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WOOD BIOCHEMICALS FOR THE PROTECTION OF HEALTH

FOCUS ON HEMICELLULOSE, STILBENOIDS AND LIGNANS

Lauri Polari

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ABSTRACT

Trees produce an enormous amount of compounds that are still scantly utilized. However, the results obtained from structurally similar biochemicals suggest that wood-derived compounds could be used for the protection of health in various applications. Polyphenols, for instance, could be extracted from wood in high quantities. Similar polyphenols to those in wood include resveratrol, found in grapes, and secoisolariciresinol, present in flaxseeds. Their consumption has been inversely associated with the incidence of various diseases, especially certain cancers and obesity-related disorders.

The aim of this study was to determine the health-promoting effects of wood-derived biochemicals. The effect of spruce hemicellulose on the growth of probiotic intestinal bacteria was studied. The results suggest that the bifidobacteria and lactobacilli can utilize hemicellulose and thus it has potential as a prebiotic compound.

In particular, the efficacy of pine polyphenols to inhibit the growth of prostate cancer was our main interest. It was found that stilbenoids and lignans inhibited the proliferation of various cancer cells, and reduced the growth of prostate cancer xenografts in mice. The polyphenol rich pine knot extract was well tolerated in diet and extract-derived polyphenols were rapidly absorbed after intake. Furthermore, we determined the effect of the dietary pine knot extract on the weight gain and the expression of aromatase gene in reporter mouse expressing the promoter region of a human aromatase gene. It was found that dietary pine knot extract alleviated the obesity-induced inflammation in adipose tissue and downregulated the expression of a human aromatase gene.

Taken together, several components of spruce and pine may have a future role as health-promoting compounds.

Keywords: aromatase, bifidobacteria, bioeconomy, gut microbiota, hemicellulose, inflammation, lignans, obesity, polyphenols, prebiotic, prostate cancer, resveratrol, softwood

Lauri Polari: PUUN BIOKEMIKAALIEN, ERITYISESTI HEMISELLULOOSAN, STILBENOIDIEN JA LIGNAANIEN, TERVEYSVAIKUTUKSET
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TIIVISTELMÄ

Puut tuottavat valtavan määrän yhdisteitä, joita kuitenkin hyödynnetään niukasti. Rakenteeltaan samankaltaisia molekyylejä esiintyy runsaasti terveellisenä pidetyssä ruokavaliossa, esimerkiksi rypäleissä ja marjoissa esiintyvä resveratroli ja pellavan lignaanit, joiden kulutus on yhdistetty käänteisesti mm. syöpien ilmenemiseen sekä lihavuuteen liittyviin haittoihin. Näin puiden kemikaaleilla voisikin olla terveysvaikutuksia esim. ihmisten ravinnossa tai lääketieteessä.

Väitöskirjatyössä arvoitiin puiden hemiselluloosan, jota ei nykyisin juurikaan hyödynnetä, terveysvaikutuksia. Työssä osoitettiin havupuiden hemiselluloosan lisäävän suoliston hyödyllisten bakteerien kasvua ja tulosten mukaan bifidobakteerit ja laktobasillit voivat käyttää kuusen hemiselluloosaa ravintonaan. Siten hemiselluloosaa voitaisiin käyttää myös ravinnossa prebioottisena yhdisteenä.

Havupuut ovat hyviä polyfenolien lähteitä, koska varsinkin niiden sisäoksissa pitoisuudet ovat erittäin korkeita. Tässä työssä tutkittiin männyn sisäoksauuteen ja sen sisältämien polyfenolien, pinosylviinien ja lignaanien, vaikutuksia syövän kasvuun. Näiden yhdisteiden osoitettiin vähentävän syöpäsolujen jakautumista ja hidastavan eturauhassyövän kasvua hiirimallissa. Männyn sisäoksauute osoittautui hyvin siedetyksi ja sen polyfenolit imeytyivät nopeasti. Lisäksi työssä määritettiin ravintoon sekoitetun männyn sisäoksa-uutteen vaikutusta ylipainoisten miesten testosteronivajeeseen yhdistettyyn aromataasigeenin ilmenemiseen. Männynoksauute alensi lihavuuteen liittyvää tulehdusta rasvakudoksessa ja laski aromataasireportterin aktiivisuutta ihmisen aromataasin promoottorialuetta ilmentävissä hiirissä. Näin ollen metsäteollisuuden sivuvirroistakin eristettävissä olevien männyn polyfenoleiden antikarsinogeenisia ja hormonien säätelyyn liittyviä ominaisuuksia voitaisiin tulevaisuudessa hyödyntää terveyden edistämiseen.

Avainsanat: aromataasi, bifidobakteerit, biotalous, eturauhassyöpä, havupuu, hemiselluloosa, lignaanit, lihavuus, polyfenolit, prebiootti, resveratroli, suoliston mikrobiootta, tulehdus

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ABBREVIATIONS

ACF	Aberrant crypt foci
AHR	Aryl hydrocarbon receptor
AMPK	Adenosine monophosphate-activated protein kinase
AP-1	Activator protein 1
AR	Androgen receptor
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Bb12	*Bifidobacterium animalis* subsp. *lactis*, strain BB-12
BMI	Body mass index
BrDU	Bromodeoxyuridine
bw	Body weight
CFBM	Carbohydrate free basal medium
CDK	Cyclin dependent kinase
CRM	Calorie restriction mimetics
CLS	Crown like structure
CHD	Coronary heart disease
COX	Cyclooxygenase
CYP	Cytochrome P450
DEX	Dexamathasone
DHT	Dihydrotestosterone
DMBA	Dimethylbenz(*a*)anthracene
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
E_2	17β-Estradiol
EFSA	European Food Safety Authority
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen responsive element
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FOS	Fructo-oligosaccharide
G3BP1	Ras-GTPase-activating protein SH3 domain-binding protein 1
GAM	Gifu anaerobic medium
GC	Gas chromatography
GF	Gonadal fat
GIT	Gastrointestinal tract
GGM	Galactoglucomannan
hARO	Human aromatase
HFD	High fat diet
HGPIN	High-grade prostatic intraepithelial neoplasia
ICAM-1	Intracellular adhesion molecule-1
IBD	Inflammatory bowel disease
IC50	Half maximal inhibitory concentration
IGF-1	Insulin-like growth factor 1
IL	Interleukin

ICAM	Intracellular adhesion molecule
IVIS	In Vivo Imaging System
LFD	Low fat diet
LPS	Lipopolysaccharide
Luc	Luciferase
MCP-1	Monocyte chemoattractant protein-1
MMP-9	matrix metalloprotease 9
MNU	N-methyl-N-nitrosourea
MS	Mass spectrometry
MRI	Magnetic resonance imaging
MOS	Mannan oligosaccharide
MTA1	Metastasis-associated protein 1
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa beta
NRF2	Nuclear factor erythroid-2-related factor 2
PMA	Phorbol 12-myristate 13-acetate
PSA	Prostate specific antigen
PBS	Phosphate buffered saline
PKE	Pine knot extract
PTEN	Phosphatase and tensin homolog
SCF	Subcutaneous fat
SEC	Size-exclusion chromatography
SERM	Selective estrogen receptor modulator
SIRT1	Silent mating type information regulation 2 homolog 1
TAG	Triacylglycerol
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UCP	Uncoupling protein
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
XOS	Xylan oligosaccharide

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-III:

I **Polari L**, Ojansivu P, Mäkelä S, Eckerman C, Holmbom B and Salminen S. Galactoglucomannan extracted from wood as a new carbohydrate source for probiotic bacteria. *J. Agr. Food Chem.*, 2012, **60** (44), 11037–11043

II **Yatkin E*, Polari L***, Laajala TD, Smeds A, Eckerman C, Holmbom B, Saarinen N, Aittokallio T, Mäkelä S. Combined biomarker analysis demonstrates anticarcinogenic efficacy of a pine knot extract in orthotopic prostate cancer xenografts. *PloS One*, 2014, **9** (4), **e93764**

III **Polari L**, Yatkin E, Martinéz Chacón G, Ahotupa M, Smeds A, Strauss L, Zhang F, Poutanen M, Saarinen N, and Mäkelä S. Weight gain and inflammation regulate aromatase expression in male adipose tissue, as evidenced by reporter gene activity. *Submitted manuscript* 2015

*These authors have contributed equally to this work

In addition, some previously unpublished data are presented in this thesis.

1. INTRODUCTION

The relationship between dietary habits and health has been one of the focal points of scientific investigation in recent years. Several studies have been carried out to identify the specific food components that convey health benefits. Consumption of plants rich in fiber, antioxidants, and polyphenols is often linked with favorable effects on health, including reduced risk of cancers and alleviation of obesity-related disorders (Turner-Mcgrievy et al., 2007; Giacosa et al., 2013). According to both epidemiological and preclinical studies, dietary polyphenols may reduce oxidative stress, alleviate inflammation, and modulate multiple signaling pathways, and therefore play a role in the prevention of various diseases (Baur et al., 2006; Lowcock et al., 2013; Tomé-Carneiro et al., 2013). In addition, gut microbiota can be modulated by diet and it has been suggested that they have a role in various diseases and disorders related e.g. to obesity and immunity (Kau et al., 2011).

Gut microbiota is a complex community of approximately 100 trillion microbes distributed over more than 1000 species (Bäckhed et al., 2005). It can be modulated, in addition to normal diets, by selectively increasing the activity and number of specific microbes, associated with health-promoting properties (Kleerebezem and Vaughan, 2009). Ingested microbes with favorable health properties are called probiotics, and dietary compounds inducing the growth of beneficial bacteria are referred to as prebiotics. Currently, only a few compounds fill the criteria of prebiotics and there is a need for novel prebiotic products (Kellow et al., 2014).

Hemicelluloses are common polysaccharides in plants and structurally similar to characterized prebiotics (Scheller and Ulvskov, 2010). Softwood derived hemicellulose is an untapped source of saccharides, but health-affecting properties of softwood hemicellulose (mostly galactoglucomannan) and its derivatives have not been surveyed expect in a few preclinical studies (Faber et al., 2011a; Rivas et al., 2012). Nevertheless, softwood hemicellulose is one of the most abundant molecules in the biosphere and still a mostly unexploited resource. Therefore, it is also a highly economic raw material.

Lignans and stilbenoids are small compounds belonging to polyphenols. They are widely distributed among the plant kingdom and present in the daily diet, but their average consumption is only a few milligrams per day (Hedelin et al., 2011; Zamora-Ros et al., 2006) This is a rather small amount compared to more common phenolic compounds, especially phenolic acids and flavonoids with daily intake of several hundreds of milligrams (Ovaskainen et al., 2008). Both stilbenoids and lignans have nevertheless been linked with health benefits;

especially their anticancer properties and effects on energy metabolism have aroused wide interest.

Resveratrol, which is present in grapes and red wine, has been the most studied plant stilbenoid by far. It was suggested at 1997 that resveratrol might possess various anticancer properties (Jang et al., 1997). Ever since, resveratrol and other stilbenoids have been associated with health effects linked with a wide variety of diseases, disorders, and cellular signaling pathways (Smoliga et al., 2011). However, reported data so far includes several inconsistent results and a clear picture about the effects of the intake of stilbenoids on human health is not yet possible to establish.

Dietary plant lignans are partially metabolized after intake by gut microbiota into enterolignans before being absorbed (Clavel et al., 2005; Heinonen et al., 2001). Epidemiological studies have suggested an inverse correlation between the enterolactone concentration, a major enterolignan, in serum and the risk of breast cancer (Lowcock et al., 2013). In addition to breast cancer, lignans have been associated with the prevention of other diseases, for instance prostate cancer and cardiovascular diseases (Azrad et al., 2013; Frankenfeld, 2014). It has also been proposed that lignans may have estrogenic properties and they are often classified as phytoestrogens (Valsta et al., 2003).

Coniferous trees are a good source of polyphenols, structurally similar to dietary compounds. The exceptionally high concentrations of lignans, and stilbenoids in the knots of spruce and pine exceeds all edible plants (Willför et al., 2003a, 2003b). Polyphenols in knots are mostly unconjugated which makes their extraction more economical, and also enhances their bioavailability compared with polyphenol glycosides in edible plants (Saarinen et al., 2002a). Moreover, knots and wood-derived polyphenols are mostly neglected by the pulp and paper industry and therefore they have been so far an unutilized resource.

The aim of this study was to investigate the use of wood-derived compounds, namely stilbenoids, lignans, and hemicellulose, as health-promoting agents by using bacterial, cellular and *in vivo* models. In this work, we focused on prostate and breast cancer and obesity - common and increasing health problems in a western world.

2. REVIEW OF THE LITERATURE

2.1 Wood biochemicals

2.1.1 Wood - an abundant resource of organic compounds

The earth contains roughly a trillion tons of wood. In Europe alone, the annual increment of wood is over 600 million cubic meters (Kindermann et al., 2013). Man has utilized wood for several thousands of years. In addition to conventional applications, the pulp and paper industry has employed wood during the 20th century (Fenning and Gershenzon, 2002), and therefore, nowadays the lignocellulose fraction is the most utilized wood based material. However, only about 2 % of the total wood biomass is currently used (Pauly and Keegstra, 2008).

Table 1. The chemical composition of wood.

Compound group	% Of wood dry mass
Cellulose	33-51 %
Lignin	21-35 %
Hemicellulose and other polysaccharides	27-33 %
Organic extractives*	1-5 %
Inorganic residue	<1 %

*includes: fats, waxes, alkaloids, proteins, simple and complex phenolics, simple sugars, pectins, mucilages, gums, resins, terpenes, starches, glycosides, saponins, and essential oils

Cellulose is the main component of wood (Table 1) and it is also the most prevalent biopolymer, accounting for about 40 % of the organic carbon in the biosphere. The molecular structure of cellulose remains similar among the plant kingdom consisting of a linear chain of 1.4-β-glucose units (Liu, 2010; Pettersen, 1984). Lignin and hemicellulose are the two other main fractions in wood, both of them being much more heterogeneous than cellulose. In addition, there are several minor constituents of wood, mainly organic extractives and inorganic compounds (Table 1). The exact composition of wood cannot be defined because there is considerable variability, not only between species, but also between the different parts of wood. Age, geographical location, climate, and soil all have an effect on wood's molecular composition (Pettersen, 1984; Plomion et al., 2001). Some of the molecule derived from wood such as resins from conifers, and tannins have been traditionally used for a long time, but many of the wood based molecules, present only in small quantities, have been

mostly ignored until recently. Overall, wood is a rich source of different unique molecules, but the majority of these still lack biological characterization, and their properties and potential usefulness is yet unknown (Li et al., 2008).

Woody biomass has also aroused interest as a more ecological carbon source of raw materials to replace fossil fuels, and also to as a source of discovering more value from wood material (Ragauskas et al., 2006). In future, pulp and paper mills will slowly transform into integrated bio-refineries, which can effectively convert biomass to chemicals and energy (Ferrini and Rinaldi, 2014; Liu, 2010; Martin-Sampedro et al., 2014).

The forest industry has been a significant part of the Finnish economy and industry. Currently, there have been both political and economic aims to strengthen the Finnish forest sector by shifting the industry to being the provider of raw materials to modern bio-refineries, which can then optimize the use of woody biomass as biofuels, bioenergy, and biomaterials. This transition is a part of a modern bio-economy, the purpose of which is to improve human well-being by reducing the use of non-renewable resources, greenhouse gas emissions, and the amount of waste, and increase the utilization of renewable energy and material sources (Prime Minister's Office, 2010).

During the last few decades, it has been discovered that several small molecules synthetized in wood are structurally similar to dietary compounds that are present in healthy foods. Phenolic compounds especially have been recognized as an interesting group of biochemicals. These compounds are often enriched in specific parts of the wood, namely in bark, heartwood, and especially knots, in an easily extractable form (Holmbom et al., 2003; Piispanen et al., 2008; Pohjamo et al., 2003). Phenolic wood-derived extracts include compounds like lignans, flavonoids, and stilbenoids, which have become compounds of interest for pharmaceutical, nutraceutical, and functional food industries; in addition the antioxidative and anticancer properties of these compounds have already been notified by researchers (Bylund et al., 2005; Mellanen et al., 1996; Willför et al., 2003e).

2.1.2 Wood derived material as pharmaceuticals or nutraceuticals

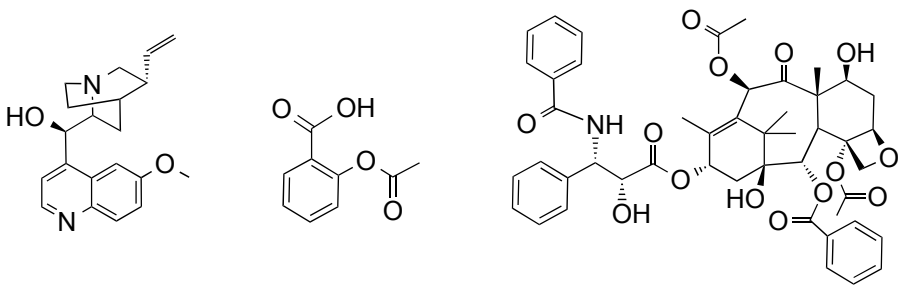

Figure 1. From left to right: quinine, aspirin and paclitaxel

While the concept of modern biorefineries is rather new, the idea of extracting health-promoting compounds from wood is not a novel one. Acetylsalicylic acid (aspirin) is one of the oldest molecules used as a pharmaceutical. The medical use of willow bark has been known since antiquity. Pure salicin was extracted from a willow (*Salix alba*) and crystallized in 1828, and commercial production of aspirin (Figure 1), a derivative of salysylic acid, started in 1899. Another historical example is quinine, which was discovered by the Incas and brought to Europe and used as a treatment for malaria already in the 17th century. A more recently discovered pharmaceutical derived from wood is paclitaxel, a compound isolated from the bark of a pacific yew tree (*Taxus brevifolia*). Paclitaxel has been used to treat several cancers including breast and ovarian cancer (Wall, 1996). In general, the versatility and capacity of wood to produce biologically active compounds is recognized, but the health promoting potential of a wide number of compounds derived from wood is not yet fully valued.

