidative stress injury induced by intestinal ischemia-reperfusion (I/R) model in mouse and to study the interaction between probiotic strains and polyphenols in berries. This was done in four papers with the following specific aims:

Paper I: to evaluate the potential of rosehips from *Rosa canina* or *Rosa pimpinellifolia*, alone or together with a mixture of eight probiotic *Lactobacillus* strains, to suppress oxidative stress in the large intestine of mice. Focus was put on antioxidative effects and phenolic profile of proanthocyanidins and anthocyanins in the cecum and colon.

Paper II: to clarify the antioxidative effects of bilberry and chokeberry fruits alone or in combination with *Lactobacillus plantarum* HEAL19 on oxidative stress in the large intestine of mice and to study the phenolic profile in the cecum and colon.

Paper III: to map the phenolic profile and evaluate the total phenolic content and antioxidative capacity of bilberry beverage inoculated with three different strains of *L. plantarum* and one strain of *Pediococcus acidilactici*, either alone or in combination with wine yeast.

Paper IV: to clarify the anti-inflammatory and antioxidative effects of bilberry fruit alone or in combination with either *L. plantarum* RESO56, *L. plantarum* HEAL19 or *Pediococcus acidilactici* on oxidative stress in the small and large intestine of mice and to study the anthocyanin-profile in different parts of the gut.

METHODOLOGY

Experimental design

The influence of polyphenol-rich diet and lactic acid bacteria (LAB) on oxidative stress caused by intestinal ischemia-reperfusion (I/R) was studied *in vivo* in papers I, II and IV. An *in vitro* experiment was conducted in paper III in order to map the phenolic profile of bilberry beverage inoculated with different LAB strains of probiotic potential, either alone or in combination with wine yeast. Studies are summarized in Table 1.

Figure 6. Bilberry beverages.

Male Balb/cJ mice were used in all three I/R studies. Mice weighed approximately 20 g and were kept under standard laboratory conditions with a controlled 12-hour light/dark cycle. The experimental designs were approved by the Ethical Committee for Animal Experimentation at Lund University. At the start of the experiment each animal was placed in its own cage with a food dish. After 7 days of acclimatization with free access to standard animal chow (R3; Lactamin, Stockholm, Sweden) and tap water, animals were fed experimental diets for 10 days. Diets were prepared every day and given to the mice ad libitum during the dark cycle when they were the most active. In all I/R studies, the standard animal chow was dissolved in water to soften the consistency prior to the addition of supplement (experimental diets). I/R-control and sham were fed only soft standard chow.

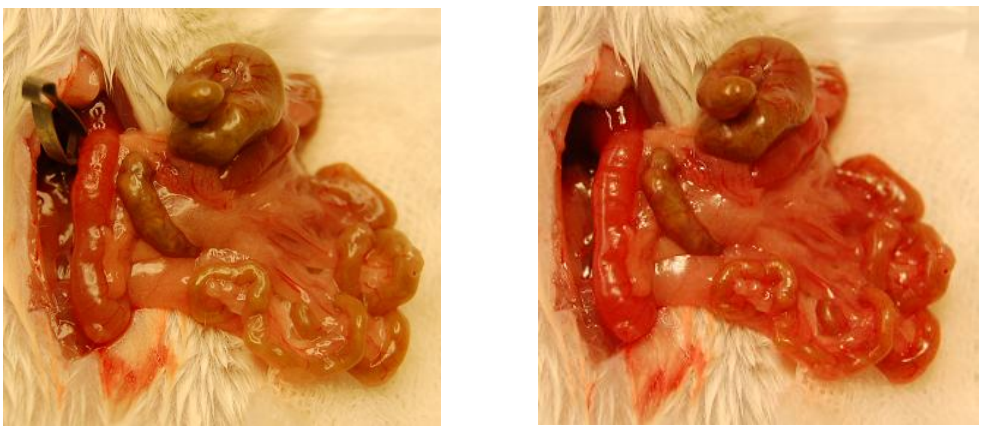


Figure 7. Intestinal ischemia followed by reperfusion.

Table 1. Overview of feed supplements, models, samples and methods used in different studies

	Feed supplement	Model	Sample	Methods used
I	Rosehips of *Rosa canina* Rosehips of *Rosa pimpinellifolia* Mixture of *Lactobacillus* strains containing: *L. plantarum* 299v *L. plantarum* HEAL19 *L. plantarum* RESO49 *L. plantarum* RESO56 *L. paraplantarum* RESO97 *L. pentosus* LP2 *L. pentosus* 9T *L. plantarum* subsp *argentoratensis* (CCUG 50787^{T})	Intestinal ischemia-reperfusion (I/R) in mouse	Cecum contents Cecal and colonic tissues	Viable count RAPD MDA Antioxidant capacity (FRAP) Total phenolics (TP) HPLC-DAD-ESI-MS
II	Bilberry (*Vaccinium myrtillus*) Chokeberry (*Aronia prunifolia*) *L. plantarum* HEAL19	Intestinal I/R in mouse	Cecal and colonic contents and tissues	Viable count MDA HPLC-DAD-ESI-MS/MS
III	Bilberry (*V. myrtillus*) beverage *L. plantarum* HEAL19 *L. plantarum* RESO56 *L. plantarum* 299v *Pediococcus acidilactici* JAM046 Yeast (*Saccaromyces cerevisiae* subsp *cerevisiae*)	*In vitro*	Beverage sample taken at incubation time 0 and after 1, 2 and 3 weeks of incubation	Antioxidant capacity (FRAP) Total phenolics (TP) HPLC-DAD-ESI-MS Ethanol content pH
IV	Bilberry (*V. myrtillus*) *L. plantarum* HEAL19 *L. plantarum* RESO56 *Pediococcus acidilactici* JAM046	Intestinal I/R in mouse	Ileal, cecal and colonic contents and tissues	Viable count MDA MPO Histology T-RFLP HPLC-DAD-ESI-MS/MS

In paper I, the anti-oxidative effects of two different species of rosehips, *Rosa canina* and *Rosa pimpinellifolia*, were compared. Freeze-dried and grounded rosehips were administered, either alone or together with a mixture of eight different *Lactobacillus* strains, to examine whether a combination may enhance the antioxidative properties of the rosehips. The bacterial mixture contained the following strains: *Lactobacillus plantarum* 299v, *L. plantarum* HEAL19, *L. plantarum* RESO49, *L. plantarum* RESO56, *L. paraplantarum* RESO97, *L. pentosus* LP2, *L. pentosus* 9T, and *L. plantarum* subsp. *argentoratensis* (CCUG 50787^{T}). The daily dose of probiotic mixture was $8*10^{8}$ cfu.

In paper II, freeze-dried and ground bilberry (*Vaccinium myrtillus*) and chokeberry (*Aronia x prunifolia*) were compared in their efficacy in preventing oxidative stress. In this study only one probiotic strain, *L. plantarum* HEAL19, was administered, either alone or together with the berries. The daily dose of *L. plantarum* HEAL19 was $1*10^{8}$ cfu.

In paper IV, freeze-dried and ground bilberry (*V. myrtillus*) was administered alone or in combination with either *Lactobacillus plantarum* RESO56, *L. plantarum* HEAL19 or *Pediococcus acidilactici* JAM046 to examine if supplementation with different strain may influence the I/R-injury in different ways. The daily dose of each probiotic strain was $1*10^{9}$ cfu.

In paper III, a bilberry beverage (*V. myrtillus*) was prepared by blending whole fruits with an equivalent amount of distilled water. Plastic bottles (500 mL) were filled with the beverage and sterilized at 121°C for 15 minutes followed by rapid cooling to room temperature before inoculation. The beverage was inoculated with one of the following four strains of LAB: *L. plantarum* HEAL19, *L. plantarum* RESO56, *L. plantarum* 299v and *P. acidilactici* JAM046, alone or together with a commercial starter culture of wine yeast (*Saccaromyces cerevisiae* subspecies *cerevisiae*), and incubated at 30°C for 3 weeks. In one series, the bilberry beverage was only inoculated with the wine yeast. Sterile, non-inoculated bilberry beverage was used as control. The yeast and bacteria were added in equivalent quantity 50:50, according to the weight of the pellet.

Intestinal ischemia-reperfusion (I/R) procedure

An intestinal I/R model was chosen to study oxidative stress since it mimics the clinical setting that occurs in critically ill patients (Stallion *et al*, 2005). Occlusion of superior mesenteric artery (SMA) in mouse results in intestinal I/R injury. Briefly, the mice were anesthetized with 7.5 mg Ketamine (Ketalar 50 mg/mL [Pfizer, UK]) and 2.5 mg Xylazine (Narcoxyl 20 mg/mL [Veterinaria AG, Schweiz]) per 100 g body weight by intraperitoneal injection. After midline abdominal incision, the SMA was isolated and occluded with a vessel clamp to obtain intestinal ischemia, confirmed by loss of pulsation and pale color of the intestines. The bowel was returned to the abdominal cavity. After the ischemic period, the clamp was removed, which resulted in immediate reperfusion, confirmed by the restoration of pulsation and color, and the abdomen was closed. After the reperfusion period, the animal was anesthetized again, sampled and sacrificed.

In papers I and II, the intestinal injury was induced by occlusion of the SMA for 30 minutes followed by 240 minutes reperfusion. In paper IV, SMA was occluded for 40 minutes followed by 120 minutes reperfusion. The sham groups were subjected to the abdominal incision but without clamping SMA, i.e. without I/R.