2.1.3 Biochemicals in edible plants and conifers

Throughout the plant kingdom, there are certain similarities in the structures and composition of plant tissues, both at the cellular and molecular level. Most of the biochemicals present in a certain plant division or class, e.g. in conifers (*Pinophyta*), can be found in other plants as well. However, there are certain aspects why some common coniferous trees can be considered as especially interesting and highly utilizable source of compounds. Phenolic compounds can be found in most parts of the tree, but they are concentrated in certain tissues. Spruce, pine, and fir knots contain a high concentration of phenols; even over 10 % of the mass can consist of phenolic compounds - exceeding all other known sources of phenolics in nature (Willför et al., 2003b). Lignans are the major group of phenolic compounds in softwood knots, but some other phenolics like flavonoids and stilbenoids can also be found (Willför et al.,

2003a, 2003b). Knots are also a convenient and bio-economical source having little value for the pulp and paper industry and can be separated from the wood mass before pulping (Holmbom et al., 2003). While knots and their lignan-containing fraction are only a very minor part of the total wood mass, due to large volumes of pulp and paper production, it is approximated that in Finland alone it is possible to produce 130 tons of spruce (*Picea abies*) lignans annually from side streams of the forest industry (Holmbom et al., 2003).

The notable chemical difference between wood and edible plants is that in most edible plants phenolic compounds occur as glycosides (Figure 2). They are also eaten as glycosides, as the majority of food processing techniques do not lead to the cleavage of the glycoside bond (Bambagiotti-Alberti et al., 1994; Meagher and Beecher, 2000; Romero-pe et al., 1999). Wood, especially knotwood in spruce and pine, is rich in free, unconjugated lignans and stilbenoids (Holmbom et al., 2003; Willför et al., 2003c). The glycosylation status may affect the chemical and biological properties of a compound like its antioxidant activity (Su et al., 2013) and absorption rate in gastrointestinal tract (GIT) (Németh et al., 2003).

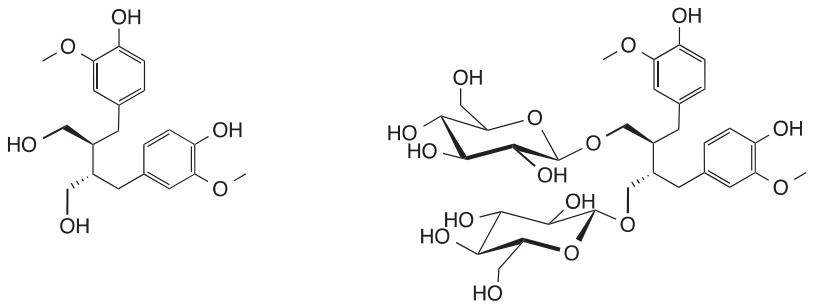

Figure 2. Structures of secoisalariciresionol (left) and its diglucoside (right)

In spite of several studies, the effect of the glycoside residues of phenolic compounds on the biological properties of molecule is still unclear. There is, nevertheless, some comparative data about the differences in biochemical properties and metabolism between phenolic compounds and their glycosides. Secoisolariciresinol and its diglucoside, SDG (Figure 2), both display antioxidant activity and intake of either of them has similar effects on body weight and serum cholesterol (Felmlee et al., 2009; Hu et al., 2007). However, the ratio of metabolites excreted in urine after digestion is different between secoisolariciresinol diglucoside and its aglycone (Saarinen et al., 2002a). Similarly, unconjugated resveratrol and its monoglucoside, piceid, have different metabolite profiles in urine and plasma, suggesting differences in absorption and metabolism of the molecule (Rotches-Ribalta et al., 2012). Piceid has higher hydroxyl radial scavenging capacity, suggesting that it could

be a more efficient antioxidant than resveratrol (Su et al., 2013). However, in the same study, resveratrol was more potent in inhibiting cell proliferation and inducing apoptosis *in vitro*. More effective transport of unconjugated resveratrol inside cells could be the main reason for its higher activity. It is also suggested that compared to resveratrol, the bioactivity of dietary piceid is significantly lower in colorectal mucosa (Kineman et al., 2007).

Results on the bioavailability of flavonoids such as daidzein, genistein, and quercetin and their glycosides are partially inconsistent (de Pascual-Teresa et al., 2006). Nonetheless, deglycosylation *in vivo* is required for absorption of flavonoids from GIT, and in cellular *in vitro* assays aglycones are usually found to be more active (Dueñas et al., 2013; Németh et al., 2003; Theil et al., 2011). Plant phenolics can also be covalently bound to macromolecules like structural cell wall components, including cellulose and lignin. Binding to macromolecules reduces the solubility of a compound, and may result in very low bioavailability in GIT (Acosta-Estrada et al., 2014). According to the same study, some macromolecules can still be fermented by microbial activity in the large intestine and bound phenolic compounds are released.

Hemicelluloses are the major constituents in plant cell walls along with cellulose, pectin, and lignin. The composition of hemicellulose varies between plant species and in conifers acetylated galactoglucomannan (GGM) is the major component of hemicellulose. GGM is present in plant cell walls and consists of about 30 % of the dry weight of the softwood mass, and therefore it is one of the most common carbohydrates in the biosphere. Nevertheless, the pulp and paper industry have not yet found any high value applications for this plentiful biopolymer (Scheller and Ulvskov, 2010; Willför et al., 2003d).

Non-woody plants are mostly composed of water and this makes their components more labile due to increased reactivity and exposure to sunlight. In contrast, due to the sturdier matrix in wood, both phenolic compounds and polysaccharides remain intact for a long time after harvesting (Holmbom et al., 2003; McDonald and Donaldson, 2003).

2.2 Gut microbiota and prebiotic compounds

2.2.1 Composition of human microbiota

A healthy human intestine is colonized by tens of trillions of bacteria and each individual microbiota includes approximately 800 species of bacteria and 7000 different strains (Bäckhed et al., 2005). Microbiota is a dynamic community

and its collective genome, also called a microbiome, may contain over 100 times the number of genes in a human genome. Microbiota affects several targets of its host including energy balance, metabolic homeostasis, and immune systems (Kau et al., 2011). In addition, microbiota can be considered as an anaerobic bioreactor, which metabolizes many otherwise indigestible compounds (Bäckhed et al., 2005). For example, microbes ferment several plant-derived saccharides into short chain fatty acids (Delzenne et al., 2011). The composition of the microbiota evolves during the aging of the host and varies with the ethnic background (Yatsunenko et al., 2012). Furthermore, some conditions like obesity or malnutrition, can have a major effect on the microbiota, which can be also modulated with dietary interventions (Cotillard et al., 2013; Ley et al., 2005). It has been shown that certain changes in the composition of microbiota are linked to obesity, for instance the gut microbiota of obese subjects includes more Firmicutes and less Bacteroidetes (Tremaroli and Bäckhed, 2012). Interestingly, germ-free mice colonized with microbiota from obese donors gained more weight due to enhanced energy harvest as those with microbiota from lean donors (Turnbaugh et al., 2006). Whether the microbiota also contributes to overweight in humans requires further investigations (Tremaroli and Bäckhed, 2012).

Human microbiota contains many bacteria, the role of which are still not known, and also bacteria that are considered as potential pathogens. Some genera of intestinal bacteria are often considered beneficial for the well being of their host (Kleerebezem and Vaughan, 2009). These bacteria are natural inhabitants of the human intestinal tract but some of them are also present in several common dietary products e.g. uncooked plants and foods that are fermented through lactic acid fermentation. Intake of bacteria with food modulates the composition and number of the microbiota and dietary products can also be supplemented with beneficial bacteria called probiotics. Probiotics, most notably lactobacilli and bifidobacteria, are endemic bacteria, mainly found in the human intestinal tract (Ferreira et al., 2011). The definition by the World Health Organization for a probiotic is a “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host”(FAO/WHO, 2001). In addition to genus and specie, the strain of a microbe has to be characterized in probiotic products since there are wide differences between the probiotic efficacy of certain strains inside the same bacteria species (Rijkers et al., 2011).

Eating prebiotic compounds that probiotic bacteria can ferment also modulates the microbiota. A prebiotic is a “nonviable food component that confers a health benefit on the host associated with the modulation of the microbiota” (Pineiro et al., 2008). In general, prebiotics are indigestible compounds that are

fermented by the microbiota. To date, the criteria for prebiotic compounds have been fulfilled only by a few oligo- and polysaccharides, most notably inulin (Roberfroid, 2007). A product containing both probiotics and prebiotics is called a synbiotic (Ferreira et al., 2011).

2.2.2 Characterization of prebiotic compounds

Plant derived fructo-oligo saccharides and inulin were the first compounds whose prebiotic properties were characterized (Mitsuoka et al., 1987; Tokunaga et al., 1993; Yazawa et al., 1978) even before the actual prebiotic concept was defined in 1995 (Gibson and Roberfroid, 1995). Inulin and fructo-oligo saccharides are both plant-derived carbohydrates and are mostly composed of fructose units. These compounds are used as a golden standard for prebiotics and are present in high concentrations in many edible plants like chicory and onion (Roberfroid, 2008). Other prebiotic compounds, the properties of which have also been characterized in clinical studies, include various indigestible saccharides like galacto-oligo saccharides (Vulevic et al., 2008) and lactulose (Tuohy et al., 2002). Several other natural compounds may also have prebiotic properties, but their presence and concentrations in food are not yet adequately characterized, and therefore no epidemiological studies about the total consumption of prebiotics are so far available.

Before clinical trials take place, prebiotic candidates are studied in microbe cultures *in vitro*. In particular, the selective stimulatory effect of prebiotics on the growth of bifidobacteria and other beneficial microbes has been of major interest (Roberfroid, 2008). Usually, the second step in studying the effect of a prebiotic compound on the modulation of a microbiota is to use an animal model or to introduce a candidate molecule to a human intestine model. A human intestine can be modeled in an artificial colon simulator or in animals with transplanted human microbiota (Turnbaugh et al., 2009; de Wiele et al., 2004). Additionally, the stimulatory effect of prebiotics on short chain fatty acid production in the gut is a principal method for assessing the efficacy of a prebiotic compound (Gibson and Roberfroid, 1995). Short chain fatty acids are fermentation products of microbiota. The increased production of short chain fatty acids, most notably acetate, lactate, propionate, and butyrate, in the large intestine affect several targets that may influence health e.g. the enhanced absorption of calcium and exclusion of pathogens (Campbell et al., 1997; Scholz-Ahrens and Schrezenmeir, 2007). Butyrate is also an energy source for colonic epithelial cells and it has been suggested to inhibit the growth and invasion of colon cancer (Emenaker et al., 2001). In general, short chain fatty acids lower fecal pH, which may prevent some pathogens from growing

(Campbell et al., 1997). Those may also affect satiety and food intake through multiple cell signaling pathways (Flint et al., 2012).

Originally, prebiotic research was mostly carried out to demonstrate the effects of a consumption of prebiotics on the composition of gut microbiota (Gibson and Roberfroid, 1995). Currently, the modulatory effect alone on the composition of gut microbiota is not sufficient to assume the compound as a prebiotic, but also the physiologic effects on the host have to be shown (Pineiro et al., 2008). Prebiotics have been suggested, based on several clinical trials, to alleviate several symptoms related to obesity and diabetes (Kellow et al., 2014). Whether prebiotics might also benefit healthy, lean subjects is a debated topic, but this has not been proven in clinical trials.

2.2.3 Prebiotic compounds and human health

Non-digestible carbohydrates are important energy source for intestinal bifidobacteria and promote their growth (Gibson and Roberfroid, 1995). In addition, reduced levels of intestinal bifidobacteria has been linked to obesity in both human trials and mouse models (Cani et al., 2007a; Kalliomäki et al., 2008; Wu et al., 2010b). Therefore, it is not surprising that the consumption of prebiotics is associated with body weight and type two diabetes. The body weight, energy intake, and satiety of overweight or diabetic individuals have been shown to improve after prebiotic interventions - even though the magnitude of effects varied (Delzenne et al., 2011; Kellow et al., 2014; Roberfroid, 2008). In addition, prebiotics may also reduce postprandial glucose and insulin levels, while the exact effects on the fasting levels of glucose have been controversial (Kellow et al., 2014).

Obesity is linked with systemic low-grade inflammation and a high fat diet has been suggested to increase the proportion of lipopolysaccharide (LPS) producing gut microbes that induce inflammation (Cani et al., 2007a; Visser et al., 1999). According to several clinical trials, diet that supplemented with prebiotic saccharides has been suggested to alleviate the low-grade inflammation (Kellow et al., 2014; Roberfroid, 2008). For instance, the levels of inflammatory biomarkers, such as LPS and C-reactive protein have been reduced in clinical trials (Dehghan et al., 2014; Vulevic et al., 2013). A few alternative mechanisms for the anti-inflammatory properties of prebiotics have also been suggested: changes in the composition of microbiota may alleviate proinflammatory cytokine and LPS production, or the exclusion of pathogens or some microbial products, such as short chain fatty acids, may interact with immune cells and enterocytes to alleviate inflammation (Roberfroid, 2008). Results from animal studies suggest that the high consumption of prebiotics

could protect against obesity-related changes in microbiota, increased inflammation, and related metabolic disorders (Cani et al., 2007b; Everard et al., 2014).

Some clinical studies propose that the consumptions of prebiotics: inulin and galacto-oligosacharides, could reduce the levels of total cholesterol and LDL, or circulating triacylglycerol (TAG) (Tovar et al., 2012; Vulevic et al., 2013). One possible mechanism could be that microbiota-produced propionate inhibits the cholesterol synthesis in the liver (Pereira et al., 2003; Trautwein et al., 1998). However, the results of different trials have been inconsistent, and the meta-analysis of clinical trials does not fully support significant effects on TAG or cholesterol levels (Kellow et al., 2014). In addition, there are studies implying that the consumption of prebiotics enhances the absorption of minerals, especially calcium (Scholz-Ahrens et al., 2007). According to the same authors, the mechanism might include short chain fatty acids and other acidic fermentation products of prebiotics. Acidic compounds lower the luminal pH of intestine, which increases the solubility and diffusion of minerals. Altogether, the beneficial effects of prebiotic compounds have been shown in several studies, even though their ultimate mechanisms of action remain unsolved.

2.2.4 Wood hemicellulose as a prebiotic compound

Wood is a rich source of different indigestible polysaccharides, some of which are suggested to possess prebiotic properties. However, cellulose, the main woody carbohydrate (Figure 3), is not a prebiotic compound (Sunvold et al., 1995). Nonetheless, cellulose is still considered as a dietary fiber (Lattimer and Haub, 2010). The second most common polysaccharide fraction in wood and in other plants is hemicellulose. It is present mainly in plant cell walls and its main role is to strengthen the cell wall by tethering the cellulose fibrils (Scheller and Ulvskov, 2010). In comparison with cellulose, hemicellulose is a much more heterogeneous group of different polysaccharides (Xiao et al., 2001). Its sugar unit composition varies between different plant taxa and may, among others, include glucose, mannose, galactose, xylose, and arabinose residues in different combinations (Schädel et al., 2010). While structural details like the saccharide composition and branching pattern of a backbone of hemicellulose vary between species and even cell types, the principal feature of hemicelluloses between plant divisions is β(1-4) –linked saccharide backbone with an equatorial configuration (Scheller and Ulvskov, 2010).

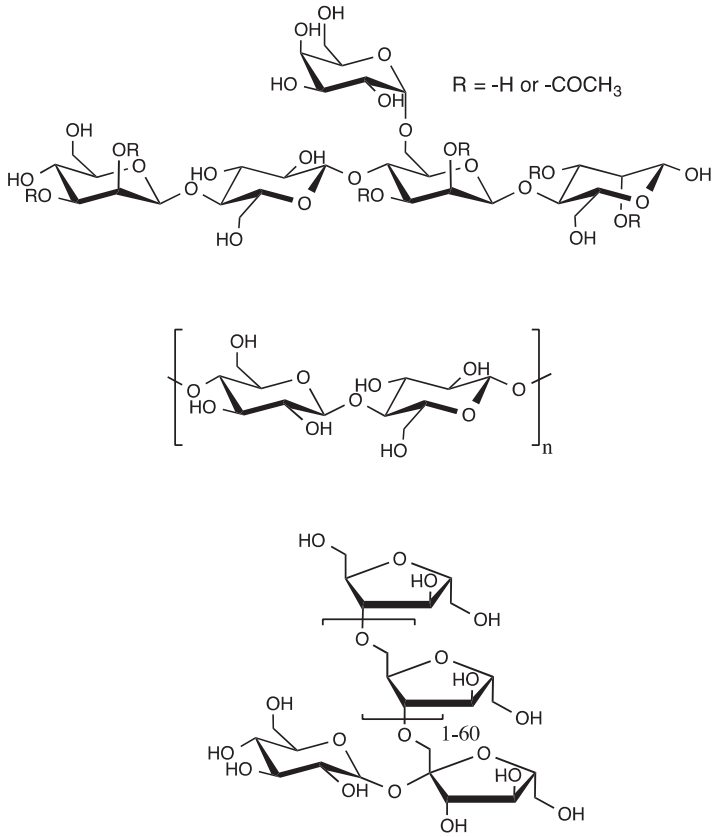

Figure 3. Top: Structure of galactoglucomannan oligomer, middle: structure of cellulose, bottom: structure of inulin

Hardwood hemicellulose is mostly composed of xylan, xylose being the dominant sugar residue in e.g. birch and eucalyptus. Xylan can be hydrolyzed to free xylose and xylo-oligo saccharides (XOS) with other minor substituents (Ban et al., 2008). Birch derived XOS has been found to promote the growth of bifidobacteria both *in vivo* and *in vitro* (Okazaki et al., 1990). In addition to bifidobacteria, XOS promotes the growth of several butyrate-producing intestinal bacteria (Scott et al., 2014) and stimulates the production of short chain fatty acids *in vivo* (Campbell et al., 1997; Mäkeläinen et al., 2010). The consumption of XOS as a dietary supplement has been shown to have beneficial effects on the GIT of elderly subjects (Chung et al., 2007). In combination with inulin (Figure 3), XOS also decreased the level of circulating LPS and the LPS-dependent induction of inflammatory interleukin 1β (IL-1β) expression in the blood of healthy subjects, suggesting that dietary XOS has anti-inflammatory properties (Lecerf et al., 2012). In addition to hardwood, xylan based hemicellulose exists in several non-woody plants like in fruits and vegetables. Therefore, it is also present in daily diet (Vazquez et al., 2000).