Analyses

Extraction of phenolic compounds

The objective of extraction prior to analysis is to liberate phenolic compounds from the plant material and other tissues. Organic solvent penetrates the cell membranes and extracts the phenolics from vacuolar compartments or food matrix (He *et al*, 2005). The factors that contribute to the efficacy of solvent extraction are type of solvent, pH, temperature, number of steps and volume of solvent, and particle size in the sample (Escribano-Bailón & Santos Buelga, 2003). The most widely used solvent for extraction of polyphenols is methanol and aqueous methanol (addition of small percentage water). The pH of the extraction medium determines the degree of solubility and solvent is often acidified with hydrochloric, acetic, formic or trifluoroacetic acids (TFA) (Escribano-Bailón & Santos Buelga, 2003). In papers I-IV, methanol and aqueous methanol acidified with TFA (paper I), formic acid (paper II and IV) and hydrochloric acid (paper III) were used as extraction solvents. Homogenization or crushing followed by ultrasound-assisted extraction is used to reduce particle size, which increases the

extractability (Escribano-Bailón & Santos Buelga, 2003). These extraction steps were applied in papers I-IV and were performed at low temperature (4°C) in order to prevent enzymatic degradation. During centrifugation, proteins (enzymes) were precipitated, which also reduces enzyme activity.

Analysis of phenolic compounds

High-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) detection is a very powerful and widely used technique for separation, screening, identification and quantitative determination of polyphenols. In papers I-IV, different phenolic groups were detected and identified by using HPLC-DAD-ESI-MS/MS. The basic principle of HPLC is that a small volume of a liquid sample is injected into a moving stream of mobile phase (liquid) that passes through a column packed with particles of stationary phase. In the column, the sample is separated into its components that have different affinities towards different phases and so exit the column at different times (retention time, Rt). As compounds leave the column, they pass through a detector where the data about each eluted compound is acquired and processed. The outcome from HPLC is presented as a chromatogram where different components of the sample are represented by peaks, and as a table showing the retention time and the height/area of each peak (Snyder *et al*, 2010).

The mobile phases usually consist of an acidified aqueous solvent (solvent A) and methanol or acetonitrile (solvent B). Acidification provides better retention and separation on the C_8- and C_{18}-RP (reversed-phase) columns, which are the most commonly used for separation of polyphenols. Acetic, formic or trifluoroacetic acids are the most commonly used acid modifiers (Merken & Beecher, 2000). Thermostatically controlled columns are normally kept at ambient temperature or slightly above (30-35°C) to improve separations and keep retention times stable. Polyphenols absorb in the ultraviolet (UV) region and are usually analyzed by UV-vis with diode array detection (DAD). The UV-vis spectrum obtained indicates which class the phenolic compound belongs to, e.g. flavonols have λ_{max} at 280 nm, hydroxycinnamic acid derivates at 300-320 nm and anthocyanins at 500-520 nm. The wavelength and intensity of absorption maximum may also provide information about substitution (glycosylation and acylation) patterns of the phenolic compound (Santos-Buelga *et al*, 2003).

However, UV-vis spectrum cannot distinguish between the compounds with similar spectral characteristics and gives no information about the identity of the conjugate (sugars or acids) attached to the aglycone. Mass spectrum obtained

under electrospray ionization (ESI-MS) is used for more detailed and accurate structural information and identification of polyphenols. Sample in a liquid phase is sprayed into a chamber with high voltage where a dry gas flows in the opposite direction to the mist causing disintegration of the drops into charged droplets. As the solvent evaporates the droplets become smaller. Eventually, solvent-free ions are produced that are passed through the mass analyzer. In the mass analyzers the ions are separated according to their mass-to-charge ratio (*m/z*) prior to detection (Downard, 2004). Ionization in both positive and negative mode is used, but the highest sensitivity is obtained using ESI in negative mode (Cuyckens *et al*, 2004). Because of the positive charge in their structure, anthocyanidins are more suitable for mass spectrometry analysis in positive mode.

Ferric reducing activity power (FRAP)

Ferric reducing activity power (FRAP) is an assay based on single electron transfer (ET). ET-based assays measure capacity of an antioxidant to reduce an oxidant, thereby causing the color change. The degree of color change is proportional to the concentration of the antioxidant in the sample. The reaction reaches the end point when color change stops (Huang *et al*, 2005). Antioxidative capacity determined by FRAP was performed in paper I and paper III according to the description by Benzie & Strain (1996). Briefly, colorless ferric tripyridiyltriazine (Fe^{3+}-TPTZ) is reduced to blue-colored ferrous form (Fe^{2+}-TPTZ) at low pH. The color shift is measured spectrophotometrically at 593 nm. Any half-reaction that has a redox potential lower than that of Fe^{3+}-TPTZ (~0.77 V) will drive the ferric (Fe^{3+}) to ferrous (Fe^{2+}) reaction. The antioxidative capacity is expressed as mmol $Fe^{\pm}$. The FRAP assay is quick, simple and inexpensive and the reaction is highly reproducible over a wide concentration range. It directly measures antioxidants in the sample. A disadvantage with the method is that absorption slowly increases after the end point of the reaction for some polyphenols.

Total phenolic content (TP)

In paper I and paper III, total phenolic content (TP) was measured by the Folin-Ciocalteu reagent. It is an ET-based assay that measures the reducing properties of phenolic compounds. In the assay, the Folin-Ciocalteu reagent (a mixture of phosphomolybdate and phosphotungstate) is reduced and the blue-colored product is measured spectrophotometrically at 765 nm (Singleton *et al*, 1999). TP is most often expressed as gallic acid equivalents in milligrams. The Folin-Ciocalteu reagent is commercially available and the procedure is rather standardized. It is a commonly accepted assay and is routinely used in dietary

antioxidant research throughout the world with a large amount of comparable data produced.

Malondialdehyde (MDA)

Oxidation of polyunsaturated fatty acids in lipoproteins leads to formation of hydro- and endo-peroxides, which are unstable and decompose to yield a broad range of reactive intermediates, including alkanals, alkenals, hydroxyalkenals and most abundantly, malondialdehyde (MDA). MDA reacts readily with amino groups in proteins, resulting in chemical modification of the protein. Mutagenic and carcinogenic adducts are formed when MDA reacts with DNA bases. Measurement of MDA is widely used as an indicator of lipid peroxidation and oxidative stress in cells and tissues (Del Rio *et al*, 2005). MDA-586™ (Oxis International Inc. Portland, Oregon, USA), a colorimetric assay, was used in papers I, II and IV to determine MDA levels in cecal, colonic and ileal tissues. The MDA-586™ assay measures free MDA and protein-bound MDA (total MDA) and minimizes the interference from other lipid peroxidation products such as alkanals, alkenals and hydroxyalkenals. The method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (NMPI), with MDA at 45°C to form intensely colored carbocyanine, which is spectrophotometrically measured at 586 nm.

Myeloperoxidase (MPO)

Myeloperoxidase (MPO) is a membrane-bound, green heme protein found almost exclusively in neutrophils and to lesser extent in circulating monocytes. The MPO adequately reflects the numbers of neutrophils in the healthy and inflamed tissue (Grisham *et al*, 1988). In paper IV, MPO was spectrophotometrically measured as a marker of neutrophil accumulation and tissue inflammation.

Histopathology

The degree of injury caused by intestinal ischemia-reperfusion is assessed by histological evaluation. The procedure for tissue preparation is formalin fixation, dehydration, paraffin embedding and cutting followed by staining with haematoxylin and eosin (H&E). In paper IV, the grading system by Chiu was used to score mucosal injury in the small intestine according to the following criteria: 0 = normal mucosa; 1 = development of subepithelial Gruenhagen´s space at the tip of the villus; 2 = extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; 3= massive epithelial lifting with a few

denuded villi, 4 = denuded villi with exposed dilated capillaries; 5 = digestion and disintegration of lamina propria, hemorrhage, and ulceration (Chiu *et al*, 1970).

Randomly amplified polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) is a PCR-based method where arbitrary, short primers (around 8-10 bases in length) are used to amplify the DNA at low stringency achieved by low annealing temperature. The DNA fragments of different sizes are separated by agarose gel electrophoresis, to give a characteristic genomic fingerprint of the organism (Power, 1996; Hadrys *et al*, 1992). The method is informative, simple, reliable and suitable for studying a large number of isolates in a short time. Different parameters such as choice and concentration of the primer, annealing temperature and concentration of the chemicals included in the PCR reaction must be standardized to avoid non-reproducible results (Power, 1996; Hadrys *et al*, 1992). RAPD was used in paper I for grouping and typing of *Lactobacillus* isolates. A 9-mer with sequence 5'-ACG CGC CCT-3' was used as primer for DNA amplification.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

T-RFLP is a method used for fingerprinting and comparing bacterial community structures. In the first step, bacterial DNA is extracted from the sample. The genes of interest are PCR amplified with primers in which one is fluorescently labeled. Purified PCR products are digested with a restriction enzyme and fragments are separated by electrophoresis. The outcome from T-RFLP is presented as an electopherogram where the profile of the bacterial community is represented by colored peaks and as a table showing the size, base pairs and the height/area of each peak. The T-RFLP profiles of the bacterial community between the samples are most commonly compared by using principle component analysis (PCA). The method is rapid, reproducible and gives high resolution (Wang, 2004). T-RFLP was used in paper IV to analyze bacterial community profile in the cecum of mice fed different diets, and comparison of the cecal microflora between the groups was obtained by PCA.

Statistics

The differences between all groups (papers I-IV) were evaluated by Kruskal-Wallis one way ANOVA on ranks. The Mann-Whitney rank sum test was used when only two experimental groups were compared. These statistical analyses were conducted in SigmaStat version 3.1 (SPSS Inc., Chicago, Ill, USA). One-way

ANNOVA was applied to the data on the number of T-RFs (paper IV) as the distribution was normal. This evaluation was performed using Minitab statistical software (release 16; State College, PA, USA). Values were considered statistically significant when $p<0.05$.

Multivariate data analysis with principal component analysis (PCA) was used to reveal the internal structure and possible correlations of the data with a large number of variables. PCA was applied in paper III to reveal the correlation pattern between different anthocyanins in bilberry beverages, and in paper IV to reveal the possible differences in cecal microbial population between the experimental groups. Multivariate data analyses were performed in SIMCA-P+12.0.1 (Umetrics, Sweden).

Modde 9.0 software (Umetrics, Sweden) was used for evaluation of experimental design, and for analysis and interpretation of all data and variables obtained in paper III.

Results and discussion

Polyphenols

As a general rule, the strongly colored fruits and berries contain high concentrations of anthocyanins as pigments and also flavonol glycosides and/or conjugated hydroxycinnamic acids (Määttä-Riihinen *et al*, 2004; Wu *et al*, 2004). Anthocyanins are suggested to possess antioxidative (Tsuda et al, 1999), anti-inflammatory (Wang et al, 1999), and antimicrobial (Puupponen-Pimiä et al, 2005) properties. The bioavailability of anthocyanins is reported to be low (Murkovic *et al*, 2001; McGhie *et al*, 2003). They are mostly absorbed and excreted unmetabolized, i.e. in the glycosylated form in which they were administered (McGhie *et al*, 2003). Polyphenols are usually characterized and quantified by HPLC-DAD-ESI-MSn, which was also used in papers I-IV.

Anthocyanins

No anthocyanins were found in animals not fed berry-supplemented diets.

In papers I, II and IV, anthocyanins were analyzed in the intestinal content of mice fed berries, while in paper III they were analyzed in bilberry beverages. Bilberry (*Vaccinium myrtillus*) contained 14 anthocyanins (papers II-IV; Table 2; Lätti *et al*, 2010; Laaksonen *et al*, 2010), chokeberry (*Aronia x prunifolia*) contained four anthocyanins (paper II; Oszmiański & Wojdylo, 2005) and one anthocyanin was found in rosehips (paper I, Hvattum, 2002). Anthocyanins Dp-3-arab and Cy-3-gal could not be separated in paper II and IV, and Pt-3-arab and Pn-3-gal were co-eluted in paper IV.

In paper I, anthocyanins were analyzed in the cecal content. Cyanidin-3-O-glucoside, the only anthocyanin found in rosehips (Hvattum, 2002), was present in significantly higher concentrations ($p<0.001$) in *Rosa pimpinellifolia*-fed groups compared to *Rosa canina*-fed groups. There was a trend that LAB supplementation to the rosehips increased the concentrations of anthocyanins in cecum. The resident microflora of mice

seemed to have higher activity towards anthocyanin-degradation than supplementation with LAB mixture.

In papers II and IV, bilberry (B) was administered, either alone or together with different strains of lactic acid bacteria (LAB). In paper II, chokeberry (Ar) was also administered, either alone or together with a LAB strain. Strains used in different papers are listed in Table 1. In paper II, cecal and colonic contents were collected for analysis of anthocyanins, and ileum contents were also analyzed in paper IV.

Chokeberry powder consisted of four anthocyanins: Cy-3-glu, Cy-3-gal, Cy-3-arab and Cy-3-xyl (xyloside). Of these, Cy-3-glu was not detected in cecal and colonic contents of chokeberry-fed mice (groups Ar and Ar+LplH19) (Paper II). In paper IV, all 14 anthocyanins from the bilberry powder were detected in ileum of the bilberry-treated animals (B, B+Lpl56, B+LplH19 and B+Ped) (Table 2). Recovery of Cy-3-glu and also Dp-3-glu was extremely low in the ileum. The low concentrations of these anthocyanins in ileum and cecum may be due to several factors. One possibility is that they were absorbed from the upper GI tract or metabolized by bacteria and enzymes in the small intestine. It has been shown that Cy-3-glu was mainly absorbed from the stomach (Passamonti *et al*, 2003) and jejunum (Matuschek *et al*, 2006; He *et al*, 2009). Furthermore, it has been suggested (Del Bó *et al*, 2010; Felgines *et al*, 2002) that Cy and Dp glycosides are subjected to methylation in the small intestine, resulting in the increase of Pn, Pt and Mv glycosides under physiological conditions. This trend was also observed in the ileum in paper IV. It has also been proposed that Cy-3-glu may be degraded to protocatechuic acid (PCA) and Cy aglycone both *in vitro* and *in vivo* (animals and humans) (Aura *et al*, 2005; Keppler & Humpf, 2005; Fleschhut *et al*, 2006; Tsuda *et al*, 1999a; Wu *et al*, 2009; Vitaglione *et al*, 2007). In paper I, Cy-3-glu was the only anthocyanin found in the rosehips, with significantly higher concentration detected in *R. pimpinellifolia* than *R. canina*. This could be expected on account of dark blue colour of *R. pimpinellifolia*. PCA was detected in the cecal content of mice fed RP and seemed to be negatively correlated with Cy-3-glu, i.e. when concentration of Cy-3-glu was high the concentration of PCA was low, and vice versa. The results suggested that PCA was a metabolite from microbial degradation of Cy-3-glu. Ingestion of anthocyanin-rich black raspberry powder in pigs resulted in high concentration of PCA in the cecum followed by ileum and colon (Wu *et al*, 2009).

In paper II, cecal content of B group and B+LplH19 group contained nine and eight anthocyanins respectively, compared to the 14 anthocyanins that were found in the bilberry powder added to the feed (Table 2). The missing anthocyanins were glucosides and galactosides of Mv, Pt and Pn. In the colon of groups B and B+LplH19, the same content of 12 anthocyanins was found out of the 14 anthocyanins in the native bilberry.

In paper IV, cecum and colon of the bilberry-treated animals (B, B+Lpl56, B+LplH19 and B+Ped) contained 10 and 13 anthocyanins respectively compared to the 14 anthocyanins that were found in the bilberry powder (Table 2). In contrast to paper II, Mv-3-glu and Pt-3-glu were detected in cecum content of mice in paper IV. Pn-3-glu was not found in colon of mice in both studies. Additionally, Pn-3-gal was missing in paper II, while it was found in the lowest concentration in colon in paper IV.

The lower numbers of bilberry-anthocyanins recovered from cecum and colon in paper II compared to the number found in paper IV may depend on variations in the individual microflora of mice. It can also be due to the fact that the animals had free access to feed, and the time of the last intake of bilberry-supplemented feed before sample collection may have had an influence on how much of the anthocyanin was found in the different parts of the intestines (Borge *et al*, 2007; He *et al*, 2009).

According to the results from papers II and IV, glucosides and galactosides of Mv, Pn and Pt in the bilberry-supplemented groups seemed to be the ones most efficiently digested by the microflora in cecum or absorbed in the body. Felgines *et al* (2002) detected low amounts of anthocyanins from blackberries in cecal contents of rats, suggesting an adaptation of microflora to anthocyanin degradation. Different authors detected Mv-3-glu and Mv-3-gal as predominant anthocyanins in different tissues and in urine after ingestion of bilberry/blueberry anthocyanins (Sakakibara *et al*, 2009; McGhie *et al*, 2003). They also found galactoside and glucoside of Pn and Pt both in tissues and urine, suggesting the higher absorption of these anthocyanins.

In papers II and IV the highest recovery in cecum and colon was seen for Mv-3-arab and Pt-3-arab in mice fed bilberry and Cy-3-arab and Cy-3-xyl in mice fed chokeberry. The results imply that they were relatively poorly degraded by the gut microflora or absorbed in the body. It has been proposed that bioavailability of

anthocyanins is modulated by both the nature of the sugar conjugate and the phenolic aglycone (Sakakibara *et al*, 2009; McGhie *et al*, 2003; Wu *et al*, 2005). Pentosides (arabinoside and xyloside) seem to be quite stable in the gut and resistant to the microbial degradation in the large intestine (He *et al*, 2005; Ichiyanagi *et al*, 2006).