Acetylated galactoglucomannan (GGM) is an abundant polysaccharide in softwoods like in spruce and pine, but is not found in edible plants in significant concentrations. GGM consists of a β(1-4) –linked chain of randomly distributed mannose and glucose units (Figure 3), with molar ratio of 4:1, and galactose residues as a single side units (Willför et al., 2003d). The polymerization rate of GGM varies between 100 and 400 monosaccharide units and GGM can be hydrolyzed to a mixture of different oligomers, similarly to XOS (Price et al., 2011; Xu et al., 2008). GGM has been recently suggested to have prebiotic properties due the fact that hydrolyzed GGM stimulated the growth of bifidobacteria and promoted short chain fatty acid production in fecal cultures (Rivas et al., 2012). Another GGM derived oligosaccharide also promoted the growth of bifidobacteria *in vivo*, increased the fecal short chain fatty acid concentration and lowered the fecal pH (Faber et al., 2011a, 2012a). Additionally, GGM derived oligosaccharides have been shown to possess immunomodulating properties by alleviating *Salmonella typhimurium* and *Eimeria acervulina* infections in chickens (Faber et al., 2012a, 2012b). Compared to XOS, which is already commercialized in China and Japan as a health promoting product and nutraceutical (Vazquez et al., 2000), the information about the efficiency of GGM as a prebiotic compound is still preliminary.

Other hemicellulose derived saccharides can also have prebiotic properties because they have a similar kind of chemical structure to xylan and GGM (Scheller and Ulvskov, 2010). They may become a valuable resource because novel prebiotic molecules are still required due that the present prebiotics being based only on a few different molecules (Jakobsdottir et al., 2014; Kellow et al., 2014). From the perspective of Finnish bioeconomy and utilization on forest biomass, xylan and GGM are interesting prebiotic candidates since they are major compounds in the most important local wood species, spruce, pine, and birch.

2.3 Stilbenoids, lignans and human health

2.3.1 Classification of plant derived phenolic compounds

Phenols, or phenolics, are a diverse group of compounds consisting of a hydroxyl group directly bound to an arene ring. Plant phenolics are phenols, which are formed in plants, in contrast to microbial, mammalian, or artificial phenols. Mammalian and microbial phenols are, however, compounds that often have a plant phenol as a precursor, and they are formed through the metabolic activity of microbes or mammals (Landete, 2012). All phenols share

certain structural similarities and common synthetic pathways implying that most of the plant phenolics are derived from a few intermediates of shikimate and polyketide pathways (Vogt, 2010).

A phenolic compound with several hydroxyl groups is called a polyphenol. The classic definition of polyphenol is rather specific, including water-solubility, molar mass of 500-4000 D, and possessing 12-16 phenolic groups per 1000 D of relative molecular mass (Haslam and Cai, 1994). This definition excludes mono- and dimeric plant phenols, e.g. lignans and flavonoids, from true polyphenols. However, a term "polyphenol" is nowadays used rather freely despite its previous meaning. Quideau et al. recently proposed a revisited definition for a plant polyphenol: "The term "polyphenol" should be used to define plant secondary metabolites derived exclusively from the shikimate-derived phenylpropanoid and/or the polyketide pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression" (Quideau et al., 2011). This definition covers significant wood derived phenolic compounds addressed in this thesis and will be used hence.

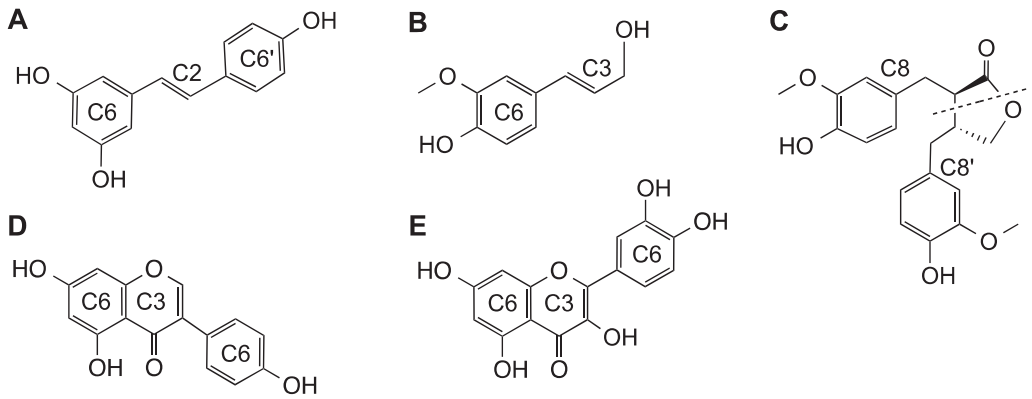

*Figure 4. Structure of (**A**) trans-resveratrol (stilbenoid), (**B**) coniferyl alcohol (monolignol), (**C**) matairesinol (lignan), (**D**) genistein (flavonoid/isoflavone) and (**E**) quercetin (flavonoid/flavonol).*

Polyphenols can be classified into different groups according to the number of phenol rings they contain and the structural elements that bind arene rings to each other. Flavonoids are probably the largest polyphenol group containing over 8000 characterized compounds so far (Del Rio et al., 2013). They have C6-C3-C6 –structure (Figure 4), where first C6 and C3 are fused to form a

benzopyran -structure. Flavonoids are classified into six major subgroup (flavone, isoflavone, flavonol, flavan-3-ol, anthocyanidin and flavanone) and several minor ones. Other major groups of plant polyphenols are tannins, which are compounds also filling the classic definition of polyphenol (Haslam and Cai, 1994), and heterogeneous group of lignin polymers. Lignins are complex aromatic polymers, which are derived from simple phenolic compounds, mainly from hydroxycinnamyl alcohols, also called monolignols (Boerjan et al., 2003). Lignans are monolignol dimers, usually bonded via central carbon with C8-C8' (Figure 4) linkage (Davin and Lewis, 2004; Umezawa, 2004). Stilbenoids are phenolic compounds with C6-C2-C6' stilbenes (Figure 4) backbone (Meier, 1992). Stilbenoids can also form a dimers similarly to monolignols (Quideau et al., 2011; Rolfs and Kindl, 1984). Plant polyphenols are generally suggested as having a functional role in plant resistance against environmental stress and different hostile organisms, including pathogens, fungi, insects and mammalian herbivores (Barbehenn and Constabel, 2011; Quideau et al., 2011). Variable polyphenol patterns between different plants are suggested to maximize their joint defense against different herbivores (Agrawal et al., 2012; Salminen et al., 2011; Treutter, 2006). Plants might also modulate their polyphenol level according to the presence of herbivores (Agrawal et al., 2012). The polyphenol content of plant specie can differ between plant varieties, growth regions, and even annually between harvests (Stervbo et al., 2007).

2.3.2 Phenolic compounds in human diet

As phenolic compounds can be found throughout the plant kingdom, they are naturally present in the daily diet. There are substantial variations in major dietary sources of phenolics between individuals and populations, but the average daily intake of phenolics in Europe is often estimated as being 800-1000 mg/day (Table 2) as aglycones (Ovaskainen et al., 2008; Perez-Jimenez et al., 2011; Scalbert and Williamson, 2000; Tresserra-Rimbau et al., 2014). Flavonoids and phenolic acids are the two major classes of phenols present in a regular human diet, accounting for the majority of total phenol intake (Scalbert and Williamson, 2000). Other phenolic compound groups in the diet are lignans and stilbenoids. While tannins as a derivatives of phenolic acids and other phenolics could also be included in the compound groups previously described, they are often discussed as a group of their own (Salminen et al., 2011; Scalbert and Williamson, 2000). Common phenolic monomers in dietary tannins include catechin, epicatechin, and gallic acid (Serrano et al., 2009).

The amount of phenolic compounds in food can be quantified either by measuring compounds from a food matrix with chromatographic techniques or by measuring the total amount of phenolic compounds based on the reduction

reaction with a Folin-Ciocalteu reagent (Scalbert and Williamson, 2000). When the Folin assay and chromatographic methods are compared (Table 2), the Folin assay leads to significantly higher concentrations than chromatographic methods. The reasons for this are the side reactions of the Folin-Ciocalteu reagent with non-phenolics like ascorbic acid and the undetectability of some compounds due to technical difficulties in chromatography (Arranz et al., 2010; Perez-Jimenez et al., 2011; Scalbert and Williamson, 2000).

Table 2. Daily intake of phenolic compounds.

Study population	Mean daily intake,g	Major sources (in order)	Method	Reference
Data collected from several studies to reflect the average polyphenol intake in Western diets	1	Fruits, beverages (juice, wine, tea, coffee, and beer), vegetables, dry legumes, cereals, chocolate	Chromatography and Folin assay	Scalbert and Williamson, 2000
Spanish adults	2,8	cereals, fruits, beverages, vegetables, legumes, nuts	Folin assay	Saura-Calixto et al., 2007
Finnish adults	0,86	coffee, bread and cereals, tea, fruits, berries	Chromatography	Ovaskainen et al., 2008
French adults	0,82	coffee, fruits, tea, red wine, cocoa, vegetables, cereals	Chromatography	Perez-Jimenez et al., 2011
	2,0	as above	Folin assay	Perez-Jimenez et al., 2011
Institutionalized elderly people in Spain	0,3	red wine, coffee, apples, oranges, green beans	Chromatography	González et al., 2014
Elderly people in Spain	0,82	coffee, oranges, apples, olives and olive oil, vegetables, red wine	Chromatography	Tresserra-Rimbau et al., 2013

In contrast to the total intake of phenolic compounds, the consumption of specific polyphenols varies more between populations (Table 2) (Manach et al., 2004). For example, in Finland, the main source of phenolics is coffee and therefore phenolic acids are the major group of phenolics in the Finnish diet (Ovaskainen et al., 2008). On the other hand, in the Spanish diet, fruits and

vegetables are more common and therefore flavonoids contribute the most of the daily intake of phenolics (González et al., 2014; Tresserra-Rimbau et al., 2013).

Certain phenolic compounds are only present in specific edible plants and their intake correlates strongly with the consumption of this food. Stilbenoid intake, for instance, correlates with the consumption of wine and grapes (Zamora-Ros et al., 2006). Stilbenoids, the most importantly resveratrol and its glycoside, are present in several dietary plants such as berries and peanuts, but grapes and red wine are the most important sources due to their high stilbenoids content (Lyons et al., 2003; Sobolev and Cole, 1999; Stervbo et al., 2007). The average intake of stilbenoids is 2-4 mg/day, but the high consumption of wine and grapes may increase the daily stilbenoid dose to over 30 mg (González et al., 2014; Perez-Jimenez et al., 2011; Stervbo et al., 2007; Tresserra-Rimbau et al., 2013). Isoflavones genistein and daidzein are another example of partly food-specific compounds. They are present in many leguminous plants, but their high intake is linked to substantial soy consumption (Kelly et al., 1993).

Similar to stilbenoids, a high lignan intake is linked with the consumption of specific foods. The main lignan sources in European countries are cereals, fruits, berries, vegetables and beverages (Hedelin et al., 2011; Tetens et al., 2013). Even though the contribution of different food groups varies between countries, the average consumption of lignans is approximately the same (1 - 4 mg/day). However, lignan content in flaxseeds and sesame seeds exceeds other edible plants by far and the high intake of flaxseed multiplies the daily lignan dose (Kurzer et al., 1995; Milder et al., 2007; Smeds et al., 2007). Only 10 grams of flaxseed contains approximately 30 mg lignans (Milder et al., 2007).

2.3.2.1 Bioavailability of polyphenols

Different phenolic compounds have different absorption rates and metabolism in GIT. Absorption through the gut barrier and metabolism by intestinal and hepatic enzymes or by intestinal microbiota are the key steps affecting the bioavailability of a dietary compound (Manach et al., 2004). Glycosylated plant polyphenols may have limited bioavailability because hydrophilic sugar moiety usually hampers the passive diffusion of molecules through the small intestine. Therefore, the absorption of glycosides is often lower than unconjugated polyphenols, as suggested e.g. for dietary flavonoids (Németh et al., 2003) and secoisolariciresinol (Saarinen et al., 2002a). Nevertheless, many glucosides are still cleaved by β-glucosidases in the small intestine and some compounds, like resveratrol, are absorbed irrespective of the glycosylation status (Goldberg et al., 2003; Németh et al., 2003).

Moreover, other factors such as food matrix and amount of food, transit time, individual microbiota, and intestinal pH can affect absorption of polyphenol molecules (Manach et al., 2004). Absorption and bioavailability of polyphenols can be estimated by measuring their concentrations, and concentrations of known metabolites, in blood and urine after intake. Common metabolites of polyphenols include mainly glucuronate and sulfate derivatives of the parental compound (Burkon and Somoza, 2008; Kroon et al., 2004). However, compound specific (and often also food specific) data is required before firm conclusions about the bioavailability can be made (Koli et al., 2010; Palafox-Carlos et al., 2011). Altogether, the process whereby food polyphenols are released from the food matrix in GIT, possibly fermented by microbiota, absorbed from the gut, metabolized further, and finally excreted from the body is very complex. Thus, more thorough research about these compounds is still needed to understand the metabolism of polyphenols.

Absorption and metabolism of stilbenoids, especially resveratrol, is very rapid. After an oral dose of 25 mg resveratrol, irrespective of the food matrix, the concentration of resveratrol in the plasma peaked half an hour after intake (Goldberg et al., 2003; Walle et al., 2004). At the time, the plasma concentrations of free resveratrol and its metabolites were 0,04 μM and 2 μM, respectively. Additionally, the intravenous dose of 0,2 mg resveratrol was metabolized to sulphate conjugates within 30 minutes in healthy subjects (Walle et al., 2004). The half-life of resveratrol and its metabolites in human plasma was around 10 hours indicating that exposure to metabolites is much higher than that for the parental compound (Boocock et al., 2007; Brown et al., 2010; Walle et al., 2004). However, it was proposed that resveratrol metabolites are restored to the parental compound inside cells in target tissues (Patel et al., 2013). This indicates that intracellular exposure for free resveratrol may be more sustained than the rapid metabolism of resveratrol in plasma indicates.

Healthy volunteers taking a dose of 5 grams of purified resveratrol had a maximum 0,3-2,4 μM concentration of free resveratrol in their plasma (Boocock et al., 2007). In the same study, the maximum concentration of the main metabolite of resveratrol, resveratrol 3-sulfate, was 3,7-14 μM. The five gram dose of resveratrol is the highest studied in humans and no serious adverse events were observed suggesting that resveratrol is a well tolerated compound (Boocock et al., 2007; Brown et al., 2010). The 2,5 g and higher doses still caused mild to moderate gastrointestinal symptoms to some subjects (Brown et al., 2010). Nevertheless, a resveratrol intake higher than 25 mg/day is not possible via a common diet (Burns et al., 2002; Stervbo et al., 2007). Pharmacokinetics of other stilbenoids resembles that of resveratrol. Piceatannol, pinosylvin, and rhapontigenin were all glucuronidated rapidly in rats after an

intravenous dose (Roupe et al., 2006). According to urinary excretion rates, the elimination half-life of each compound was over 10 hours.

Many dietary plant lignans are metabolized by gut microbiota to enterolactone and enterodiol, also referred to as mammalian lignans or enterolignans (Clavel et al., 2005; Setchell et al., 1980; Stitch et al., 1980). Of these, enterolactone concentration in serum is often used as a marker of high lignan consumption (Heald et al., 2007; Kilkkinen et al., 2001). Other lignan metabolites synthetized by gut microbiota include 7-hydroxyenterolactone and related compounds (Clavel et al., 2005; Heinonen et al., 2001). The conversion rate of plant lignans to enterolignans is not 100 %, and unmodified plant lignans are also absorbed in the gut (Peñalvo et al., 2004).

Metabolism of enterolignans is slow compared to stilbenoids and most people have a constant enterolactone concentration of around 0,01 – 0,03 μM in blood (Johnsen et al., 2004; Kilkkinen et al., 2001; Zhang et al., 2008). However, basal enterolactone concentration in blood depends heavily on diet (Johnsen et al., 2004). Enterodiol and enterolactone concentrations in blood are increased 8–10 h after the consumption of the parent lignan and in blood the half-life of enterolactone is 10-15 h and enterodiol 4-10 h (Kuijsten et al., 2005a, 2005b; Setchell et al., 2014; Udani et al., 2013). The concentration of absorbed dietary plant lignans peak in the blood 5-7 h after intake and their half-life is 2-7 h (Penalvo et al., 2005; Setchell et al., 2014; Tomimori et al., 2013). However, dietary lignans are usually glycosylated, which delays their metabolism and absorption. Unconjugated 7-hydroxymatairesinol was suggested to be absorbed much faster than glycosylated dietary lignans and its concentration in plasma peaked only one hour after administration (Udani et al., 2013).

The high consumption ground flaxseed (0,3 g/kg per bodyweight) increased levels of enterolactone and enterodiol in plasma to 0,17 μM and 0,10 μM (Kuijsten et al., 2005b). The daily intake of 500 mg secoisolariciresinol diglucoside elevated the average enterolactone concentration in serum to 0,40 μM (Hallund et al., 2006) and the secoisolariciresinol diglucoside intake of 600 mg/day for four months increased levels of enterolactone, enterodiol and secoisolariciresinol in plasma to 0,44 μM, 0,88 μM and 0,45 μM, respectively (Zhang et al., 2008).