The opposite seemed to be true for *in vitro* studies. In paper III, bilberry beverages were incubated with one of the three LAB strains (Table 1), either alone or in combination with wine yeast, over 3 weeks at 30°C. All 14 anthocyanins present in the native bilberry were detected in the beverages. In addition, Pt aglycone was found in the samples. All anthocyanins decreased during incubation, but Pt aglycone, Mv-3-arab and co-eluted Pt-3-arab and Pn-3-gal were strongly correlated and showed the highest reduction in beverages, especially those inoculated with only LAB. The degradation of anthocyanins seemed to be due to the exogenic factors and not due to the bacterial activity, since non-inoculated, sterile bilberry-control and LAB-inoculated beverages showed the same reduction of anthocyanins. Beverages were autoclaved prior to inoculation in order to inactivate both bilberry enzymes and contaminating microorganisms. They were prepared and incubated in plastic bottles sealed with screw cap with a water seal. Bottles were not completely filled and had a certain headspace. Heat, pH, oxygen, light and various storage conditions have marked effects on anthocyanin stability (Kalt *et al*, 2000). Heat applied during autoclavation, or oxygen present in the headspace or diffusing through the packaging material or during sampling, may have influenced the stability of anthocyanins and especially Pt aglycone, Mv-3-arab and co-eluted Pt-3-arab and Pn-3-gal. Anthocyanins conjugated with sugar pentoses (arabinosides) are less stable during heating and processing than anthocyanin with galactoside or glucoside (Yue *et al*, 2008; Howard *et al*, 2010). It has been shown that Mv-3-arab, but also Pt-3-arab, were very sensitive to oxygen during the storage in oxygen-permeable packaging materials (Trošt *et al*, 2008; Giovanelli *et al*, 2007). Plastic packages allow a certain permeability of oxygen (Trošt *et al*, 2008; Giovanelli *et al*, 2007).

Addition of wine yeast to the beverages resulted in much higher recovery of anthocyanins (Paper III). The protective effect may have been due to the high yield of CO_2 during alcoholic fermentation, which decreased oxygen levels in the bottles and preserved anthocyanins from oxidation. Furthermore, anthocyanins may have been retained by the yeast cell walls during the incubation period and then released during extraction. Results conflict on the influence of anthocyanin´s polarity on its adsorbtion by yeast. Some authors (Lubbers *et al*, 1994; Morata *et al*, 2003) suggested that more hydrophobic anthocyanins (Mv, Pn and even Pt) are

adsorbed by yeast to a greater extent while others (Mazauric *et al*, 2006) showed that anthocyanins may not be adsorbed in relation to their sole polarity. However, malvidin derivates were the most extracted anthocyanins from yeast lees (Lubbers *et al*, 1994; Morata *et al*, 2003; Mazauric *et al*, 2006).

Table 2. Anthocyanins found in native bilberry and in contents of different parts of the intestines.

Bilberry (powder/beverage)	**MS (MS^2) ions (*m/z*)**	**Paper II detection site**	**Paper IV detection site**
Delphidin-3-O-galactoside (Dp-3-gal)	465 (303)	Cecum, colon	Ileum, cecum, colon
Delphidin-3-O-glucoside (Dp-3-glu)	465 (303)	Cecum, colon	Ileum, cecum, colon
Delphidin-3-O-arabinoside (Dp-3-arab)	435 (303)	Cecum, colon	Ileum, cecum, colon
Cyanidin-3-O-galactoside (Cy-3-gal)	449 (287)	Cecum, colon	Ileum, cecum, colon
Cyanidin-3-O-glucoside (Cy-3-glu)	449 (287)	Cecum, colon	Ileum, cecum, colon
Petunidin-3-O-galactoside (Pt-3-gal)	479 (317)	Colon	Ileum, colon
Cyanidin-3-O-arabinoside (Cy-3-arab)	419 (287)	Cecum, colon	Ileum, cecum, colon
Petunidin-3-O-glucoside (Pt-3-glu)	479 (317)	Colon + cecum in B-group	Ileum, cecum, colon
Petunidin-3-O-arabinoside (Pt-3-arab)	449 (317)	Cecum, colon	Ileum, cecum, colon
Peonidin-3-O-galactoside (Pn-3-gal)	463 (301)	ND	Ileum
Peonidin-3-O-glucoside (Pn-3-glu)	463 (301)	ND	Ileum, colon
Malvidin-3-O-galactoside (Mv-3-gal)	493 (331)	Colon	Ileum, colon
Malvidin-3-O-glucoside (Mv-3-glu)	493 (331)	Colon	Ileum, cecum, colon
Malvidin-3-O-arabinoside (Mv-3-arab)	463 (331)	Cecum, colon	Ileum, cecum, colon

ND = not detected

Phenolic compounds other than anthocyanins

In papers I-III, other polyphenolic groups than anthocyanins were analyzed, i.e. flavonols, flavanols, proanthocyanins, phenolic acids and phenolic metabolites. In animals not fed berry-supplemented diets, no phenolic compounds were found.

In paper I, phenolic compounds analyzed were catechin (*m/z* 289), metabolite I (*m/z* 291), proanthocyanidin-monomer-glycoside (*m/z* 451), proanthocyanidin-dimer-glycoside (*m/z* 739), proanthocyanidin-dimer-diglycoside (*m/z* 901). These compounds were previously detected in rosehips (Salminen *et al*, 2005). Significantly higher concentrations of these compounds were detected in cecal content of mice fed *Rosa canina* than *Rosa pimpinellifolia*. Furthermore, there was a trend towards higher concentration of phenolic compounds when LAB supplemented the rosehip-diet. The LAB mixture may have modified the composition of colonic microflora, probably increasing the metabolic activity towards higher degradation of proanthocyanidins into monomers and dimers, or certain strains in the mixture themselves may have contributed to the degradation of polyphenols.

In paper II, five aromatic acids were detected in cecum of bilberry-fed groups (B and B+LplH19) (Table 3). Two isomers of 3,4-dihydroxyphenylacetic acid (diHPA) and 3-hydroxyphenylpropionic acid (3-HPP) were identified while two compounds could not be identified. Additionally, in colon of bilberry-supplemented animals, three compounds were detected but could not be identified. The detected aromatic acids were probably produced during microbial degradation of different polyphenols present in the berries (Rusell *et al*, 2007). Identified isomers of diHPA are suggested to be metabolites from the microbial degradation of quercetin or proanthocyanidin-dimer (Aura *et al*, 2002; Wu *et al*, 2009; Gonthier *et al*, 2003b). Catechin, epicatechin, proanthocyanidins/procyanidins, as well as chlorogenic acid, have been proposed as precursors of 3-HPP (Rios *et al*, 2003; Gonthier *et al*, 2003b; Gonthier *et al*, 2003c). Procyanidins (epicatechin-monomers linked with A-type and B-type bonds), quercetin glycosides and chlorogenic acid have been identified in the bilberry (Prior *et al*, 2001; Määttä-Riihinen *et al*, 2004).

In cecum and colon of chokeberry-supplemented groups (Ar and Ar+LplH19), 4-hydroxyphenylacetic acid (4-HPA) and 3-hydroxyphenylacetic acid (3-HPA) respectively were identified (Paper IV). Dehydroxylation of diHPA at meta and para positions results in formation of 3-HPA and 4-HPA (Rios *et al*, 2003). diHPA

has been described as an intermediate between flavanols and the more dehydroxylated 3-HPA and 4-HPA. Since procyanidins have been identified as the major class of polyphenols in chokeberries (Kulling & Rawel, 2008), they were probably degraded by microflora, firstly into diHPA and then into 3-HPA or 4-HPA.

More than 80% of procyanidins in chokeberries have a higher polymerization degree than 10-mers and only around 4% are monomers, dimers and trimers (Kulling & Rawel, 2008). As the degree of procyanidin polymerization increases, the yield of microbial metabolites decreases due to the reduced accessibility of the substrate or interaction of procyanidin with proteins in the gut lumen (Gonthier *et al*, 2003b). The higher content of procyanidin polymers in chokeberry compared to bilberry may have been the reason that fewer metabolites were detected in the cecum and colon of chokeberry-treated groups.

In general, large individual differences in concentration and detectable levels of different phenolic compounds between different individuals were observed in papers I and II.

Table 3. Aromatic acid metabolites detected in cecum and colon of mice fed bilberry (B) or chokeberry (Ar), either alone or together with *Lactobacillus plantarum* HEAL19 (LplH19) (Paper II).

MS (*m/z*)	MS^2 (*m/z*)	Compound	Detection site and group
167	123	3,4-Dihydroxyphenylacetic acid	Cecum and colon B and B+LplH19
167	123	3,4-Dihydroxyphenylacetic acid	Cecum and colon B and B+LplH19
223	123	Non-identified I	Cecum and colon B and B+LplH19
223	123	Non-identified II	Colon B and B+LplH19
223	123	Non-identified III	Colon B and B+LplH19
151	107	4-Hydroxyphenylacetic acid	Cecum Ar and Ar+LplH19
151	107	3-Hydroxyphenylacetic acid	Colon Ar and Ar+LplH19
289	271	Non-identified EC	Colon B and B+LplH19
190	146	Non-identified	Cecum and colon B, B+LplH19, Ar, Ar+LplH19
165	121	3-Hydroxyphenylpropionic acid	Cecum and colon B and B+LplH19

In paper III, phenolic compounds detected in all bilberry beverages were gallic acid (*m/z* 169), protocatechuic acid (*m/z* 153), chlorogenic acid (*m/z* 353), caffeic acid (*m/z* 179), quercetin (*m/z* 301) and quercetin-3-glucoside (*m/z* 463). Presence of these phenolics in bilberry has been previously confirmed (Riihinen et al, 2008; Hokkanen *et al*, 2009; Laaksonen *et al*, 2010). Two compounds with UV spectra characteristic of hydroxycinnamic acids (UV_{max} at 311-313 nm) and fragmentation pattern *m/z* 535→371→165 and *m/z* 411→163→119 respectively were identified as coumaric acid derivatives (Hokkanen *et al*, 2009). During three weeks of incubation at 30°C, detected phenolics were quite stable in beverages

inoculated with different LAB (Table 1), either alone or in combination with yeast. Chlorogenic acid and also quercetin aglycone and glycosides in apple juice were not much influenced by storage conditions, oxygen pressure or thermal processing (Van der Sluis *et al*, 2005). Furthermore, LAB strains added to the bilberry beverage seemed to lack the metabolic activity towards phenolic acids in the tested conditions. Metabolic activity was recorded in studies where LAB strains were cultivated in different growth media containing a certain concentration of pure phenolic acid such as caffeic, *p*-coumaric, ferulic, *m*-coumaric, gallic or protocatechuic acid (Calvin et al, 1993; Rodríguez et al, 2008).