2.3.3 Stilbenoids and lignans modulate multiple cellular targets

The most studied plant stilbenoid, resveratrol, was isolated for the first time, and characterized, from the extract of white hellebore (*Veratrum album*) in 1939 (Takaoka, 1939). It is a simple molecule with two isomers; a *trans*-isomer

is usually dominant in plants (Figure 5). All stilbenoids discussed in this thesis are *trans*-isomers by default, unless mentioned otherwise. In 1976, resveratrol was found in grapevine (*Vitis vinifera*) and its antifungal properties were also described (Langcake and Pryce, 1976). Interest in resveratrol as a bioactive compound in wine increased after it was suggested that a high wine consumption might explain why mortality due to coronary heart diseases (CHD) in France was relatively low (so-called "French paradox") in spite of having a relatively high amount of saturated fats in diet (Renaud and de Lorgeril, 1992).

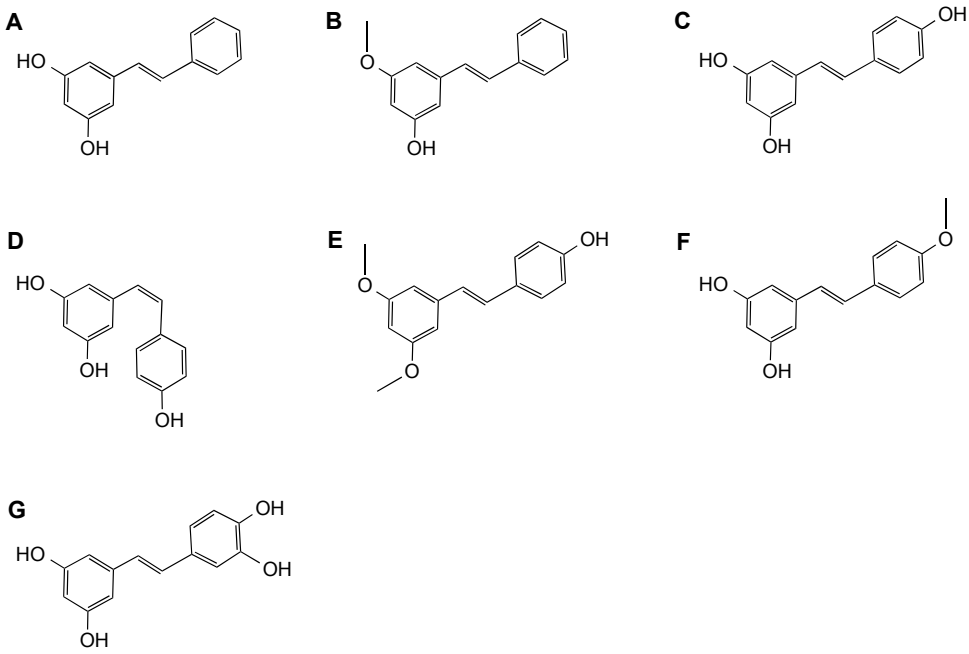

*Figure 5. Structures of natural stilbenoids: (**A**) pinosylvin, (**B**) monomethyl ether of pinosylvin, (**C**) trans-resveratrol, (**D**) cis-resveratrol, (**E**) pterostilbene, (**F**) rhapontigenin and (**G**) piceatannol.*

Resveratrol is probably one of the most studied polyphenols in the world during the last 15 years. In 2015, a PubMed search generated over 7000 articles about resveratrol and the number has increased annually. The key findings that raised interest were that topically administered resveratrol prevented the formation of skin tumors in mice (Jang et al., 1997), and the molecular basis how resveratrol could reduce the risk of cardiovascular diseases (Bertelli et al., 1995; Pace-Asciak et al., 1995). Later, the majority of resveratrol-related articles has focused on potential health-promoting properties and in addition to cancer and heart diseases, resveratrol has been suggested to affect several targets including

metabolism and obesity (Zang et al., 2006), longevity (Howitz et al., 2003), the inhibition of virus replication (Docherty et al., 1999; Evers et al., 2004) and the risk of Alzheimer's disease (Lindsay et al., 2002; Luchsinger et al., 2004). Moreover, the biological activity of other plant stilbenoids including piceatannol, pterostilbene, and pinosylvin (Figure 5) has aroused interest.

Lignans are a diverse group of phenolic compounds (Figure 6). Interest in dietary lignans and their impact on health originated in the discovery of enterolactone in human urine (Setchell et al., 1980; Stitch et al., 1980). It was found that enterolactone was a metabolite of the plant lignans matairesinol and secoisolariciresinol, and also another major lignan metabolite, enterodiol, was characterized (Axelson et al., 1982). Thereafter, the possible favorable effects of circulating lignans on human health have been discussed in multiple studies; with breast, prostate, and colon cancers being of special interest (Adlercreutz, 2007).

Secoisolariciresnol, lariciresinol, matairesinol, and pinoresinol and their glycosides contribute the majority of dietary lignan intake of humans (Milder et al., 2007). Other dietary lignans include medioresinol, isolariciresinol, sesamin, and syringaresinol (Hedelin et al., 2011; Penalvo et al., 2005). However, the first medically utilized lignan was podophyllotoxin, found in the extract of American mayapple (*Podophyllum peltatum*) and it is not present in the daily diet (Kelly and Hartwell, 1954). It was used to treat warts, but more importantly it has been a precursor to widely used anticancer drugs e.g. etoposide (Hande, 1998). Nevertheless, its properties are outside of the scope of this thesis, as is honokiol, another lignan also used in traditional medicine but not found in the common diet (Lee et al., 2011).

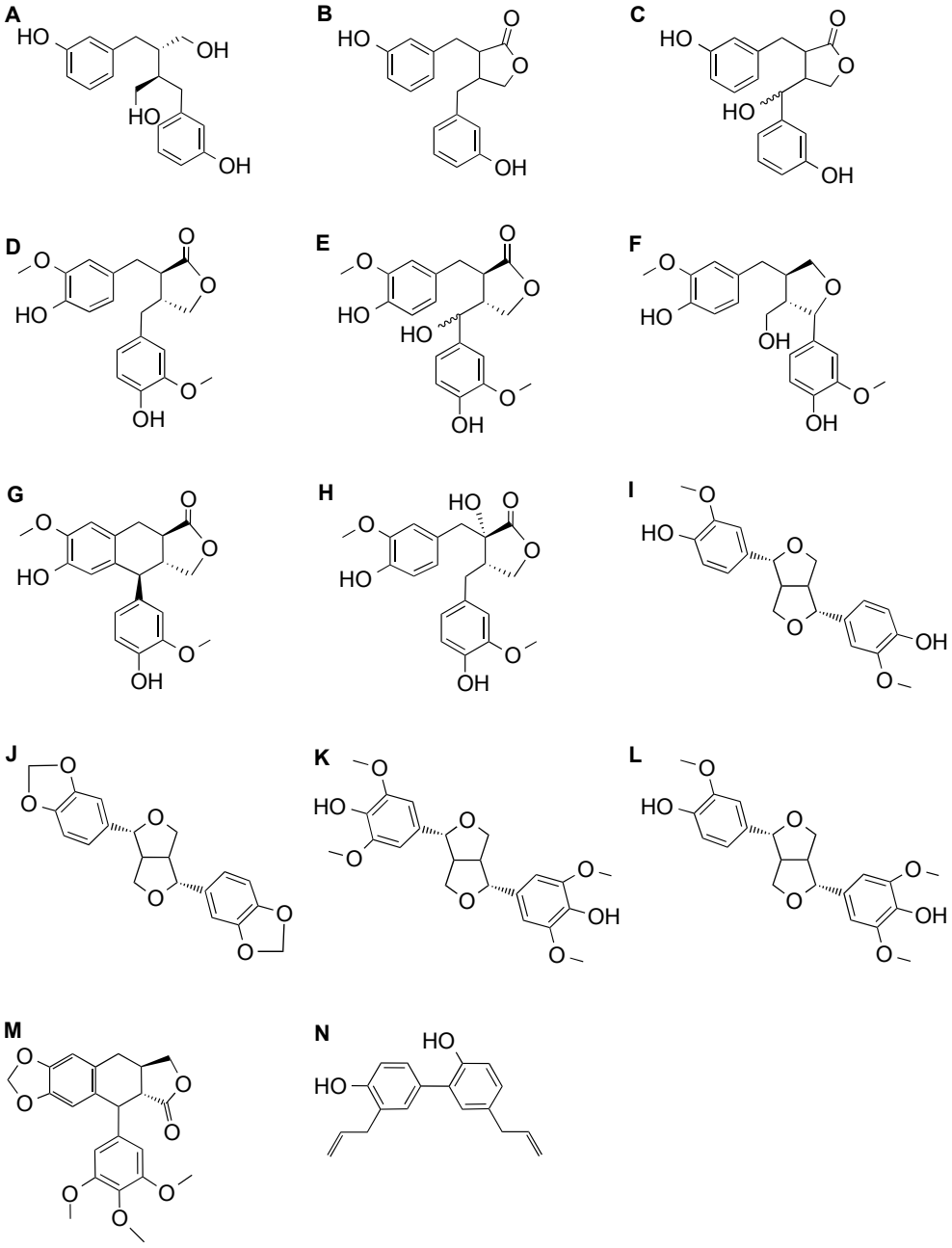

*Figure 6. Structures of lignans: (**A**) enterodiol, (**B**) enterolactone, (**C**) 7-hydroxy enterolactone, (**D**) matairesinol, (**E**) 7-hydroxy matairesinol, (**F**) lariciresinol, (**G**) conidendrin, (**H**) nortrachelogenin, (**I**) pinoresinol, (**J**) sesamin, (**K**) syringaresinol, (**L**) medioresinol, (**M**) podophyllotoxin and (**N**) honokiol.*

2.3.3.1 Antioxidant activity of polyphenols

Many polyphenols have antioxidative properties and their concentration in food surpasses the concentration of other antioxidants e.g. vitamin C and E (Manach et al., 2004). While the consumption of phenolic compounds has been suggested to be a protective factor against several chronic diseases associated with oxidative stress, it has been discussed that one of the major molecular mechanisms behind polyphenol-mediated effects *in vivo* may be the antioxidative activity of these compounds (Fox et al., 2012; Ghanim et al., 2010; Lim et al., 2007; Willför et al., 2003e; Wu et al., 2006). Cited studies show that many polyphenols, including lignans and stilbenoids, are very efficient antioxidants; they suppressed the generation of reactive oxygen species, scavenged free radicals, and inhibited lipid peroxidation.

Oxidative stress is linked with cancer and cardiovascular and neurodegenerative diseases and it can damage different biomolecules including cell membranes, DNA, and many proteins (Avery, 2011; Liu and Xu, 2011). While alleviation of oxidative stress is an important effect of dietary polyphenols, it is not reasonable to exclude mechanisms also related to other factors, for instance to inflammation, microbiota, and metabolic responses (Biasi et al., 2014; Yiannakopoulou, 2013). After all, direct molecular targets of polyphenols and especially their interactions *in vivo* are still poorly understood. Nevertheless, there are several suggested targets of polyphenols that will be discussed in the following chapters.

2.3.3.2 Stilbenoids and lignans as a modulators of energy metabolism, and obesity-associated inflammation

Obesity is a risk factor in different diseases and reduces life expectancy. The prevalence of obesity and being overweight are rising in several countries and obesity is one of the leading preventable causes of death worldwide (Mokdad et al., 2004; Ng et al., 2014). Polyphenols, especially resveratrol, have been suggested as being able to target several signaling pathways that affect energy metabolism and may alleviate obesity-related inflammation (Baur et al., 2006a; Sajish and Schimmel, 2014; Um et al., 2010).

The association between obesity and calorie restriction is rather intuitive and calorie restriction has been known for a long time as an efficient way to increase the lifespan of rodents (Weindruch and Walford, 1982). Calorie restriction mimetics (CRM) are compounds that modulate signaling pathways involved in calorie restriction without actual reduction in energy intake. This could, in theory, lead to the extension of a lifespan (Guarente, 2000; Hursting et al., 2003). Resveratrol has been among the most hyped CRM candidates since

2003, when it was suggested that resveratrol increases the lifespan of budding yeast (*Saccharomyces cerevisiae*) 70 % by activating Sir2 (Howitz et al., 2003). Sir2 is a homolog of mammalian sirtuin, SIRT1 (silent information regulator 1) the activity of which is linked to low calorie intake (Guarente, 2000). Sirtuins are a group of NAD-dependent deacetylases with a wide array of substrates and functions, reviewed by (Yuan et al., 2013).

In addition to budding yeast, resveratrol extended the lifespan of short lived species including roundworm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*) and turquoise killifish (*Nothobranchius furzeri*) (Valenzano et al., 2006; Wood et al., 2004). Dietary resveratrol also prolonged the lifespan of overweight mice fed with high caloric diet by 20%, and shifted their physiology toward mice fed with a standard diet (Baur et al., 2006a). However, the lifespan of lean, genetically normal rodents was not extended by resveratrol (da Luz et al., 2012; Pearson et al., 2009; Strong et al., 2013). Nevertheless, dietary resveratrol has modulated several biomarkers linked with obesity and aging, for instance insulin sensitivity and telomerase activity (Baur et al., 2006a; Liu et al., 2013; Pearce et al., 2008). Under energy restriction, no beneficial effects were observed in resveratrol fed rats (Alberdi et al., 2014). This suggests that the effects of calorie restriction and resveratrol are not cumulative.

Further studies have validated that resveratrol is a direct activator of SIRT1 (Hubbard et al., 2013; Olholm et al., 2010). Nonetheless, in clinical trials the effects of resveratrol intake on energy metabolism have fluctuated. Favorable effects were observed in obese men taking 150 mg/day resveratrol for 30 days on e.g. glucose homeostasis, systolic blood pressure, the markers of systemic inflammation, and the expression of SIRT1 (Timmers et al., 2011). Yet in two other clinical studies, no significant physiological responses to resveratrol supplementation with doses of 75 and 500 mg/day were observed (Poulsen et al., 2013; Yoshino et al., 2012).

Only a few studies have been conducted regarding the role of lignans in SIRT1 activation. Recently, syringaresinol and sesamin were associated with upregulation and activation of SIRT1 (Cho et al., 2013; Su et al., 2014). Lignans may still have other favorable effects on energy metabolism due to the fact that in epidemiological studies both lignan intake and enterolactone level in serum were associated with reduced cardiometabolic risk factors and lower obesity prevalence (Frankenfeld, 2013, 2014; Peñalvo et al., 2012). However, consumption of sesame (approximately 50 mg lignans daily) for five weeks did not change cardiometabolic risk markers nor the markers of inflammation in obese men and women (Wu et al., 2009). In mice, a dietary secoisolariciresinol

attenuated high fat diet (HFD) -induced weight gain and fat accumulation (Fukumitsu et al., 2008; Tominaga et al., 2012).

Besides the activation of SIRT1, other molecular pathways involved in energy metabolism and obesity-related pathologies are also targeted by resveratrol. Activated 5' AMP-activated protein kinase (AMPK) is the master regulator of energy metabolism and it upregulates fatty acid oxidation and increases the uptake of glucose (Ruderman et al., 2013). It has even been proposed that AMPK is mandatory for resveratrol-mediated signaling in energy metabolism (Price et al., 2012; Um et al., 2010). The resveratrol-mediated activation of AMPK is observed in liver, muscles, and white adipocytes, both *in vivo* and *in vitro* (Timmers et al., 2011; Wang et al., 2011; Zang et al., 2006). In addition, stilbenoids were suggested to affect energy metabolism and weight gain by inhibiting fat accumulation and by suppressing adipogenesis-related cell signaling (Gomez-Zorita et al., 2013, 2014; Kim et al., 2011a; Rayalam et al., 2008), and by increasing heat production in the mitochondria of brown fat and muscles (Alberdi et al., 2013; Andrade et al., 2014; Do et al., 2012).

Increased inflammation is associated with metabolic diseases, for instance type 2 diabetes and various cancers (Gupta et al., 2011; Sell et al., 2012). Chronic low-grade inflammation is one of the key features of obesity-related pathogenesis in adipose tissues (Sell et al., 2012). Obesity-associated inflammation is characterized by abnormal cytokine production and macrophage infiltration into adipose tissues forming characteristic crown-like structures (CLS) around adipocytes (Cinti et al., 2005). Adipose tissue derived monocyte chemoattractant protein 1 (MCP-1) chemotactically recruits more monocytes to the sites of inflammation in white adipocytes (Lê et al., 2011). Resveratrol attenuated the tumor necrosis factor α (TNFα) -induced MCP-1 gene expression and secretion from adipocytes *in vitro*. The effect was mediated by repressing the nuclear factor kappa beta (NF-κB) activity (Zhu et al., 2008). NF-κB is considered as a key mediator of inflammation. Dietary resveratrol also reduced TNFα concentration in serum and macrophage infiltration in adipose tissue *in vivo* (Jeon et al., 2012). In a one year clinical trial, type 2 diabetic patients took resveratrol-enriched grape extract (daily resveratrol dose was 8 mg) (Tomé-Carneiro et al., 2013). As a result, the expressions of several pro-inflammatory cytokines were downregulated including interleukin (IL) 1β, IL-6 and TNFα.

Overall it can be said that while the described effects of stilbenoids on metabolic parameters are still partly controversial in the literature, current findings already suggest that dietary resveratrol may be an effective anti-obesity agent and protect against adipose tissue inflammation. The role of polyphenols

in the alleviation of cancer-related inflammation will be discussed more closely in chapter 2.3.5.4.

2.3.4 Stilbenoids, lignans, and cancer risk

The relationship between the consumption of polyphenols and cancer risk has been intensively studied, for instance the effects of consumption of tea and soy on cancer risk has been assessed in multiple studies (Yang et al., 2009) (Fritz et al., 2013). However, the focus of this thesis work is the health promoting effects of polyphenols, namely lignans and stilbenoids that are present in high concentrations in conifers, especially in pine and spruce. The role of other polyphenols in the prevention of diseases will therefore not be further discussed here.