Antioxidative capacity and total phenolic content

In papers I and III, antioxidative capacity was measured using the ferric reducing activity power (FRAP) assay, while total phenolic content (TP) was determined with Folin-Ciocalteau reagent. Both are electron transfer (ET) based assays that measure the reducing properties of phenolic compounds.

In paper I, antioxidative capacity and total phenolic content were measured in cecum contents of mice fed either of two different *Rose* species alone or in combination with a mixture of *Lactobacillus* strains (Table 1). In paper III, antioxidative capacity and total phenolic content were determined in a bilberry beverage inoculated with either of three different LAB strains alone or in combination with yeast.

LAB supplementation to the rosehips tended to increase the concentrations of total phenolic contents and antioxidative capacity in cecum in paper I. Antioxidative capacity was 6.21 mmol Fe^{2+}/100g in the *Rosa canina* (RC) fed group and 6.95 mmol Fe^{2+}/100g in combination with *Lactobacillus* mixture (RC+LAB). Corresponding values were 5.47 mmol Fe^{2+}/100g in *Rosa pimpinellifolia* (RP) fed group and 6.45 mmol Fe^{2+}/100g in combination with *Lactobacillus* mixture (RP+LAB). Total phenolic content values in groups RC and RC+LAB were 373 and 428 GAE/100 g respectively. Corresponding values in groups RP and RP+LAB were 395 and 423 mg GAE/100 g respectively.

In paper III, antioxidative capacity and TP decreased during 3 weeks of incubation in all bilberry beverages, i.e. both with different LAB strains alone and together with yeast. After 3 weeks of incubation, the average FRAP values were 3.0 mmol Fe^{2+}/100g in beverage supplemented with LAB and 3.3 mmol Fe^{2+}/100g in beverage supplemented with LAB and yeast. After the same incubation period, the

average TP value of beverages incubated with LAB was 284 mg GAE/100 g and beverages incubated with LAB plus yeast was 306 mg GAE/100 g.

The results obtained in paper I differed from the results in paper III. Results in paper I indicate that supplementation with the LAB mixture has a different influence on the degradation of polyphenols than the indigenous microflora. *Lactobacillus* strains in the mixture demonstrated tannase activity. The higher concentrations of phenolic compounds were also found in groups where rosehips were supplemented with LAB mixture.

In paper III, decreases in antioxidative capacity and TP in all bilberry beverages were probably due to the decrease of phenolic compounds and especially anthocyanins. Anthocyanins are suggested to be strong antioxidants, but are quite unstable during processing and storage (Wang *et al*, 1999; Kader *et al*, 1997; Buckow *et al*, 2010; Skrede *et al*, 2000).

Polyphenols differ in their behavior and stability between *in vitro* and *in vivo* systems. Many factors, such as interactions with metal ions, proteins and other antioxidants, as well as distribution between the lipid and water phase and exposure to the complex microflora in the GI tract, may affect the action of an antioxidant in *in vivo* models compared to *in vitro* models. The concentration of phenolics and pH of the environment may also be crucial factors.

Inflammatory markers

Ischemia-reperfusion

In rodents, the blood supply to the intestines is regulated by the superior mesenteric artery (SMA) (Leung *et al*, 1992). Occlusion of the SMA results in ischemia and upon removal of the clamp reperfusion is observed. In papers I and II, the time-interval for ischemia was 30 min and 240 min for reperfusion. This time-interval was chosen according to Riaz *et al* (2002) and Håkansson *et al* (2006) since it gave the highest lipid peroxidation (MDA) in colon of mice. In paper IV, time-intervals for I/R were changed to 40 min ischemia and 120 min reperfusion to obtain more severe intestinal damage. Increases in the ischemic time were associated with comparable decreases in the tickness of the mucosal layer and severity of the intestinal damage (Boros *et al*, 1995). After 40-50 min of

ischemia, the intestinal damage becomes irreversible (Guan *et al*, 2009). When a maximum ischemic stimulus is reached, the injury becomes independent of reperfusion time (120 or 240 min) (Redel *et al*, 2008). In paper IV, the ischemic duration of 40, 45 and 50 min was tested (data not shown). Since the mortality of mice increased after 40 min ischemia, an ischemic period of 40 min was chosen in order to reduce the mortality. A relatively short reperfusion period of 120 min was chosen in order to avoid the influence of eventual healing processes.

Malondialdehyde (MDA)

In papers I, II and IV, malondialdehyde (MDA) was measured as an indicator of lipid peroxidation in the tissue, verifying the oxidative damage. MDA was determined in cecal and colonic tissues in papers I and II. In paper IV, ileal and colonic tissues were analyzed. In agreement with other studies (Håkansson *et al* 2006; Ozkan *et al*, 2009; Muià *et al*, 2005), MDA was significantly lower in the sham group (p=0.026 in cecum and p=0.038 in colon in paper I; p=0.014 in colon in paper II; p=0.035 in ileum and colon in paper IV) compared to the I/R-control. Different supplements to the diet had different effects on the MDA values.

In paper I, supplementation with LAB mixture or *Rosa pimpinellifolia* (RP) significantly reduced MDA in colon (p=0.007 and p=0.035 respectively; Figure 8). RP was shown to possess significantly higher concentration (p<0.001) of anthocyanins than *R. canina*. Antioxidative and anti-inflammatory properties of polyphenols in RP probably contributed to the observed lowering effect of MDA (Daels-Rakotoarison *et al*, 2002). Certain strains of LAB have been shown to possess antioxidative activity, chelate metal ions and scavenge reactive oxygen species (ROS) (Lin & Yen, 1999a; Kullisaar *et al*, 2002; Songisepp *et al*, 2005). *L. paraplantarum* RESO97 was isolated in higher frequency from the LAB group than groups where LAB was combined with rosehips and it can be speculated that *L. paraplantarum* RESO97 possesses higher antioxidative and scavenging abilities towards ROS than the other LAB strains, providing protection against lipid peroxidation. A strikingly low median value of MDA was observed when *R. pimpinellifolia* and LAB mixture were administered in combination but, because of the large spread within the group, no significance was reached.

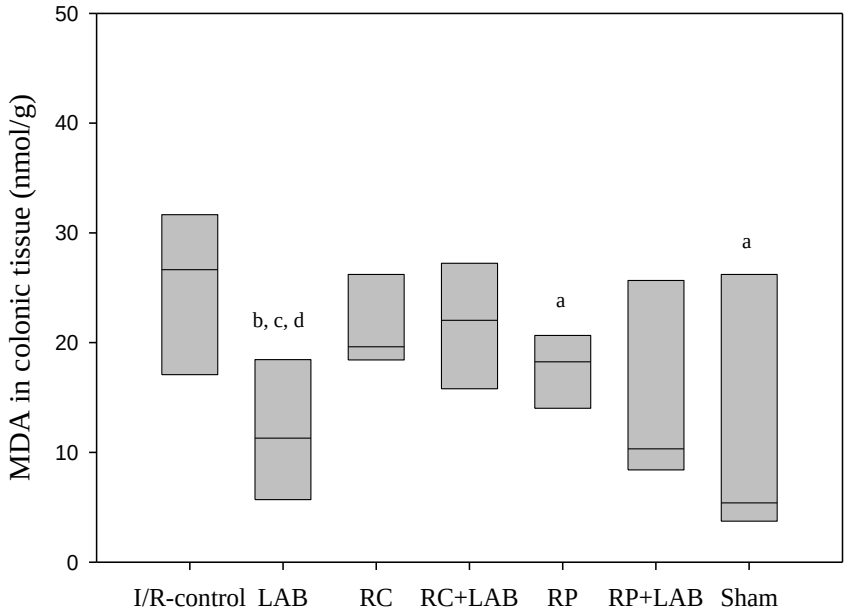


Figure 8. Malondialdehyde (MDA) after I/R in colonic tissue of mice administered different diets supplemented with rosehips (RC and RP) and *Lactobacillus* mixture (LAB). **a** and **b** denote $p<0.05$ and $p<0.01$ respectively compared to the I/R-control group; **c** denotes $p<0.05$ compared to the RC group; **d** denotes $p<0.05$ compared to the RC+LAB group. Figure taken from paper I.

In paper II, MDA was significantly lower in the colonic tissue of groups supplemented with bilberry (B), alone or together with *Lactobacillus plantarum* HEAL19 (B+LplH19) (p=0.030 and p=0.021 respectively), compared to the I/R-control group (Figure 9). The B group also showed a significantly lower MDA value than the group supplemented with chokeberry (Ar) (p=0.016). Other authors (Håkansson *et al*, 2009; Osman *et al*, 2008) confirmed the reducing effects of the diet supplemented with bilberry and probiotics on colonic MDA levels during intestinal inflammation. Lipid peroxidation is mediated by ROS and lowering effects of MDA by bilberries are probably due to their antioxidative and anti-inflammatory properties (Zheng *et al*, 2003; Russell *et al*, 2007).

Chokeberry-fed groups (Ar and Ar+LplH19) did not have reducing effect on lipid peroxidation which was in contrast with previous results (Valcheva-Kuzmanova *et*

al, 2004; Valcheva-Kuzmanova *et al*, 2005). Different chokeberry species (*Aronia x prunifolia* powder versus *A. melanocarpa* juice) as well as different administration methods (ad libitum versus stomach intubation) were used in the studies, which may have influenced the results. Factors such as cultivar, fertilization, maturation of the berries, harvest date and habitat may also contribute to the differences in quantity and profile of phenolics in chokeberry fruits and hence antioxidant properties (Kulling & Rawel, 2008).

Although chokeberry was shown to possess higher total phenolic content (Kähkönen *et al*, 1999) and higher antioxidative capacity *in vitro* (Zheng *et al*, 2003) than bilberry, it did not have antioxidative effects in colon of mice subjected to intestinal I/R (Paper II). As previously mentioned, *in vivo* systems are more complex than *in vitro* systems and it may affect the action of an antioxidant. Polymeric procyanidins are the major polyphenols found in chokeberries. More than 80% of these procyanidins have a polymerization degree higher than 10-mers (Wu *et al*, 2004; Kulling & Rawel, 2008), which may decrease their absorption, cause interaction with proteins in the gut lumen and also make them less accessible for the microbial degradation (Gonthier *et al*, 2003b). It could be noted in paper II that fewer metabolites were found in the cecum and colon of mice fed chokeberry than in bilberry-fed mice. Aromatic acid metabolites may be more efficient antioxidants than the parent phenolic compounds (Gao *et al*, 2006; Kim *et al*, 1999; Monagas *et al*, 2009). They are proposed to be more easily absorbed from the large intestine into the circulation and may contribute not only to the local but also to the systemic protection against oxidative stress injury.

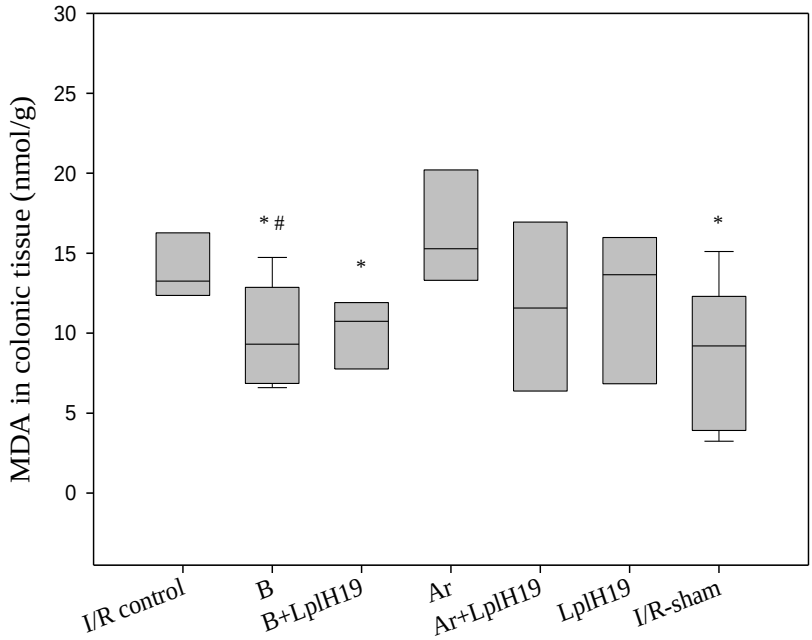


Figure 9. Malondialdehyde (MDA) after I/R in colonic tissue of mice administered different diets supplemented with bilberry (B) or chokeberry (Ar), either alone or together with *Lactobacillus plantarum* HEAL19 (LplH19). Asterisk (*) denotes $p<0.05$ compared to the I/R-control; hash (#) denotes $p<0.05$ compared to the Ar group. Figure taken from paper II.

In paper IV, MDA was significantly reduced ($p<0.001$) in the ileal tissue of mice fed bilberry (B) compared to the I/R-control (Figure 10A). The B group also showed significantly ($p<0.002$) lower MDA than the bilberry in combination with *Lactobacillus plantarum* RESO56 (B+Lpl56). MDA in B group was also lower than bilberry in combination with *L. plantarum* HEAL19 (B+LplH19) and *Pediococcus acidilactici* JAM046 (B+Ped) but the difference did not reach statistical significance ($p=0.054$). Bilberries are a rich source of polyphenols, and especially anthocyanins which comprise about 90% of phenolic compounds (Kähkönen *et al*, 2003). In paper IV, ileum contained the highest concentration of anthocyanins. The suggested antioxidant, anti-inflammatory and radical-scavenging abilities of anthocyanins in bilberries may have contributed to the decrease of MDA in the ileum of mice fed bilberry (B group) (Zheng *et al*, 2003; Wang *et al*, 1999; Kähkönen *et al*, 2003). It can also be speculated that bilberry

antioxidants may decrease the amount of ROS-generating enzyme xanthine oxidase (XO) accumulated in ischemic ileal tissue and thus prevent lipid peroxidation in the ileum (Grisham & Granger, 1988).

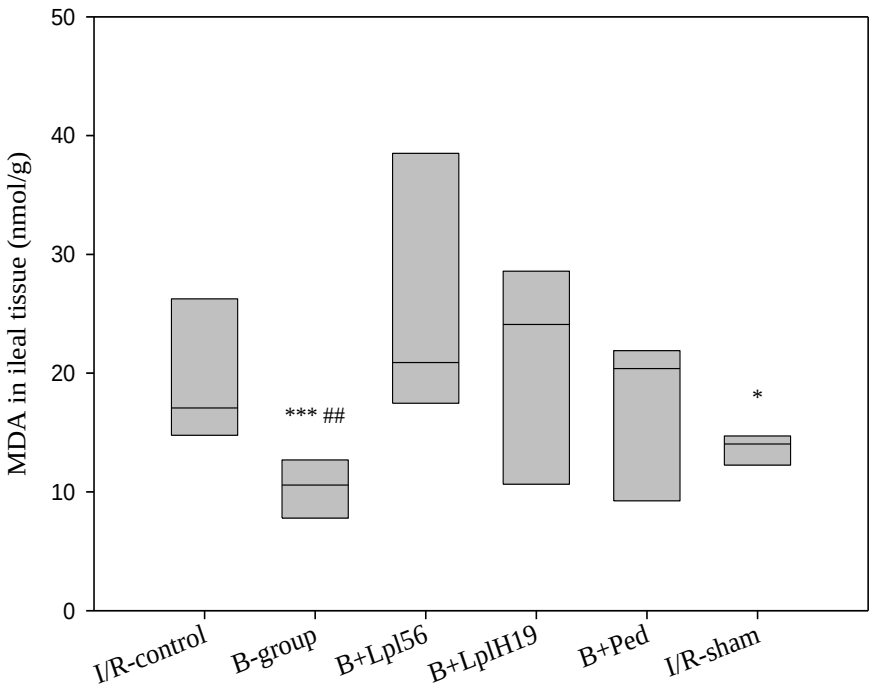
Supplementation with bilberry together with either LplH19, Lpl56 or Ped could not reduce MDA in the ileum. One explanation may be that lactobacilli exposed to high oxygen levels during reperfusion overproduced hydrogen peroxide (H_2O_2) from O_2 via NADH-dependent oxidase or pyruvate oxidase contributing to oxidative stress (Murphy *et al*, 1984; De Angelis & Gobbetti, 2004). Another explanation may be that LAB stimulated the immune system of the small intestine by activating the phagocytes and their production of ROS, which could further contribute to the increased oxidative stress and hence lipid peroxidation (Schriffrin *et al*, 1995). Then again, the lack of effect on MDA in the ileum upon addition of LAB may be due to changes in the food due to growth of the supplemented LAB prior to the consumption rather than *in vivo*. Feed was given to the mice ad libitum and was replaced approximately every 24 hours. It can be speculated that during this time LAB can have started to catabolize food components as well as producing, for example H_2O_2. Consumption of this feed may have had a negative effect on the oxidative state of the small intestinal tissue. The variation between the MDA values of the different animals was strikingly high within the bacterial groups. Individuals may have had naturally different antioxidant capacity of mucosa and different predisposition for handling intestinal inflammation. Durak *et al* (2000) found inflamed intestinal mucosa to possess higher antioxidant value, i.e. superoxide dismutase, glutathione peroxidase and catalase enzyme activities, and lower MDA value than healthy mucosa in the same individual with ulcerative colitis.

Lactobacillus plantarum is not an aggressive bacterium and has been previously shown to suppress intestinal and liver injuries when ingested alone and together with bilberry (Paper II; Osman *et al*, 2007; Osman *et al*, 2008). In these studies the protective effects were seen in the colon, and ileum was not tested. There are physiological and environmental differences between the small intestine and colon, e.g. the oxygen tension is higher in the small intestine than in the colon. It has been suggested that the small intestine is more susceptible to ischemia-reperfusion injury than colon (Stallion *et al*, 2005; Leung *et al*, 1992b).