Breast cancer is the most common cancer in women. In Finland, over 4000 new breast cancers are diagnosed annually and 800 deaths occur due to breast cancer (NORDCAN, 2011). Breast cancer risk is partially associated with dietary habits and dietary interventions are an opportunity to lower the risk for the development and recurrence of breast cancer (Fritz et al., 2013; Saarinen et al., 2007; Umar et al., 2012). The role of resveratrol in cancer prevention, especially as a part of Mediterranean diet in wine, grapes, berries and peanuts, has been studied intensively (Giacosa et al., 2013; Stervbo et al., 2007; La Vecchia and Bosetti, 2006). It has been suggested that breast cancer risk is inversely related to dietary resveratrol intake (Levi et al., 2005). Interestingly, the same authors have found that an inverse relationship is observed only when grapes, but not wine, are the main source of resveratrol in diet. This suggests that alcohol intake increases cancer risk and decimates the protective effect of resveratrol. Alcohol consumption, irrespectively of beverage type, has been previously linked to increased breast cancer risk (Ellison et al., 2001; Tjønneland et al., 2007).

Intake of lignan rich food has also been associated with reduced breast cancer risk (Cotterchio et al., 2008). In this study, flaxseed was the main lignan contributor accounting for 88% of the total intake. When results were adjusted by individual body mass index (BMI), risk reduction was only seen in overweight women. High flaxseed consumption alone was associated with 20-30 % reduction in breast cancer risk (Lowcock et al., 2013). Irrespective of diet, increased BMI has been associated with elevated risk for postmenopausal breast cancer (World Cancer Research Fund / American Institute for Cancer Research, 2010).

High plasma enterolactone level and intake of lignans have been linked to reduced breast cancer risk in both pre- and postmenopausal women (Fink et al., 2007; McCann et al., 2004; Piller et al., 2006; Sonestedt et al., 2008). However, results in different studies are not fully consistent. According to meta-analyses on the association between lignans and breast cancer risk, high lignan exposure may reduce the breast cancer risk, especially in postmenopausal women (Buck et al., 2010). However, enterolactone concentration in plasma may reflect mainly the short-term intake which only weakly correlates with the total intake of plant lignans according to dietary questionnaires (Piller et al., 2006). In addition, total consumption of lignans has often been estimated on the low side, due to dietary questionnaires sometimes lacking the lignan rich food, especially flaxseed, and some fairly common lignans, for instance pinoresinol (Buck et al., 2010). These factors have probably increased the heterogeneity of results between studies.

Prostate cancer is the most common cancer in men. In Finland, 4000 - 5000 new prostate cancers are diagnosed annually and the incidences have increased significantly during the previous decades as the number of elderly people has increased (NORDCAN, 2013). Prostate cancer risk is also associated with diet and lifestyle, but knowledge about the exact mechanisms is still lacking (Mandair et al., 2014; Møller et al., 2014). Prostate cancer usually grows slowly but finally it may progress to a non-curable state. The relationship between dietary habits and prostate cancer was observed in 1989, when high consumption of fruits and vegetables was associated with a decreased prostate cancer risk for the first time (Mills et al., 1989). In other studies, prostate cancer risk was inversely related also to high intake of legumes and certain vegetables, and high concentration of polyphenols in patients' serum was associated with decreased prostate cancer risk (Kolonel et al., 2000; Lee et al., 2003; Ozasa et al., 2004).

Increased serum enterolactone concentration, and intake of lignans were conversely correlated with prostate cancer risk, which suggests that lignan intake could have a protective role against prostate cancer (Heald et al., 2007; Mccann et al., 2005b). However, in some other studies association between serum enterolactone and reduced prostate cancer risk has not been found (Travis et al., 2009; Ward et al., 2008, 2010). A reason for these conflicting results can be that enterolactone is a poor biomarker for long-term lignan intake. It has also been suggested that lignan doses required for the inhibition of carcinogenesis may exceed those obtained from the diet (Saarinen et al. 2010).

The consumption of red (but not white) wine was proposed to be inversely associated with prostate cancer risk (Rybicki et al., 2011; Schoonen et al.,

2005). In particular, risk of more aggressive prostate cancer was suggested to be decreased by high red wine intake (Schoonen et al., 2005). However, in some other studies no clear association between moderate red wine consumption and prostate cancer risk was found (Chao et al., 2010; Sutcliffe et al., 2007). Otherwise, high alcohol consumption is linked to a slight increase in prostate cancer risk, but unlike in breast cancer, there are no conclusive results about the association of low alcohol consumption and prostate cancer risk (World Cancer Research Fund / American Institute for Cancer, 2014).

2.3.5 Anticarcinogenic activity of stilbenoids and lignans

Numerous studies have pointed out the anticancer potential of stilbenoids, resveratrol being by far the most studied compound. After the first study, describing the anticancer properties of resveratrol (Jang et al., 1997), the interest of cancer researchers in stilbenoids has increased massively. The anticancer activity of resveratrol is mediated through many cell signaling pathways that regulate e.g. inflammation, cell proliferation, resistance to cell death, invasion, metastasis, and tumor angiogenesis, all of which are recognized as the hallmarks of cancer (Hanahan and Weinberg, 2011).

Links between the consumption of dietary plant lignans and cancer have been investigated since mammalian lignans were characterized (Setchell et al., 1981). Effects of a lignan rich diet and high circulating enterolactone concentration on the reduction of cancer risk and related cellular mechanisms have been of special interest in several studies (Adlercreutz, 2007).

2.3.5.1 Effects of stilbenoids on cell survival

In vitro studies have shown that stilbenoids interact with multiple molecular targets that may affect cell survival and viability. The required resveratrol concentration to suppress cell survival *in vitro* has usually been 5 - 100 µM, depending on cancer cell type (Aggarwal et al., 2004; Calabrese et al., 2010). This concentration range is rather high in comparison with serum concentrations found *in vivo* in clinical studies (chapter 2.3.2.1). However, the comparison between active resveratrol concentrations *in vitro* and effective dosage *in vivo* has proved to be complicated and the roles of many metabolites are poorly understood (Baur and Sinclair, 2006; Patel et al., 2013). It is suggested that cancer cells are more sensitive to stilbenoids than normal cells and therefore resveratrol could be a promising compound to prevent the growth of tumors (Aziz et al., 2006; Benitez et al., 2007; Fulda and Debatin, 2004; Losa, 2003). In addition, the antioxidative activity of stilbenoids may protect cells from events associated with tumor initiation (Jang et al., 1997; Losa, 2003).

Resveratrol (5 -100 μM) inhibited the growth of cancer cells by inducing a cell cycle phase G1/S arrest *in vitro* in a concentration-dependent manner (Benitez et al., 2007; Hsieh and Wu, 1999; Wang et al., 2010a; Yang et al., 2013). Cell cycle arrest could be explained by resveratrol-mediated downregulation of the modulators of cell cycle and senescence including cyclin D1 and cyclin-dependent kinases (CDK) (Benitez et al., 2007; Wang et al., 2010a; Yang et al., 2013). Resveratrol was suggested to activate extracellular signal-regulated kinases (ERK) 1/2 and p53, which are upstream modulators of cyclin/CDK complexes (Gao et al., 2004; Hsieh et al., 2011; She et al., 2001). Tumor-suppressor protein p53 modulates multiple cellular processes and p53-mediated cell cycle arrest is one of the crucial anticancer mechanisms of cells; it arrests cells on G1/S phase by transactivating CDK inhibitor 1 (p21) (Bieging et al., 2014). ERK1/2 is a key mediator in a complex signaling cascade in the immune response and the modulation of ERK1/2 activity also contributes to the p53-mediated cell cycle arrest in cancer cells (Hsieh et al., 2011; Huang et al., 2010; She et al., 2001). The effect of stilbenoids on the activation of ERK-pathway might be tissue or cell type specific but detailed mechanism of how resveratrol modulates ERK 1/2 is not confirmed (Eo et al., 2014; Gao et al., 2004; Hsieh et al., 2011; She et al., 2001). A recent study suggests that resveratrol may bind to the active site of tyrosyl transfer-RNA synthetase triggering the activation of signaling pathways that stimulate the expression of several discussed targets of resveratrol, e.g. p53, SIRT1 and AMPK (Sajish and Schimmel, 2014).

Other stilbenoids, e.g. pinosylvin and piceatannol, have also been suggested to inhibit the proliferation of cancer cells (Park et al., 2013; Piotrowska et al., 2012). In addition, it was proposed that Ras-GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) could be the direct target of resveratrol and the binding of resveratrol to G3BP1 might active p53 (Oi et al., 2014; Yuan et al., 2010). G3BP1 is an enzyme which is involved in several signaling pathways and is often overexpressed in cancer cells (Barnes et al., 2002; Oi et al., 2014).

Stilbenoids also modulated the activity of p53 via the downregulation of metastasis-associated protein 1 (MTA1) (Kai et al., 2010; Li et al., 2013b). The expression of MTA1 in cancer cells correlates with tumor aggressiveness and downregulation of MTA1 was proposed to acetylate p53, which leads to its increased transcriptional activity (Li et al., 2009). Resveratrol and pterostilbene inhibited MTA1 signaling *in vivo* in DU145 prostate cancer xenografts (Li et al., 2013b). The silencing of MTA1 also increased the apoptotic efficiency of resveratrol (Kai et al., 2010). Nevertheless, the role of p53 in stilbenoid-triggered apoptosis, programmed cell death, is still somewhat controversial. Resveratrol induced apoptosis in a concentration-dependent manner only in

fibroblasts with wild type p53, but no induction was seen in p53-deficient fibroblast. Resveratrol activated p53-dependent transcription in a dose-dependent manner in JB6 reporter cells (Huang et al., 1999). However, resveratrol also induced apoptosis in p53-deficient or mutated cells, e.g. in PC-3 and DU145 prostate cancer cells, suggesting that resveratrol induces apoptosis via multiple mechanisms (Benitez et al., 2007; Gill et al., 2007).

Resveratrol promoted caspase-mediated apoptosis by enhancing the expression of pro-apoptotic Fas-ligand, and reducing levels of antiapoptotic proteins Bcl-2 and Bcl-X_L (Clément et al., 1998; Estrov et al., 2003). Resveratrol treatment also upregulated anti-apoptotic members of the Bcl-2 protein family, e.g. Bid and Bax (Aziz et al., 2006). In addition to *in vitro* studies, this was also observed in PC-3M-M2 prostate cancer xenografts (Sheth et al., 2012). Apoptosis can be also triggered through the pathway that targets mitochondria and resveratrol has been shown to stimulate the mitochondrial apoptosis in different cancer cells (Opipari et al., 2004; Tinhofer et al., 2001). The exact mechanism of action in resveratrol-stimulated mitochondrial apoptosis is still uncertain and may be dependent on cancer cell type (Gill et al., 2007).

Resveratrol sensitized several cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through the p53-independent induction of p21 (Fulda and Debatin, 2004). TRAIL is an endogenous, proapoptotic ligand of death receptors 4 and 5 (DR4 and DR5) and it induces apoptosis in various types of cancer cells without significant toxicity toward normal cells (Gonzalvez and Ashkenazi, 2010). Resveratrol sensitized cancer cells to apoptosis *in vivo* by enhancing the expression of TRAIL specific death receptors (DR4 and DR5) and inhibiting the expression of anti-apoptotic Bcl-2 in PC-3 prostate cancer xenografts (Ganapathy et al., 2010) Resveratrol may sensitize cancer cells to TRAIL by activating FOXO transcription factors (Chen et al., 2010; Ganapathy et al., 2010). FOXO transcription factors regulate several pathways that play a role in cell survival, and activity of FOXOs can be attenuated with the activation of a protein kinase Akt signaling pathway (Fu and Tindall, 2008). Interestingly, resveratrol inhibited the activation of the Akt pathway in different types of cells, followed by the activation of the death receptor mediated apoptosis (Gill et al., 2007; Haider et al., 2002; Roy et al., 2011). The inhibition of Akt with resveratrol may also contribute to the previously discussed modulation of the expression of apoptosis-related Bcl-2 protein family (Aziz et al., 2006; Sheth et al., 2012). Taken together, resveratrol has been shown to activate p53 and to inhibit Akt in different cancer cells, but it also has other pro-apoptotic targets. The exact role of FOXO in resveratrol-mediated apoptosis needs still further investigations.

In addition to apoptosis, resveratrol induced autophagic cell death in cancer cells (Li et al., 2013a; Opipari et al., 2004). Autophagy is a catabolic process in which a cell degrades its own organelles. It protects cells against both starvation and intracellular pathogens. It may have a protective role against cancer in that autophagy suppresses tumorigenesis by limiting the accumulation of p62 (Mathew et al., 2009). p62 is a protein which controls bone and metabolic homeostasis, but it is also required for tumor formation and its accumulation linked to enhanced tumorigenesis (Moscat and Diaz-Meco, 2012). Resveratrol activates autophagy in cancer cells by activating independently both the production of phagophores via the inhibition of the mammalian target of rapamycin (mTOR) and the induction of p62 expression, which elongates phagophores (Puissant et al., 2010). Phagophore is the membrane structure, which in autophagy sequesters cell organelles to be degraded. mTOR regulates multiple cellular processes and is also involved in calorie restriction and aging. Resveratrol-mediated activation of SIRT1 (Sirtuin type 1) in cancer cells may also promote autophagy (Li et al., 2013a). The role of resveratrol in the activation of sirtuins and calorie restriction is discussed more closely in a chapter 2.3.3.2.

2.3.5.2 Lignans and cell survival

The resemblance of the effects of dietary lignans and their metabolites on cell survival to those of stilbenoids and lignans have been reported targeting some of the previously discussed pathways, including p53 and Akt (Chen et al., 2007) (Peuhu et al., 2010). Both enterolactone and enterodiol were shown to inhibit the growth of different prostate cancer cells (Lin et al., 2001). The IC50 concentrations of enterolignans for cell proliferation were 50-100 μM, enterolactone being a more potent growth inhibitor. Similar effects of enterolignans have also been measured on breast and colon cancer cells *in vitro* (Mousavi and Adlercreutz, 1992; Qu et al., 2005; Welshons et al., 1987). However, these lignan concentrations are rather high in comparison with observed circulating concentrations after the high intake of lignans (chapter 2.3.2.1).

Plant lignans secoisolariciresinol diglucoside, matairesinol, hydroxymatairesinol, and sesamin inhibited cancer cell proliferation and arrested the cell cycle (Ayella et al., 2010; Harikumar et al., 2010; Miura et al., 2007; Peuhu et al., 2010; Yokota et al., 2007). Sesamin, secoisolariciresinol diglucoside, and enterolactone were suggested to arrest the cell cycle by downregulating the expressions of cyclin A and D1 in cancer cells (Ayella et al., 2010; Chen et al., 2009b; Harikumar et al., 2010; Qu et al., 2005; Yokota et al., 2007). Enterolactone, pinoresinol, and sesamin upregulated the expression

of p53, and similarly induced apoptosis in cancer cells (Chen et al., 2007; Deng et al., 2013; Fini et al., 2008; Sepporta et al., 2013). Pinoresinol was associated with the p53-independent overexpression of the CDK inhibitor p21, which arrested the cell cycle (Sepporta et al., 2013).

Several lignans including enterolactone, nortrachelogenin, and matairesinol were suggested to inhibit Akt activity *in vitro* in cancer cells (Chen et al., 2009b; Peuhu et al., 2010, 2013). Inhibition of Akt-mediated survival signaling with 40 μM lignans sensitized the cancer cells to TRAIL-triggered apoptosis (Peuhu et al., 2010, 2013). It was indicated that lignan-mediated inhibition of Akt activity is a result of inhibition of the insulin-like growth factor 1 (IGF-1) receptor (Chen et al., 2009b; Peuhu et al., 2013). IGF-1 receptor mediates the cell survival and the growth of various cancer cells and it is often upregulated in tumors, especially in prostate cancer (Hellawell et al., 2002). The secoisolariciresinol-supplemented diet was also associated with the downregulation of IGF-1 receptor in mice with MCF-7 breast cancer xenografts (Saggar et al., 2010).

2.3.5.3 Inhibition of angiogenesis and metastatic activity

Formation of new blood vessels is a critical step in the growth of tumors, and angiogenesis inhibiting compounds are being used in anticancer therapies (Ebos and Kerbel, 2011; Hanahan and Weinberg, 2011). Resveratrol inhibited angiogenesis by preventing both the expression of vascular endothelial growth factor (VEGF) and its stimulatory effect on endothelial cells (Kimura and Okuda, 2001; Tseng et al., 2004). VEGF is a pro-angiogenic protein and its overexpression in tumors is linked to increased growth and formation of metastasis. Therapeutic efficacy of several anti-VEGF drugs has already been shown (Ebos and Kerbel, 2011). Resveratrol inhibited the secretion of VEGF and reduced the microvessel density in MDA-MB-231 breast cancer xenografts (Garvin et al., 2006). Similarly, VEGF expression was downregulated in PC-3 prostate cancer xenografts in resveratrol-fed mice (Ganapathy et al., 2010).

Another angiogenesis-related target of resveratrol is the intracellular adhesion molecule-1 (ICAM-1) (Baldwin, 2001). An elevated ICAM-1 level is suggested to enhance the adhesion capability of cancer cells and promote the tumor vascularization (Charnaux et al., 2012; Gho et al., 1999). Resveratrol reduced ICAM-1 expression in cancer cells through the inhibition of NF-κB activation and inhibited the expression of ICAM-1 to a basal level in stimulated endothelial cells and monocytes (Ferrero et al., 1998; Kim et al., 2011b; Park et al., 2009). The role of NF-κB in cancer will be discussed more closely in the next chapter.

Lignans may also prevent the neovascularization of tumors and the formation of metastases. Sesamin inhibited the expression of ICAM-1, VEGF and matrix metalloprotease 9 (MMP-9) *in vitro* (Harikumar et al., 2010). MMP-9 is collagenase, involved in the breakdown of the extracellular matrix. It has been shown to contribute to the invasion and formation of metastasis in cancer (Woo et al., 2004). Dietary secoisolariciresinol diglucoside, equivalent to 5-10 % flaxseed diet, reduced the number and volume of B16BL6 melanoma metastases in lungs ((Li et al., 1999). Lariciresinol (20 and 100 mg/kg bw), enterolactone (16,8 mg/kg bw) and a 10 % flaxseed diet decreased the microvessel density in MCF-7 breast cancer xenografts (Lindahl et al., 2011; Saarinen et al., 2008). Enterolactone also decreased the amount of extracellular VEGF, derived from stroma and MCF-7 cells, in mice (Saarinen et al., 2010b). Matairesinol was suggested to suppress VEGF-stimulated angiogenesis *in vitro* and to reduce vascularization *in vivo* (Lee et al., 2012). In the same study, matairesinol inhibited the secretion of VEGF from HeLa cells. In general it can be said that dietary lignans and stilbenoids have been shown to inhibit angiogenesis in tumors and angiogenesis-related cell signaling.

2.3.5.4 Anti-inflammatory properties

Resveratrol has been suggested to suppress the activity of the transcription factor NF-κB in cancer cells (Banerjee et al., 2002; Manna et al., 2000; Zhong et al., 1999), similar to that of adipose tissue as discussed in chapter 2.3.3.2. The increased activity of NF-κB protects cancer cells from apoptosis and stimulates cell proliferation thus promoting the cell survival (Perkins, 2007). NF-κB also regulates the production of inflammatory compounds, e.g. several cytokines, which may promote tumor growth (Hanahan and Weinberg, 2011).

Resveratrol was suggested to inhibit NF-κB activity and NF-κB –dependent gene expression by blocking IκB kinase, which is a regulator of NF-κB activation (Holmes-Mcnary and Baldwin, 2000). Resveratrol inhibited the expression of cyclooxygenase-2 (COX-2), which is induced by NF-κB (Banerjee et al., 2002; Kang et al., 2009; Subbaramaiah, 1998; Wu et al., 2010a). Cyclooxygenases catalyze the formation of prostaglandins, and expression COX-2 is enhanced by proinflammatory response (Cerella et al., 2010). COX-2 also has an important role in tumorigenesis and it is constitutively overexpressed in a variety of cancers. In addition to COX-2, resveratrol reduced the production of MMP-9 through inhibition of NF-κB and activator protein-1 (AP-1) (Banerjee et al., 2002; Woo et al., 2004). It has been proposed that resveratrol may alleviate inflammatory responses by modulating the previously discussed activation of the ERK1/2 pathway (El-Mowafy and White, 1999; Eo et al., 2014; Harper et al., 2007; Kang et al., 2009). Recently, it

was suggested that resveratrol also modulates inflammatory responses via estrogen receptor (see also chapter 2.3.5.6) (Nwachukwu et al., 2014; Srinivasan et al., 2013).

In addition to resveratrol, other natural *trans*-stilbenoids possess comparable anti-inflammatory properties. Pterostilbene inhibited the activation of NF-κB, AP-1 and MMP-9 in HepG2 liver cancer cells and in addition, downregulated VEGF expression (Pan et al., 2009). Piceatannol suppressed the NF-κB activation and COX-2 expression in breast epithelial cells (Son et al., 2010). Similarly, pinosylvin inhibited the expression of MMP-9 and COX-2 in cancer cells (Park et al., 2012). Furthermore, lignans have also been associated with the inhibition of NF-κB activity. Enterolactone, enterodiol, and sesamin suppressed the NF-κB-driven transcription, and the production of inflammatory cytokines *in vitro* (Corsini et al., 2010; Harikumar et al., 2010).

2.3.5.5 Inhibition of colon carcinogenesis

Colon cancer is a promising target for dietary interventions. Dietary compounds reach the colon in effective concentrations and therefore they would be assumed to exert their possible anticancer effects there (van Breda et al., 2004). Some food metabolites with anticancer properties, such as enterolignans, are also produced in the intestinal tract (Clavel et al., 2005). High consumption of vegetables, fruits and especially dietary fiber has been linked with a decreased colon cancer risk (World Cancer Research Fund / American Institute for Cancer Research, 2011)

Formation of aberrant crypt foci (ACF) in the colon can be used as a biomarker to study the early colon carcinogenesis (Jenab and Thompson, 1996; Takahashi et al., 2013). ACF are abnormal clusters of glands in the colon and rectum, and they are among the earliest changes observed, possibly leading to colon cancer. Resveratrol (daily 0,2 mg/kg bw in drinking water, and 8 and 23 mg/kg in diet) significantly reduced the number of ACF and expression of cell cycle-mediating p21 in the colon of rodents (Kineman et al., 2010; Sengottuvelan et al., 2006; Tessitore et al., 2000). Furthermore, sesame-derived lignan sesaminol reduced the formation of ACF in rats and a lignan-rich rye-bran diet inhibited the formation of intestinal polyps in mice (Mutanen et al., 2000; Sheng et al., 2007). An enterolactone rich diet (1-10 mg/kg bw/day) inhibited the growth of Colo 201 colon cancer xenografts in mice (Danbara et al., 2005).

Changes in Wnt signaling in colonic tissue have been linked to the development of colon cancer (Klaus and Birchmeier, 2008). Activation of a Wnt-pathway increases the accumulation β-catenin in cytoplasm. Translocated β-catenin acts

as a transcriptional co-activator and activates the transcription of several Wnt target genes which function e.g. in cell differentiation, signaling, and proliferation. Resveratrol inhibited Wnt signaling in both colon cancer cells and normal mucosa-derived cells (Hope et al., 2008; Ji et al., 2013). In the same studies, also nuclear localization of β-catenin was decreased by resveratrol. In addition, resveratrol had a direct inhibitory effect on the expression of MALAT1, a long non-coding RNA overexpressed in clinical colorectal cancer tissues (Ji et al., 2013). Also another stilbenoid, pinosylvin, inhibited the translocation of β-catenin and transcription of the target genes of β-catenin in colon cancer cells (Park et al., 2013).

The effects of resveratrol have been investigated clinically in colon cancer patients. In one study, colon cancer patients received daily for 14 days either a 3,9 or 15,5 mg dose of resveratrol or 80 g of grape extract (equivalent to 0,07 mg of resveratrol, but including also other polyphenols) (Nguyen et al., 2009). Dietary resveratrol decreased the expression of the Wnt target in tissue biopsies of normal mucosa, but not in those of cancer tissue (Nguyen et al., 2009). In spite of the markedly lower resveratrol dose, the effect on Wnt target genes was higher with grape powder than pure resveratrol. This suggests that the effect on Wnt signal throughput is the result of the joint effect of resveratrol with other bioactive grape-derived polyphenols. The daily intake of 5 grams of resveratrol induced apoptosis in tumor metastases (Howells et al., 2011). In this study, resveratrol was given to colon cancer patients with hepatic metastases for 14 days. After the intervention, patients were scheduled to undergo liver resection. In tissue analysis, resveratrol was found both in cancer and normal tissue, the average concentrations being 5 and 2 nmol/g, respectively. In liver metastases there was also an increase in the expression of cleaved caspase 3, a marker of apoptosis. This indicates that dietary resveratrol can reach tissues outside GIT in active, apoptosis inducing concentrations (Howells et al., 2011).

2.3.5.6 Modulation of estrogen receptor activity and effects on breast carcinogenesis

Several polyphenols including lignans and stilbenoids have been suggested as interacting with the estrogen receptors α (ERα) and β (ERβ) and are thus often referred to as phytoestrogens (Fritz et al., 2013; Hughes, 1988; Patisaul and Jefferson, 2011). A phytoestrogen is defined by the Committee on Toxicity (2000) as “any plant-derived compound or its metabolite that can mimic the action, or modulate the binding, metabolism or production of endogenous estrogens in the body. Phytoestrogens may also have additional biological activities not mediated through estrogen specific pathways”.

Both estrogen receptors are potential targets for breast cancer therapy. ERα is expressed in over 70 % of breast tumors and its activity is linked with tumor growth. ERβ is associated with tumor suppression and its agonists could therefore be used to prevent the growth and progression of cancer (Deroo and Korach, 2006) (Dey et al., 2013). ERα and ERβ may have a role also in other cancers such as prostate and ovarian cancer (Chan et al., 2014; Dey et al., 2013). In addition to steroid hormones, variable compounds (Figure 7) including tamoxifen and diethylstilbestrol, can bind and modulate the activity of ERα.

Estrogenic properties of dietary polyphenols, for instance genistein, and their effects on breast cancer risk have been studied for several decades (Biggers and Curnow, 1954; Welshons et al., 1987). Synthetic ER agonist diethylstilbestrol can be classified as stilbenoid according to its structure (Figure 7). This has aroused interest about the potential estrogenic properties of natural stilbenoids (Gehm et al., 1997; Levenson et al., 2003; Serrero and Lu, 2001). According to a recent structural modeling study, resveratrol and diethylstilbestrol actually bind in a similar though not identical way to ERα (Chakraborty et al., 2013).

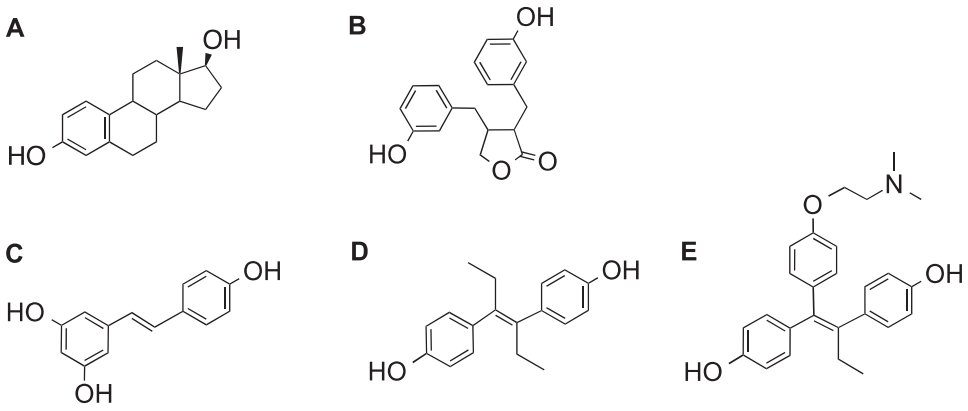

Figure 7. Structures of steroid hormone 17β-estradiol (A) and mammalian lignan enterolactone (B) that have been suggested to share similarities in structural elements. Resveratrol (C) has common structural elements, like a stilbene-backbone, with synthetic nonsteroidal estrogenic compounds diethylstilbestrol (D) and tamoxifen (E)

Estrogenic properties of lignans have been studied since enterolactone was found in the urine of women (Setchell et al., 1980; Stitch et al., 1980). Most of the estrogenicity studies have concentrated on a few lignan metabolites instead of plant lignans (Adlercreutz, 2007). Results to date are still inadequate for justifying the classification of all lignans as phytoestrogens. The estrogenic

properties, for instance, of common dietary plant lignans, secoisolariciresinol and matairesinol, have not been demonstrated.

The binding affinity of resveratrol to ERα was 7,7 μM (IC50, a ligand concentration yielding 50% inhibition of binding of fluorescein-labeled estradiol to ERα), indicating only weak interaction (Mueller et al., 2004). In the same study, the binding affinity of enterolactone was 6,7 μM. Compared with the biding affinities of 17β-estradiol (E_2) and diethylstilbestrol, IC50 values of enterolactone and resveratrol were less than a thousandth of those and therefore their potency to activate ERα was much weaker (Mueller et al., 2004). The binding affinities of resveratrol and enterolactone to ERβ were even weaker (IC50 are 30 and 40 μM, respectively) than those to ERα. Resveratrol in 10-50 μM concentrations was suggested to activate the ERα-mediated transcription *in vitro* (Bhat et al., 2001; Bowers et al., 2000; Gehm et al., 1997; Levenson et al., 2003). However, the activation of ERα was 7000-fold lower in comparison with E_2. This suggests that resveratrol is a relatively weak agonist of ERα. Additionally, piceatannol has been suggested to induce weak ERα-mediated transcriptional activity (Maggiolini et al., 2005). A few studies proposed that in addition to weak agonism resveratrol may modulate ERα indirectly through the activation of ERK1/2 and p53 (De Amicis et al., 2011; Bowers et al., 2000).

Enterolactone and enterodiol (1-25 μM) were proposed to activate both ER *in vitro* (Carreau et al., 2008; Penttinen et al., 2007; Saarinen et al., 2000; Welshons et al., 1987). Of the major enterolignans, enterolactone was the most potent activator of both ERs (Saarinen et al., 2000; Welshons et al., 1987).

Estrogen-responsive breast cancer cells are used to study estrogenic properties of compounds. MCF-7 and T-47D cells especially have been utilized in such studies. It was suggested that resveratrol (10-25 μM) stimulate estrogen-responsive cell proliferation (Basly et al., 2000; Gehm et al., 1997; Matsumura et al., 2005). However, the estrogenicity of resveratrol is still inconsistent, while in a few other studies the growth stimulation was not observed (De Amicis et al., 2011; Bove et al., 2002; Serrero and Lu, 2001). Resveratrol concentration of 30 μM or higher actually inhibited the cell growth, similarly to other cancer cells (De Amicis et al., 2011; Levenson et al., 2003; Lin et al., 2010; Nwachukwu et al., 2014; Serrero and Lu, 2001). A recent study suggested that resveratrol does not stimulate proliferation but modulates the inflammatory response via binding with ERα in MCF-7 and T-47D cells (Nwachukwu et al., 2014; Srinivasan et al., 2013).
Enterolactone (1-10 μM) stimulated the growth of estrogen-responsive breast cancer cells (T-47D and MCF-7) (Mousavi and Adlercreutz, 1992; Sathyamoorthy et al., 1994; Wang and Kurzer, 1997; Welshons et al., 1987).

However, the stimulatory effect on cell growth was only observed *in vitro* and the growth of estrogen-dependent MCF-7 xenografts in mice was not stimulated in the absence of estradiol (Bergman Jungeström et al., 2007; Power et al., 2006; Saarinen et al., 2006). In the presence of E_2, both dietary flaxseed and subcutaneously administrated enterolignans (15 mg/kg bw) reduced the growth of MCF-7 breast cancer xenografts in mice.

The uterotrophic assay is used to determine the estrogenic properties of chemicals *in vivo* by measuring their effects on the uterus (OECD series on testing and assessment no. 21 detailed review paper, 2002). Daily doses of 0,03–70 mg/kg bw resveratrol did not have any effect on the estrous cycle or uterine morphology in rats, suggesting that resveratrol has little or no estrogenic activity in reproductive estrogen-responsive tissues (Bove et al., 2002; Slater et al., 1999; Turner et al., 1999). The intake of different lignans (secoisolariciresinol diglucoside: 3 mg/kg bw, 7-hydroxymatairesinol: 50 mg/kg bw, enterolactone: 1-10 mg/kg bw or 10 % flaxseed diet) did not affect uteri either (Orcheson et al., 1998; Saarinen et al., 2000, 2002b). In estrogen-responsive reporter mice (Lemmen et al., 2004) the intraperitoneally injected dose of 10 mg/kg bw enterolactone activated the gene expression in tissues selectively, e.g. it was activated in the uterus and vagina, but not in bone (Damdimopoulou et al., 2011; Penttinen et al., 2007). In addition, lignan-rich diets modulated the E_2-induced ER activity in mice (Penttinen-Damdimopoulou et al., 2009). Based on these results enterolactone could be a selective estrogen receptor modulator (SERM). Similar systematic *in vivo* studies about the effects of stilbenoids and plant lignans on ER-signaling are still lacking.

The effects of resveratrol on breast carcinogenesis have been studied in different preclinical models. Resveratrol (0,2 to 100 mg/kg bw) suppressed the tumor formation in rodents, including 7,12-Dimethylbenz(*a*)anthracene (DMBA) and N-methyl-N-nitrosourea (MNU)-induced mammary carcinogenesis in rats and spontaneous tumor formation in HER-2/neu-transgenic mice (Banerjee et al., 2002; Bhat et al., 2001; Chatterjee et al., 2011; Provinciali et al., 2005). According to a recent study, resveratrol treatment might prevent estrogen-induced mammary cancer (Singh et al., 2014). In this study, it was suggested that resveratrol upregulates the expression of nuclear factor erythroid 2-related factor 2 (NRF2) in mammary tissues. NRF2 regulates genes that are involved in protection against oxidative DNA damage (Reddy et al., 2007). A lignan-rich flaxseed diet suppressed mammary carcinogenesis and inhibited the growth of DMBA-induced tumors and the formation of proliferative terminal end buds (Chen et al., 2003; Khan et al., 2007; Serraino and Thompson, 1991; Thompson et al., 1996; Ward et al., 2000). The reduction in the number of terminal end bud structures in the developing mammary gland

correlates with lower incidence of mammary cancers in rodents (Russo and Russo, 1978). Similarly, purified lignans including secoisolariciresinol diglucoside, 7-hydroxymatairesinol, sesamin, and enterolactone were proposed to reduce DMBA-induced mammary carcinogenesis (Chen et al., 2003; Hirose et al., 1992; Saarinen et al., 2000, 2002b, 2008; Thompson et al., 1996).

On the whole, the estrogenic properties of stilbenoids *in vivo* are yet to be confirmed and current knowledge does not uniformly justify the classification of resveratrol as a phytoestrogen or SERM. Resveratrol might still have other beneficial roles in breast cancer prevention and inhibition of tumor growth. Enterolactone might be a weak SERM, but estrogenic activity of plant lignans is yet to be shown. However, enterolignans and their precursors have been shown to reduce tumor formation and growth and consequently high lignan consumption may therefore reduce breast cancer risk.

2.3.6 Effects of stilbenoids and lignans on androgen receptor and prostate cancer

The androgen receptor (AR) is a ligand-dependent nuclear transcription factor that is activated by androgens, testosterone, and dihydrotestosterone (DHT). While AR has several physiological functions, it is also associated with prostatic diseases, most notably prostate cancer (Mills, 2014). The growth of prostate cancer is dependent on androgens and therefore AR is an important clinical target in prostate cancer therapies. Many novel strategies targeting the activation of AR have been used for treating prostate cancer, for example, enzalutamide that efficiently prevents androgen binding to AR (Beer et al., 2014; Gauthier et al., 2012). The effect of polyphenols on activity and expression of AR has also been investigated in several studies.