In paper IV, MDA showed a tendency to decrease in the colonic tissue of animals fed bilberry alone as well as together with different LAB, but the decrease was not significant (p=0.055) compared to the I/R-control (Figure 10B). In paper II,

administration of bilberry, either alone or in combination with *L. plantarum* HEAL19 (LplH19), significantly decreased MDA in the colonic tissue after I/R injury. The difference between these studies was the duration of ischemia and reperfusion. In paper IV, ischemia lasted for 40 min followed by 120 min reperfusion, compared to the 30 min ischemia and 240 min reperfusion in paper II. After an ischemic period of 40-50 min (long ischemia) irreversible damage occurs and reperfusion can not restore normal epithelial cell structure and function (Guan *et al*, 2009). When a maximum ischemic stimulus (long ischemia) is reached, the time of reperfusion (120 or 240 min) has no influence on the severity of the damage (Redel *et al*, 2008).

A)



B)

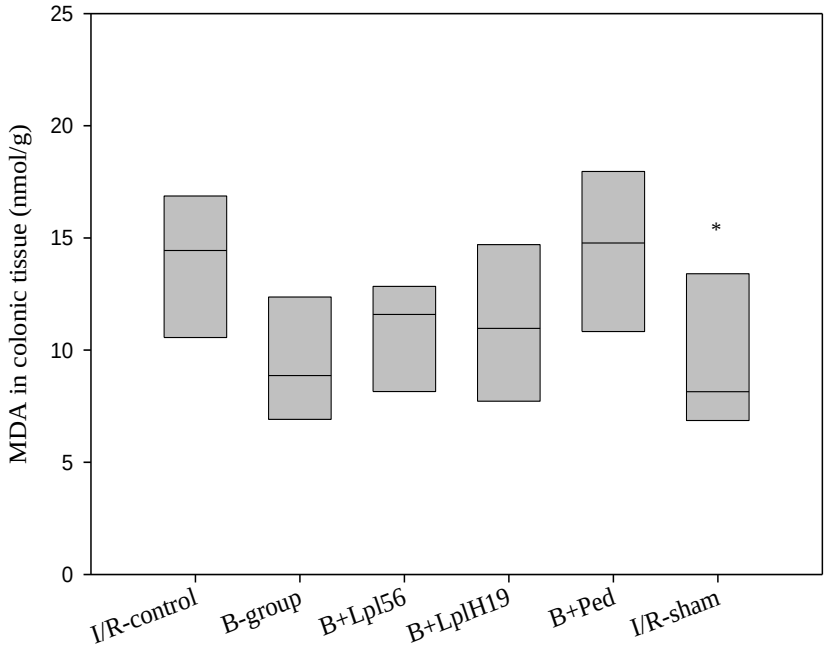


Figure 10. Malondialdehyde (MDA) after I/R in A) ileal tissue and B) colonic tissue of mice administered different diets supplemented with bilberry (B), either alone or together with *Lactobacillus plantarum* RESO56 (Lpl56), *Lactobacillus plantarum* HEAL19 (LplH19) or *Pediococcus acidilactici* JAM046 (Ped). Asterisks (*) denote $p<0.05$ or (***) $p<0.001$ compared to the I/R-control; hashes (##) denote $p<0.01$ compared to the B+Lpl56 group. Figure taken from paper IV.

Myeloperoxidase (MPO)

In paper IV, myeloperoxidase (MPO) was measured in the small intestine (ileum) after I/R, as an indicator of neutrophil infiltration. Prior to the I/R, mice were fed diet supplemented with bilberry (B), either alone or together with a lactic acid bacterium listed in Table 1. MPO was significantly lower in the sham ($p=0.040$) compared to the I/R-control. The increased MPO after intestinal I/R was confirmed by others (Muià *et al*, 2005; Ozkan *et al*, 2009). The lowest median value was observed in the bilberry-fed group (B), but none of the dietary supplements significantly reduced MPO, probably due to the large individual variations within the same group.

Histopathology

Histological damage that occurs after intestinal I/R is characterized by shortening and loss of intestinal villi, necrosis and neutrophil infiltration (Chiu *et al*, 1970). In paper IV, the most severe mucosal damage was observed in the I/R-control, with complete loss of villi and marked congestion. The median value of the mucosal injury score of the I/R-control was 4.5, and this was significantly ($p<0.001$) higher than that of the sham (0.25). No significant differences were recorded in the diet-supplemented groups. The median values of the injury scores were 2.5, 3.5, 4.0 and 3.3 for the groups B, B+Lpl56, B+LplH19 and B+Ped respectively. Pretreatment with bilberry alone (B group) showed the lowest median valuefor the mucosal integrity and morphology even if the difference in injury score was not significant. This coincides with the observations that the B group also showed a lower MDA (significant) and MPO values (non-significant) in ileum than the LAB groups. As previously suggested, the protective effects of the bilberry may be due to the high content of polyphenols with antioxidative and anti-inflammatory properties. In histopathological analysis large individual variations within the same group were also observed, suggesting that different individuals may have differed naturally in terms of antioxidant capacity of the mucosa and were not equally sensitive to the I/R damage (Durak *et al*, 2000).

Bacteriology

Lactobacilli and Enterobacteriaceae

In papers I, II and IV, the lactobacilli and *Enterobacteriaceae* counts were analyzed in colonic tissue, and in paper IV also in cecal tissue.

In paper I, the lactobacilli count of colonic tissue varied between 10^5 and 10^6 cfu/g. Diets supplemented with either *Rosa canina* (RC) or *Rosa pimpinellifolia* (RP) alone (median values 5.0 log cfu/g colonic tissue) reduced the number of viable lactobacilli compared to the diets supplemented with rosehips in combination with a mixture of *Lactobacillus* strains (median values 6.22 and 6.41 log cfu/g colonic tissue for RC+LAB and RP+LAB respectively), but no significant differences were found between the groups. All eight *Lactobacillus* strains in the mixture (Table 1) could be re-isolated from the colonic tissue of groups administered LAB (RC+LAB, RP+LAB and LAB). The most frequently occurring strain was *L. plantarum* 299v followed by *L. plantarum* RESO56, *L. paraplantarum* RESO97 and *L. plantarum* HEAL19. *L. paraplantarum* RESO97 was re-isolated to a higher

degree in the LAB group compared to groups fed a combination of rosehips and LAB.

In paper II, the lactobacilli count of colonic tissue varied between 10^4 and 10^7 cfu/g. Groups supplemented with either bilberry (B) or chokeberry (Ar) alone showed significantly (p=0.002 and p=0.008 respectively) lower count of lactobacilli compared to the group supplemented with *L. plantarum* HEAL19 (LplH19) alone. Daily administration of LplH19 together with berries prevented the decrease of lactobacilli.

In paper IV, the lactobacilli count of colonic tissue varied between 10^5 and 10^6 cfu/g. All groups fed bilberry showed a non-significant decrease in lactobacilli compared to the IR-control and sham groups. In the cecal tissue, lactobacilli count varied between 10^5 and 10^7 cfu/g. Bilberry supplementation significantly (p=0.030 and p=0.003 respectively) reduced the count of lactobacilli compared to the supplementation with bilberry in combination with either *L. plantarum* RESO56 (B+Lpl56) or *P. acidilactici* JAM046 (B+Ped).

In agreement with the results obtained in papers I, II and IV, Håkansson *et al* (2009) reported that supplementation with bilberry decreased the total lactobacilli count in the cecum of rat while combination of bilberry and probiotic mixture prevented the decrease. There are contradictory results on the influence of polyphenols on lactobacilli. Some authors (Lee *et al*, 2006; Tzounis *et al*, 2008; Puupponen-Pimiä *et al*, 2005a) claim that unabsorbed polyphenols and their metabolites can stimulate the growth of lactobacilli while others (Rodríguez *et al*, 2009; Parkar *et al*, 2008) suggest that several polyphenols, of which some are present in bilberry, have concentration-dependent inhibitory effects on the growth of *L. plantarum* and reduce adhesion ability of probiotic *L. rhamnosus* to human gut cell line. Generally, it appears that some species or strains of lactobacilli may be more susceptible to polyphenols than others.

In papers I, II and IV, the *Enterobacteriaceae* count in colonic and cecal tissues of all animals was generally low, and varied between $<10^2$ cfu/g and 10^4 cfu/g. There were no significant differences in *Enterobacteriaceae* count between the groups.

The composition of the bacterial flora

In paper IV, the microbiota of the cecal content of each animal was analyzed by T-RFLP. A total of 40 T-RFs were recorded for the whole cohort of animals. There

were no significant differences in the number of T-RFs between the groups. Furthermore, when bacterial diversity of the microbiota was calculated with the Shannon-Wiener diversity index and compared between the groups, no significant differences were obtained. However, supplementing the diet with bilberry seemed to have changed the composition of the cecal microbiota. It has been shown by others that a diet rich in proanthocyanins, catechin or anthocyanins can inhibit the growth of gram-positive *Clostridium leptum* cluster, *Clostridium histolyticum*, *Staphylococcus* spp., *Salmonella* spp., *Helicobacter pylori*, and *Bacillus cereus* while it can increase the proportions of *Enterobacteriaceae* and *Bacteroides*, *C. cocoides-Eubacterium rectale*, *Lactobacillus*, *Bifidobacterium* and *Escherichia coli* (Smith *et al*, 2004; Tzounis *et al*, 2008; Hara *et al*, 1995; Dolara *et al*, 2005; Puupponen-Pimiä *et al*, 2005).

Conclusions

The main conclusions drawn from this work are presented below.