Resveratrol was suggested as inhibiting both the expression and transcriptional activity of AR (Gao et al., 2004; Narayanan et al., 2009; Shi et al., 2009; Wang et al., 2010a). However, resveratrol does not affect the transcriptional activity of AR through binding the ligand-binding domain of a receptor (Gao et al., 2004; Harada et al., 2007; Wang et al., 2008a). Resveratrol downregulated AR-mediated transcription independently of the expression of AR in androgen-dependent LNCaP prostate cancer cells (Mitchell et al., 1999; Shi et al., 2009; Wang et al., 2008a). Neither the AR nuclear translocation nor the AR binding with DNA were affected *in vitro* (Shi et al., 2009). The inhibitory mechanism of transcriptional activity of AR was hypothesized to be either the direct deacetylation of AR through SIRT1 activation or an alteration in the recruitment of AR cofactors (Fu et al., 2006; Shi et al., 2009). It was suggested that resveratrol could modulate the transactivation of AR by activating the Raf–MEK–ERK signaling pathway (Gao et al., 2004), which is a complex signal

cascade and modulation of which by resveratrol is also observed in other cancer cells (chapter 2.3.5.1).

Tumor suppressor phosphatase and tensin homolog (PTEN), that is characterized as a growth suppressor in cancer cells, was suggested to regulate the AR activity in prostate cancer cells (Lin et al., 2004; Wang et al., 2010b). The inactivation of PTEN may induce an Akt pathway and therefore promote cell survival (Chen et al., 2010). In prostate cancer cells, resveratrol inhibited cell proliferation in both AR-dependent and -independent mechanisms by inducing PTEN through the inhibition of AR activity and directly binding with an epidermal growth factor receptor (EGFR), rapidly inhibiting its phosphorylation (Wang et al., 2010a, 2010b). Inactivation of EGFR results in decreased activity of the Akt pathway and thus reduced cell growth. Resveratrol arrested the cell cycle and induced apoptosis similarly in both androgen responsive and non-responsive prostate cancer cells (Benitez et al., 2007; Hsieh and Wu, 1999; Sgambato et al., 2001). Resveratrol was also suggested as elevating PTEN protein levels in other cancer cells and thus inhibiting cell proliferation (Waite et al., 2005).

In PTEN knockout mice, dietary resveratrol decreased the development of high-grade prostatic intraepithelial neoplasia (HGPIN) lesions and the number of adenocarcinomas in prostate (Li et al., 2013a; Narayanan et al., 2009). HGPIN is suggested as preceding the development of prostate cancer in humans (Li et al., 2013a). According to these results, resveratrol affects prostate cancer cells through both AR-dependent and -independent mechanisms. It may suppress the activity of AR, activate the tumor suppressor PTEN, and protect against the activation of the Akt pathway in PTEN- deficient cells. In addition, diet-derived resveratrol also downregulated the expression of insulin-like growth factor-1 (IGF-1) (Harper et al., 2007; Klink et al., 2013). Increased IGF-1 pathway signaling is associated with the androgen independent growth of prostate cancer with a poor prognosis (Ryan et al., 2007). Other natural stilbenoids, pterostilbene and pinostilbene, had similar effect as resveratrol on the transcriptional activity of AR and cell cycle arrest in prostate cancer cells (Wang et al., 2010a).

A resveratrol supplemented diet (50-100 mg/kg diet) delayed the tumor growth and reduced AR activity in mice with LNCaP prostate cancer xenografts (Wang et al., 2008a). However, resveratrol worsened the survival of mice with androgen sensitive LAPC-4 prostate cancer xenografts (Klink et al., 2013). A possible reason for this might be that cells with mutated p53 were not sensitive to resveratrol-triggered apoptosis. It was hypothesized that resveratrol could stimulate oncogenic pathways that may overcome its protective effects in some

type of prostate cancers (Klink et al., 2013). Nevertheless, resveratrol has been shown efficient in several preclinical models of prostate cancer. However, the observed cellular targets linked with stilbenoids are not specific only for prostate cancer, but similar mechanisms are found in different types of cancer.

The role of lignans and a lignan-rich diet in prostate cancer prevention are also being studied, but no clear conclusions about the mechanism of action have yet been made (Adlercreutz, 2007; Mccann et al., 2005a; Ward et al., 2010). As with resveratrol, lignans have been linked to several anticancer effects including cell cycle arrest and increased apoptosis rate in prostate cancer cells (Chen et al., 2009b; Lin et al., 2001; Peuhu et al., 2010). It has been suggested that enterolactone and plant lignans could suppress the proliferation and migration of prostate cancer cells through inhibition of IGF-1/IGF-1 receptor signaling, and induce apoptosis by inhibiting the Akt pathway (Chen et al., 2009b; Peuhu et al., 2010).

Enterolactone, secoisolariciresinol and a few other plant lignans inhibited prostate specific antigen (PSA) secretion in LNCaP prostate cancer cells (Han et al., 2008; McCann et al., 2008). These results indicated that lignans might suppress AR signaling pathways due to the fact that the expression of PSA is regulated by androgens and PSA secretion is positively associated with AR activity (Ruizeveld de Winter et al., 1994). Dietary 7-hydroxymatairesinol (0,15 % and 0,30 % in the diet) and enterolactone (0,01 % in the diet) inhibited the growth of LNCaP xenografts in mice (Bylund et al., 2005; Laajala et al., 2012). However, PSA secretion was not affected by enterolactone treatment, suggesting that growth inhibition *in vivo* was mediated by other mechanisms than inhibition of AR activity (Laajala et al., 2012).

In a clinical study, the daily dose of 30 g of flaxseed was associated with both reduced PSA level in serum and cell proliferation rate in prostatic epithelium (Demark-Wahnefried et al., 2004). Similarly, tumor cell proliferation was reduced in prostate cancer patients consuming 30 g daily dose of flaxseed for one month (Azrad et al., 2013; Demark-wahnefried et al., 2001; Demark-Wahnefried et al., 2008). Overall it can be concluded that lignans might have beneficial effects on prostate cancer prevention and treatment. While some of the studies indicate that they might inhibit AR activity or expression, current knowledge does not uniformly support that lignans are AR modulators in prostate cancer cells.

2.3.7 Modulation of aromatase by stilbenoids and lignans

Aromatase is a member of the wide cytochrome P450 (CYP) enzyme superfamily, and it catalyzes the biosynthesis of estrogens from androgens (Ghosh et al., 2009; Thompson and Siiteri, 1974). Aromatase is encoded by the aromatase gene (*CYP19A1*), which is expressed in gonads and in several extragonadal organs like in bone (Shozu et al., 1998), skin (Harada, 1992), adipose tissues (Mahendroo et al., 1993) and brain (Honda et al., 1994). The CYP19A1 gene consists of a coding region with 9 coding exons and a regulatory region including at least 11 untranslated first exons (Demura et al., 2008). Aromatase expression is regulated in a complex tissue-specific manner by alternative non-coding first exons, flanked by tissue-specific promoter regions (Figure 8) (Simpson et al., 2002). However, translated exons are identical in different tissues (Bulun et al., 2003; Irahara et al., 2006). The structure of the expressed protein is also similar from species to species, but the distribution in different organs and the regulatory area of the gene have a major interspecies divergence (Mitsuyo and Callard, 2001; Sahmi et al., 2014; Silverin et al., 2000; Zhao et al., 2009). For instance, the regulatory region of the aromatase gene is much less complex in rodents and the expression in adipose tissue is very low in comparison with humans.

Aromatase is an important clinical target and aromatase inhibitors have already been utilized as a frontline therapy for postmenopausal ERα positive breast cancers (Baum et al., 2002; Johnston et al., 2013). About 70 % of postmenopausal breast cancers are ERα positive and reducing the estrogen production can inhibit their growth. After menopause, adipose tissue is the main organ of aromatization and there the local estrogen production is also increased by obesity and obesity-related inflammation (Bulun et al., 2012). It is suggested that parallel increase in the levels of aromatase expression or activity with obesity and adipose tissue inflammation is a possible mechanism for the increased incidence of ERα -positive breast cancer in obese, postmenopausal women (Subbaramaiah et al., 2011). However, aromatase is mostly studied in women and only a few studies have been carried out about the regulation of aromatase in male adipose tissue (McTernan et al., 2000, 2002).

Obesity in men is associated with decreased androgen concentrations and an elevated estradiol/testosterone (E_2/T) ratio in serum, also referred to as hypogonadism (Dobs et al., 2001; Ferrini and Barrett-Connor, 1998; Wu et al., 2008; Yeap et al., 2014). While decline in testosterone level is often linked with aging, an obesity-associated decrease in testosterone level can be already observed in adolescent males (Mogri et al., 2013; Tsujimura, 2013). Obesity was also proposed to increase aromatase activity and/or expression resulting in low testosterone and increased estrogen levels in men (Kley et al., 1980;

Zumoff et al., 2003). Among the possible symptoms of low testosterone levels are infertility and decreased muscle mass (Huhtaniemi and Forti, 2011).

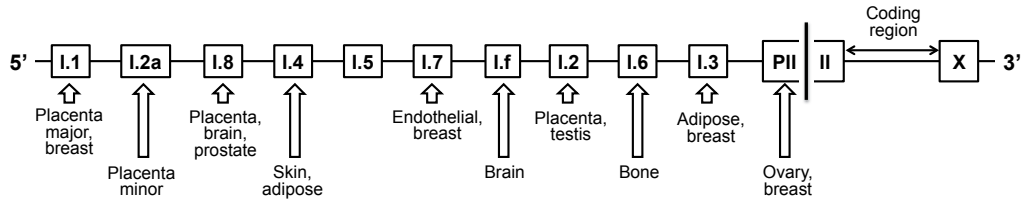

Figure 8. Structure of the human aromatase gene and the expression of alternatively used untranslated first exons in various tissues. Figure modified from Demura et al., 2008.

Some natural phenolic compounds, including enterolignans and resveratrol, have been suggested to inhibit aromatase enzyme activity (Adlercreutz et al., 1993; Bulun et al., 2005; Saarinen et al., 2002b; Wang et al., 1994, 2006). However, the inhibitory effect is only seen *in vitro.* In addition to the inhibition of the enzyme activity, reduction of aromatase expression could inhibit the biosynthesis of estrogens (Brown, 2014). The complex nature of the promoter area of the human aromatase gene may even enable the tissue specific inhibition of estrogen production by targeting the expression of aromatase promoters (Figure 8) (Bulun et al., 2009). The published data on the role of polyphenols in the modulation of human aromatase expression and E_2/T ratio in serum are very limited. However, the enterolactone level in serum and urine of healthy men was positively associated with increased androgen concentration in the serum (Low et al., 2005). Resveratrol intake increased the serum testosterone concentration in mice on high-calorie and high-cholesterol diet (Wang et al., 2014).

Resveratrol inhibited aromatase expression in placental choriocarcinoma cells by reducing the activation of aromatase promoter I.1 (Figure 8) and in MCF-7 breast cancer cells (Wang and Leung, 2007; Wang et al., 2006, 2008b). In healthy breast tissue half of the aromatase expression is derived from the activation of promoter I.4 and the rest from promoters I.3 and PII (Brown, 2014). It was suggested that resveratrol may inhibit the activation of those promoters in breast cancer cells (Wang et al., 2006). In addition, resveratrol reduced estrogen production in co-culture of T47D breast cancer cells and breast adipose fibroblasts (Chottanapund et al., 2014).

NF-κB-mediated IL-1β, TNFα as well as COX-2 and its product prostaglandin E_2 (PGE_2) are known inducers of aromatase expression in breast and female

adipose tissue (Chen et al., 2009a; Subbaramaiah et al., 2012). Resveratrol inhibited the expression of these pro-inflammatory compounds in aromatase expressing THP-1 cells (Jakob et al., 1995) and aromatase expression was also reduced in similar manner *in vitro* (Subbaramaiah et al., 2013). This suggests that resveratrol attenuates aromatase expression through the inhibition of the NF-κB pathway, which is also discussed in chapters 2.3.3.2 and 2.3.5.4. A resveratrol-rich polyphenol mix zyflamend reduced the expression of proinflammatory cytokines and mouse aromatase in mammary gland *in vivo*, but due to major differences between human and mouse aromatase genes, the result is not fully relevant in humans (Subbaramaiah et al., 2013). Polyphenol-rich grape seed extract was, however, associated with the suppression of aromatase gene transcription through downregulation of promoters I.3/PII and I.6 in human breast cancer cells (Kijima et al., 2006). Altogether, due to the lack of both relevant clinical and preclinical studies there is no sufficient knowledge as yet to conclude whether stilbenoids and lignans regulate the expression of aromatase, and how their intake modulates estrogen and androgen concentration in serum.

3. AIMS OF THE STUDY

The main objective of the present study was to assess the possible health-promoting properties of selected wood components with special emphasis on prebiotic, anticancer and endocrine effects. Wood derived hemicellulose saccharides and stilbenoids and lignans were the main groups of chemicals studied.

The specific aims were:

1) To obtain information on the potential of hemicellulose to influence the growth of probiotic bacteria. Selected lactic acid bacteria and bifidobacteria were assessed for their ability to utilize spruce (*Picea abies*) derived galactoglucomannan.

2) To determine the anticancer properties of pine (*Pinus sylvestris*) knot extract (PKE) and its main polyphenol components (pinosylvin and its methyl ether, nortrachelogenin, matairesinol), with an emphasis on prostate cancer by assessing:

- the effects of PKE and its main components on cell proliferation and apoptosis of (selected) human cancer cell lines in vitro.
- the bioavailability of orally adminstrated PKE
- the effect of PKE on the growth of human prostate cancer in a mouse xenograft model.

3) To study the effects of PKE on obesity-induced inflammation and endocrine dysregulation in adipose tissue, with a focus on:

- obesity-related metabolic parameters including weight gain, fat content, fasting glucose level and the leptin concentration
- obesity-induced adipose tissue inflammation
- regulation of the aromatase gene

4. MATERIALS AND METHODS

4.1 Preparation of extracts and working solutions

Knots from freshly cut pine trees were carved out by hand. Samples were freeze-dried, ground in a cutting blade mill to pass through a 10-mesh screen, and stored at -20 °C until analyzed. Extractions were performed with a Dionex Accelerated Solvent Extractor. Lipophilic extractives were removed by hexane extraction with three cycles at 90 °C and discarded. Hydrophilic extractives were collected by extraction with ethanol with three cycles at 100 °C, evaporated to dryness, and stored at -20 °C. The compositions of knot extracts were analyzed by a GC-flame ionization detector (FID), GC-MS, and by high-performance size-exclusion chromatography (Smeds et al., 2007, 2012).

Nortrachelogenin, matairesinol, enterolactone, pinosylvin, and pinosylvin methylether (purities > 95 %) were prepared in the Laboratory of Wood and Paper Chemistry at Åbo Akademi (Smeds et al., 2006, 2007). Resveratrol and enterodiol were from Sigma-Aldrich Co (St. Louis, MO). The stock solutions of knot extracts and phenolic compounds were prepared by dissolving compounds in dimethylsulphoxide (DMSO) in 100 mM or µg/ml concentrations, stored at -20 °C. Stock solutions were diluted 1:1000 for working solutions used in cellular assays with 0,1 % DMSO vehicle.

Galactoglucomannan (GGM) was extracted from ground spruce (*Picea abies*) by pressurized hot water at 170 °C and later precipitated with ethanol. Hemicellulose fractions were analyzed by size-exclusion chromatography (Song et al., 2013). GGM-derived mannooligosaccharide (MOS) was prepared by the controlled cleavage of the spruce-derived GGM extract with β-endo-mannanase (Megazyme, Wicklow, Ireland). Fructo-oligosaccharide (FOS) Orafti P95 was obtained from Beneo Orafti (Tienen, Belgium) and xylo-oligosaccharide (XOS) from Shandong Longlive Biotechnology (Qingdao, Shandong, China). GGM and other saccharides were dissolved in water in 6 % solutions and sterile filtered before administration in the growth medium of bacteria.

4.1.1 Determination of antioxidant activity

Three separate biochemical assays were used to study the antioxidative properties of phenolic compounds. Wood-derived compounds were compared with Trolox (Vitamin E), a known dietary antioxidant (Manach et al., 2004). The peroxyl radical scavenging activity (TRAP) of extracts and substances was measured by using a method based on the radical formation in thermal

decomposition of 2,2′-azobis(2-amidinopropane)-hydrochloride in phosphate buffer (pH 7,4) with luminol (Ahotupa et al., 1997). The amount of peroxyl radicals was determined according to the chemiluminescence, measured with a Bio-Orbit 1251 Luminometer (Bio-Orbit, Turku, Finland).

Second, the potency of polyphenols to reduce lipid peroxidation was measured in rat liver microsomes (Ahotupa et al., 1997). Rat liver microsomes (1.5 μg protein/ml) were added to an aliquot of buffer in the luminometer cuvette followed by addition luminol solution and the test compound. The reaction was initiated by adding *tert*-butylhydroperoxide solution at 33 °C, that induced lipid peroxidation. The chemiluminescence was then monitored for 45 min in 1-minute cycles, and the area under the curve calculated. Third, the capacity of polyphenols and extracts to prevent human low-density lipoprotein (LDL) oxidation in the phosphate buffer (pH 7,4) with $CuCl_2$ was measured (Ahotupa et al., 1996). After 3 hours oxidation process with Cu^{2+} at 37 °C lipids were extracted with chloroform:methanol (2:1, v/v), dried under nitrogen, and re-dissolved in cyclohexane. The total concentration of conjugated dienes as a marker of LDL oxidation was measured with spectrophotometer (PerkinElmer Corp., Norwalk, CT).

4.2 Culturing of cells and bacteria

4.2.1 Cancer cells

Immortalized cancer cells (PC-3, PC-3M-Luc and VCaP prostate cancer cells, MCF-7 breast cancer cells, Hep G2 hepatocellular carcinoma cells and U2OS osteosarcoma cells) were maintained in a phenol free Dulbecco's modified eagle medium (DMEM, Life Technologies Ltd, Carlsbad, CA) with 10 % inactivated bovine serum (EU approved origin, Life Technologies Ltd.). In addition, VCaP cells were maintained in a presence of dihydrotestosterone and MCF-7 cells in a presence of 17β-estradiol. Cells were split and cultured according to the instructions of the American Type Culture Collection (www.atcc.org). To avoid the possible growth stimulatory estrogenic effect of phenol red on cells (Berthois et al., 1986) we only used free phenol red free media in this work.