Diet supplementation with bilberry (*Vaccinium myrtillus*), *Rosa pimpinellifolia* rosehips, or a mixture of eight different *Lactobacillus* strains can decrease the lipid peroxidation in the colon of mice. A corresponding supplementation with chokeberry (*Aronia x prunifolia*) or *Rosa canina* rosehips did not have this effect. Furthermore, bilberry supplemented to the diet can reduce the lipid peroxidation in the ileum of mice subjected to a more severe ischemia-reperfusion injury.

Anthocyanins can be detected in the GI tract of mice fed bilberry, chokeberry and rosehips. Anthocyanins, especially malvidin and petunidin, with arabinoside sugar seem to be more resistant to the absorption and microbial degradation than glucosides and galactosides. Cyanidin-3-O-glucoside and delphinidin-3-O-glucoside were mostly absorbed in the upper part of the small intestine, while malvidin-3-O-galactoside, peonidin-3-O-glucoside, peonidin-3-O-galactoside and petunidin-3-O-galactoside were among the ones most efficiently digested by the microbiota, or absorbed in the cecum. Malvidin and petunidin arabinosides were the least stable anthocyanins during storage of billberry products. Addition of wine yeast may improve their stability.

Aromatic acids such as 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid and 3-hydroxyphenylpropionic acid were detected as products of microbial degradation in the large intestine of mice fed bilberry or chokeberry. Protocatechuic acid was found to be the main product of cyanidin-3-O-glucoside degradation by cecal microflora after administration of *R. pimpinellifolia* rosehips.

Bilberry, chokeberry and rosehips supplemented to the diet reduced the count of lactobacilli in the large intestine. Daily administration of the lactic acid bacteria together with berries prevented the decrease of lactobacilli. The bilberry

supplementation also strongly influenced the cecum microbiota, while no clear differences could be seen by the additional supplementation with different strains of lactic acid bacteria.

Populärvetenskaplig sammanfattning

Yttre faktorerna i omgivningen som UV-strålning från solen, rökning, läkemedel, maten och påfrestande livssituationer kan leda till att kroppens celler börjar överproducera molekyler som kallas syrefria radikaler. Dessa molekyler reagerar kemist mycket lätt med omgivande ämnen. När syrefria radikaler bildas i kroppen i normala mängder ingår de som en del av immunförsvaret och medverkar i att döda mikroorganismer som virus och bakterier, men också cancerceller. Överskott av syrefria radikaler i kroppen leder till en så kallad oxidativ stress vilket innebär att de reagerar med DNA, proteiner och lipider i cellen och orsakar skada. Detta sätter igång inflammation, som om den blir långvarig kan leda till sjukdomar som t.ex. cancer, hjärt- och kärlsjukdomar, och typ 2 diabetes. För att skydda cellerna producerar kroppen ämnen som reagerar med de syrefria radikalerna och förhindrar de att reagera med DNA, lipid eller protein i cellen. Sådana skyddsämnen kallas antioxidanter. Även om kroppen själv producerar antioxidanter så räcker de inte alltid till för att skydda cellerna från skador. Däremot så finns det mycket antioxidativa ämnen i vissa livsmedel, t.ex. frukt och bär och tanken är att man genom att äta antioxidanter även kan skydda kroppen från skador orsakade av oxidativ stress. Till antioxidanter räknas bland annat vitaminerna C och E, karotenoider och polyfenoler. Speciellt intressanta är polyfenoler eftersom de utöver antioxidativa effekter även kan ha anti-inflammatoriska och antimikrobiella egenskaper. Det senare gör att de kan påverka bakterieflora i tarmen. En ofördelaktig sammansättning av tarmfloran där andelen med sjukdomsframkallande bakterier är hög (t.ex. bakterier som hör till familjen *Enterobacteriaceae*) och där andelen inflammationsdämpande bakterier är låg (t.ex. laktobaciller och bifidobakterrier) kan leda till inflammation och andra kroppsliga störningar. Tarmfloran är förmodligen viktig för att vi på rätt sätt skall kunna tillgodogöra oss många polyfenoler då det är svårt för kroppen att ta upp dessa innan de brutits ner till mindre molekyler. Bakterierna i tarmarna måste oftast hjälpa till i denna process.

Mjölksyrabakterier, oftast laktobaciller, är viktiga för tarmhälsan och har länge använts i livsmedel för att skapa balans i mag-tarmkanalen. Sådana bakterier som äts i levande form och har positiva hälsoeffekter på konsumenten har fått namnet

probiotika. Probiotiska bakterier normaliserar bakteriefloran i tarmen genom att konkurrera ut sjukdomsframkallande bakterier, stimulerar immunförsvaret, stärker tarmens barriärfunktion och höjer biotillgänglighet av näringsämnen i kroppen. Vissa laktobaciller har antioxidativa egenskaper och kan skydda mot oxidativ stress. Vidare kan en del laktobaciller omvandla och bryta ner antioxidativa polyfenoler i frukter och bär till mindre metaboliter, hanterbara för kroppen, och möjligen härigenom förstärka bärens antioxidativa effekter i tarmen.

Oxidativ stress och tarminflammation har studerats i en så kallad ischemia-reperfusion (I/R) modell i mus. I/R innebär att tarmen har varit utsatt för syrebrist p.g.a. avstängt blodflöde (ischemia) under en viss period och när blodflödet återställs (reperfusion) tillförs inflammatoriska mediatorer som t.ex. neutrofiler (vita blodkroppar) och syrefria radikaler. Detta leder till oxidativ stress och s.k. lipid peroxidation (vilket innebär en cellskada).

Målsättning med detta avhandlingsarbete har varit att studera om olika frukter och bär med högt innehåll av polyfenoler i kombination med potentiellt probiotiska mjölksyrabakterier kan dämpa och motverka oxidativ stress och tarminflammation i I/R-modellen. Mjölksyrabakteriernas förmåga att konvertera polyfenoler i en blåbärsdryck har studerats. Dessutom undersöktes om närvaro av jäst kunde påverka konverteringen av polyfenoler.

Tillsats av blåsvarta nypon av pimpinellrosa (*Rosa pimpinellifolia*) eller en blandning av olika laktobaciller till musfoder har minskat cellskada och oxidativ stress i musens tjocktarm. Ett kosttillskott av stenros-nypon (*Rosa canina*) gav inte samma skyddande effekt. Det var en allmän trend att nyponen i kombination med den probiotiska bakterieblandningen ökade koncentration av polyfenoler och deras antioxidativ kapacitet i tjocktarmen. Den grupp av polyfenoler som ger frukter och bär deras blå, röd och lila pigment benäms i kemiska termer anthocyaniner. Ett högt innehåll av anthocyanin och en nedbrytningsprodukt av dessa återfanns i tarmar hos möss som hade ätit pimpinell-nypon.

Cellskada och oxidativ stress i I/R-modellen i mus har också visats kunna dämpas när musfodret blandades med blåbärspulver eller blåbärspulver i kombination med en potentiell probiotisk laktobacill. Ett kosttillskott av aronia skyddade emellertid inte tarmcellerna. Blåbär visade sig ha en mer komplex sammansättning av polyfenoler, framförallt av olika anthocyaniner (pigmentämnen), än aronia. I tjocktarmen hos möss som hade ätit blåbär spårades ett större antal nedbrytningsprodukter av anthocyaniner än hos möss som ätit aronia. Dessa nedbrytningsprodukter är ett resultat av att tarmens bakterier har modifierat och delvis brutit ner de ursprungliga polyfenolerna i bäret.

Vi ändrade sedan I/R-modellen lite för att försöka öka skadan av den oxidativa stressen och studerade då även skadan i tunntarmen. Blåbärspulver blandad med

musfodret skyddade även tunntarmen mot cellskada. När blåbär gavs i kombination med var och en av tre olika mjölksyrabakterier, uteblev emellertid den skyddande effekten i tunntarmen. Tunntarmens innehåll hade högre koncentration av anthocyaniner jämfört med innehållet i tjocktarmen eftersom en del anthocyaniner bryts ner och absorberas på vägen ner. Den kemiska strukturen hos olika anthocyaniner påverkade deras absorption och nedbrytning i tarmen. Ett kosttillskott av blåbär förändrade bakterieflorans sammansättning i tjocktarmen.

När blåbärsdryck, baserad på krossade bär blandade med vatten, fick stå med olika mjölksyrabakterier under 3 veckor i 30°C, minskade koncentrationen av olika anthocyaniner i drycken. Nedgången var beroende av anthocyaninernas kemiska struktur och vissa minskade mer än andra. Tillsats av jäst ihop med mjölksyrabakterier ökade anthocyaninernas stabilitet så att negången blev mindre påtaglig.

Sammanfattningsvis, kan sägas att en tillsats av piminellrosens nypon eller av blåbär till musfoderet dämpade cellskadan och den oxidativa stressen i tjocktarmen. Skyddseffekterna var tydliga även när bären gavs tillsammans med laktobaciller. När modellen utformades för att öka den oxidativa stressen och tydliggöra cellskada visade sig blåbär fortfarande ha en skyddseffekt. Den tarmskyddande effekten av blåbär och pimpinellrosens nypon beror sannolikt på det höga innehållet av antioxidativa polyfenoler, framförallt pigmentämnena (anthocyaniner), och deras förmåga att inaktivera syrefria radikaler men kanske också på anthocyaninernas påverkan på tarmens bakterieflora i en positiv riktning.

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