4.2.2 Mesenchymal stromal cells

Mesenchymal stromal cells (MSC) were isolated from the bone marrow of the tibias and femurs of male hARO-Luc reporter mice (Strauss et al., 2013). Bones were cleaned and connective tissue removed in phosphate buffered saline (PBS) and then the marrow was flushed with MSC-medium (Phenol red free

Minimum Essential Medium alpha, 15 % inactivated Fetal Bovine Serum (USA origin), 10 mM HEPES, 50 IU/ml penicillin, 50 µg/ml streptomycin, 2.5 mM L-glutamine and 0.25 µg/ml Amphotericin B (all components from Life Technologies Ltd), supplemented with 10 nM dexamethasone (DEX, SERVA Electrophoresis GmbH, Heidelberg, Germany). MSC were incubated on petri dishes in MSC-medium at 37 °C in 5% CO_2. Free-floating cells were removed and cells split 1:3 just before confluency.

4.2.3 Anaerobic bacteria

Anaerobic gut microbes (*Bifidobacterium animalis* subsp. *lactis* Bb12 (DSM 15954), *B. longum* (JCM 1217), and *L. rhamnosus* GG (ATCC 53103), *B. bifidum* (JCM 1254), *B. pseudocatenulatum* (JCM 1200), *B. infantis* (DSM 20088), *B. adolescentis* (JCM 1275) and *B. breve* (JCM 1192)) were maintained in a Gifu anaerobic broth medium (GAM, Nissui Pharmaceutical Co., Tokyo, Japan) in an anaerobic atmosphere (10 % H_2, 80 % N_2, and 10 % CO_2) at 37 °C. Bacteria were removed to a fresh broth medium after two days in 1 % inoculum. Before the growth assays, bacteria were collected by centrifuging and washed with PBS to remove medium-derived saccharides. Growth assays were carried out in a carbohydrate free basal medium (CFBM) presented by (Ruas-Madiedo et al., 2008). All CFBM components were provided by Sigma.

4.2.4 Bacterial growth assays

The washed pellet of anaerobic bacteria inoculum was suspended in CFBM, immediately supplemented with 1 % (w/v) prebiotic candidates, and incubated for one to four days. Glucose was used as a positive and water as a negative growth control. The optical density of broth cultures was measured daily at the wavelength of 600 nm. The total number of viable bifidobacteria in the broth medium was measured by the plate count method on GAM agar plates with a 48 h incubation time (24 h for *L. rhamnosus*). In the GGM-adaptation assay, bifidobacteria were grown for two weeks in CFBM with 1% (w/v) galactoglucomannan preparation. The medium was changed every second day with 1 % inoculum.

4.3 Mammalian cell culture assays

4.3.1 Proliferation assay

To study the effects of polyphenols on the cancer cell proliferation, cells were incubated with treatments *in vitro* on a 96-well plate in serum-free medium. First, the cells were seeded on a plate, immediately followed by polyphenols or

extracts mixed in the medium. After 2-4 days incubation, cells were labeled with thymidine analog bromodeoxyuridine (BrdU), followed by the incorporation of BrdU specific antibody conjugated with peroxidase, followed by its substrate. Cell Proliferation ELISA BrdU immunoassay kit was provided by Roche Diagnostics (Mannheim, Germany) The reaction with the substrate was stopped with sulfuric acid and the growth response was measured optically with a spectrophotometer at $\lambda = 450$ nm (Gratzner, 1982). Relative changes in cell proliferation rates were calculated according to the absorbances.

The estrogenicity of extracts and polyphenols was observed by measuring the growth of estrogen-responsive MCF-7 cells in the presence of the study compounds. Assays were carried out with and without 17β-estradiol in serum-free medium. Cells were pre-cultured for 48 h in a growth medium containing only charcoal stripped serum (5 % w/w) to remove estrogenic compounds before the treatments. Cell proliferation was measured with a BrDU assay, and relative proliferation rates with and without estradiol were compared on order to detect possible agonism or antagonism on ERα. Androgen-responsive VCaP cells were growth like MCF-7 cells, but instead of E2, we used 0,1 nM dihydrotestosterone to maintain AR-dependent cell proliferation.

4.3.2 Apoptosis and cell cycle assay

Near confluent cell cultures on 96-well plates, were introduced to treatments (e.g. polyphenols, pine knot extract). After a 48 or 96 h treatment period, cells were detached and disrupted with propidium iodide buffer (40 mM sodium citrate, 0,3 % Triton X-100, propidium iodide 50 mg/ml; all from Sigma). Propidium iodide labels cell nuclei and nucleus fragments quantitatively to the amount of DNA present (Krishan, 1975). The number of cell fragments and cells in the different phases of the cell cycle were measured with a BD LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ). Cell fragments (sub G0/G1 phase events) were quantified as a marker of apoptosis and the numbers of nuclei of a specific area as a measure of cells in the specific cell cycle phase (G1/G0, S and G2/M). For assessment of TRAIL sensitivity, cancer cells were treated simultaneously with polyphenols and human TRAIL/Apo2L (Promokine, Heidelberg, Germany).

4.3.3 Multiplex immunoassays

Samples were centrifuged to remove debris, and analyte concentrations were measured by 96-well plate multiplex assays according to the manufacturer's (Millipore, Billerica, MA) instruction. The manufacturer specifically customized assay kits for the required set of analytes. Analysis was carried out with Luminex 200 system (Luminex Corporation, Austin, TX) and results

calculated with xPONENT 3.1 software (Luminex Corporation). Results from serum samples were used as such. Results from tissue homogenates were adjusted according to the total protein concentration in the suspension.

The total protein content in the tissue samples was measured optically with a bicinchoninic acid (BCA, Thermo Fisher Scientific, Rockford, IL) protein assay at a wavelength of 562 nm. Standard protein solutions for quantitative BCA assay were prepared from bovine serum albumin (BSA). The multiplex results of fat tissue secretion samples were adjusted according to the number of white adipocytes in a sample.

4.4 *In vivo* models

4.4.1 Animals

Mice in all experiments were maintained in controlled conditions (22-24 °C, 50 % relative humidity, 12 hour light cycle). They were fed with soy free chow pellets (RM3, Special Diets Services, Essex, UK), unless stated otherwise, and supplied with tap water *ad libitum*. Experimental procedures were approved by the national Animal Experiment Board in Finland and carried out according to the Finnish Act on Animal Experimentation and laws, guidelines and recommendations in the European union.

4.4.2 Diet induced obesity and PKE-rich diet

hARO-Luc male mice with FVB/N background (Strauss et al., 2013) were kept 8 weeks on a high fat diet (HFD, providing 60 % of calories from fat) to induce obesity, starting at the age of 6 weeks. HFD and its control diet, low fat diet (LFD, 10 % of calories from fat), were manufactured by Research Diets Ltd (New Brunswick, NJ). Diet manufacturer premixed PKE with HFD (1600 mg of PKE in 1 kg of HFD).

At the beginning of the experiment, the mice were randomized into different dietary groups (LFD, HFD and HFD-PKE) with similar body weights and adiposities. Adiposity was measured by Echo-MRI (EchoMRI LCC, Houston, TX) analysis (Taicher et al., 2003). During the experiment, weight gain and adiposity were followed weekly. Food intake was measured twice a week by weighing both the remaining food in the cages and fresh food added. Blood glucose was measured from tail vein samples collected after a 4-hour of fasting, just before sacrifice. After terminal anesthesia, blood samples were collected by a cardiac puncture followed by cervical dislocation. Serum samples were stored

at -70 °C. Concentrations of phenolic compounds in the food and serum were analyzed by GC-MS techniques (Smeds et al., 2005, 2006).

4.4.3 Luciferase activity assay and adipose tissue methods ex vivo

Luciferase reporter gene activity in tissue supernatans was measured with a luciferase assay kit (BioThema AB, Handen, Sweden) as described by (Strauss et al., 2013). Briefly: hARO-Luc mice tissues were collected, snap frozen and kept at -70 °C until being homogenated and the supernatants collected. Luciferase assay was measured with a Victor^2 plate reader (PerkinElmer, Turku, Finland), according to the manufacturer's instructions. Results were adjusted according to the total protein concentration in supernatant (measured optically with BCA protein assay).

Adipose tissue explants, approximately 100 mg each, were cut from fresh gonadal and subcutaneous fat pads after the sacrifice. Fat pad sections were incubated for 24 h in a cell culture medium with 50 units/ml penicillin and 50 mg/ml streptomycin on a 24-well plate at 37 °C in a humidified atmosphere with 5% CO_2 (McGillicuddy et al., 2011). After incubation, media samples were frozen and analyzed for cytokine and adipokine concentration by magnetic multiplex assay (see: chapter 4.3.3).

Fat explants from hARO-Luc mice, fed for 8 weeks with HFD, were also used to study the modulation of human aromatase gene expression. The incubation media of adipose explants was supplemented *ex vivo* with substances (All from Sigma unless stated otherwise) including DEX (SERVA), prostaglandin E_2, TNFα, forskolin, phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides and various phenolic compounds and pine knot extract. Tissue samples were collected and frozen after 24 h incubation at 37 °C, and their luciferase activity was measured as described in the previous paragraph. The luciferase activity of supernatants was adjusted according to the mass of fresh adipose tissue explants before incubation.

To study the expression of the luciferase reporter gene in cultured MSC, cells from passage 2-3 after the isolation from the bone marrow of HFD-fed hARO-Luc males were seeded into 12-well plates (40 000 cells per well in 1 ml of MSC-medium without DEX). When the cell cultures were nearly confluent (four to six days incubation), similar treatments to the fat explants were used to modulate the luciferase activity. Substances in stock solutions were premixed with DMEM/F12 growth medium with 1 mg/ml BSA, 1 µg/ml insulin and 10 µg/ml transferrin (all from Sigma) (Lindahl et al., 2011). After 24 h incubation at 37°C, the cells were washed with PBS, scraped from the well in a lysis buffer

(Tropix lysis solution, Applied Biosystems, Foster City, CA) with 0.5 mM dithiotreitol, Sigma) and collected in suspension for the Luciferase activity assay. The luciferase activity of bone marrow derived MSC was measured similarly as from the fat explants. Results were adjusted according to the total protein concentration in cell suspension.

4.4.4 Orthotopic prostate cancer xenograft study

PC-3M-luc2 prostate cancer cells were maintained in a DMEM 11880 medium supplemented with 50 units/mL penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, and 10 % heat-inactivated fetal bovine serum (EU approved origin, all medium components from Life Technologies Ltd). Cells were collected from 80-95 % confluent cell culture petri dishes (⌀ 15 cm), and suspended in serum free DMEM with 2 % of green food coloring solution to help to aim the inoculation. Cell suspension ($1{,}0\times10^6$ cells in 20 µl per animal) was injected in dorsolateral prostate of athymic Nude – $Foxn^{1nu}$ male mice under isoflurane anesthesia.

One week after the cell injection, tumor bioluminescence was imaged and mice randomized to three treatment groups: vehicle (control), low dose of PKE (32 mg/kg) or high dose of PKE (160 mg/kg). Dry pine knot extract (PKE) was dissolved in a solution containing 10 % (v/v) of absolute ethanol and 90 % (v/v) corn oil. This emulsion was shaken before it was gavaged daily *per os* to animals. Fresh emulsion was prepared at least once a week and treatments period lasted for three weeks.

Tumor bioluminescence to follow the tumor growth was measured *in vivo* by injecting mice with D-luciferin (Xenogen, Oregon, USA) solution, administered as 150 mg luciferin/kg bw. Injected mice were anesthetized in an isofurane chamber and immediately transferred to IVIS Lumina system (Caliper Life Sciences, Runcorn, UK) and imaged for luminescence (Contag et al., 1995). Results were quantified using the Living Image program (Xenogen), total flux being measured by combining each defined area of interest on the tumor.

Urine samples were collected three weeks after randomizing the mice into dietary groups. Mice were kept 24 hours in metabolic cages, 3-6 mice per metabolic cage. Urine samples were collected in jars that contain sodium azide and ascorbic acid as preservatives. Phenolic compounds in urine were analyzed as described in chapter 4.4.2. After four weeks the mice were sacrificed with CO_2 suffocation followed by cervical dislocation. Blood was collected via heart puncture and the serum was separated and stored at -70 °C. The tumors were

dissected and weighed, and the tumor size was measured with a caliper. The tumor volume was calculated using the formula:
volume = (length×width×height)×(π/6).
The tumors were fixed in 10% neutral formalin and embedded in paraffin for immunohistochemical analyses. In paraffin, they were cut in 5 μm thick sections.

4.5 Histological Methods

4.5.1 Von Willbrand factor 8 (vWF8)

The vWF8 staining of endothelial cells is used to quantitate prostate tumor angiogenesis (Evans et al., 1997). First paraffin embedded tumor sections were rehydrated. After a water rinse the sections were incubated with BSA and labeled with vWF8 (dilution: 1:4000, ab6994-100, Abcam, Cambridge, U.K.) antibody. Peroxidases in samples were blocked with 3 % hydrogen peroxide and the samples were labeled with a secondary antibody. Sections were stained with diaminobenzidine, followed by Mayer hematoxylin, and dehydration. The density and length of vessels were quantified and their total length per area calculated from randomly selected areas of every tumor.

4.5.2 Phosphohistone-H3 (pH-H3)

pH-H3 stains mitotic figures, and stained sections can be used to study tumor cell proliferation (Bossard et al., 2006). Paraffin-embedded tumor samples were rehydrated. After a water rinse sections were incubated with a pH-H3 antibody (dilution: 1:200, Ser10, Cell Signaling Technology, Danvers, MA), peroxidase activity was blocked and the samples labeled with a secondary antibody (Dako, Glostrup, Denmark). Sections were stained with diaminobenzidine, followed by Mayer hematoxylin, and dehydration. The Quva Company (Tampere, Finland) quantified the density of proliferating cells on the randomly selected areas of stained tumor sections.

4.5.3 Morphometric analysis of fat tissue

Infiltration of macrophages into adipose tissue was measured from histological samples. Gonadal, mesenteric, and subcutaneous fat sections were deparafinized, rehydrated, and stained with HE. Stained adipose sections were scanned with an Olympus BH2 virtual microscope (Digital Virtual Microscope, Soft Imaging System, Olympus, Germany). Four 1 mm^2 areas were randomly selected for each HE-stained tissue. Density of white adipocytes and crown like structures (CLS) formed by macrophages were quantitated from these areas and

the CLS densitiy and average adipocyte size (=1/number of adipocytes per 1 mm^2) were calculated. A single white adipocyte surrounded by macrophage ring was counted as a one CLS (Cinti et al., 2005). Presence of macrophages in CLS was confirmed by macrophage selective mac3 (Clone M3/84) – immunohistochemical staining. The number of white adipocytes (n) in each fat section was calculated according to the mean cell size (area, A) on tissue section. It was presumed that white adipocytes are globular and they fill the substantial majority of WAT (density=0,92 g/ml; Farvid et al., 2005). Their mean volume (V) and the number of cells in a sample were calculated using the formulas:

$V_{cell}=(4/3)\pi\times r_{cell}^3 =(4/3)\pi\times(\sqrt{(A_{cell}/\pi^3)})$

$n_{cell}=V_{sample}/V_{cell}=(m_{sample}/0{,}92\ g/ml)/V_{cell}$

4.6 Statistical analysis

Statistical analyses were performed by using Microsoft Excel for Mac 2011 Version 14.1 (Microsoft Corporation, Redmond, WA) and GraphPad Prism version 6.0c for Mac OS X (GraphPad Software Inc., San Diego CA). Mann-Whitney test using two-tailed distribution was used to measure the growth stimulatory effects of various saccharides on bacteria.

For other *in vitro* studies and *in vivo* and *ex vivo* studies, the one-way analysis of variance and Dunnett's or Tukey's post-hoc test were used in normally distributed data. Otherwise the Kruskal-Wallis or Mann-Whitney test was used. Linear regression was used to test the relationship between two variables. Values of $P\leq0{,}05$ were considered statistically significant.

5. RESULTS AND DISCUSSION

5.1 Prebiotic properties of hemicellulose derived molecules

The ability of gut microbes to utilize various oligo- and polysaccharides varies greatly among the species of *Bifidobacterium* genera and even among strains from the same species. In this study, various bifidobacteria and one lactobacilli species, commonly used as probiotic bacteria, were found to utilize GGM. Both the growth rate and maximum concentration of bacteria were enhanced in a presence of GGM. In addition to GGM, results here suggest that these microbes may utilize GGM-derived MOS and xylan hemicellulose-derived XOS.

5.1.1 Galactoglucomannan stimulates the growth of bifidobacteria and Lactobacillus rhamnosus GG (I & unpublished data)

The presence of galactoglucomannan or hemicellulose derived oligosaccharides MOS and XOS in an otherwise saccharide-depleted growth medium increased the density and growth rate of bifidobacteria. Depending on the bifidobacteria species, the increased growth was observed after 2-3 days of incubation. The stimulatory effect of MOS and XOS on the growth of *L. rhamnosus* GG was, however, already observed after 24 h of incubation due to the faster growth rate compared with bifidobacteria.

The growth response of seven *Bifidobacterium* species on GGM was determined, including *Bifidobacterium animalis* subsp. *lactis* Bb12, *B. longum, B. bifidum*, *B. pseudocatenulatum*, *B. infantis*, *B. adolescentis* and *B. breve* (Figure 9). Each strain used was isolated from human intestine or feces (Kurdi, 2003). GGM (1 % w/v) in otherwise carbohydrate poor medium stimulated the growth of all studied Bifidobacteria, which strongly suggest that these bacteria were able to utilize GGM. Similarly, *B. animalis*, *B. longum, B. bifidum*, *B. pseudocatenulatum*, *B. infantis* and *B. adolescentis* had a growth response to XOS, and *B. animalis*, *B. longum, B. breve, B. pseudocatenulatum,* and *B. adolescentis* responded to MOS (Figure 9). All of the studied microbes displayed enhanced growth in the presence of the studied saccharides; no toxicity or adverse effects on growth were witnessed (unpublished data).

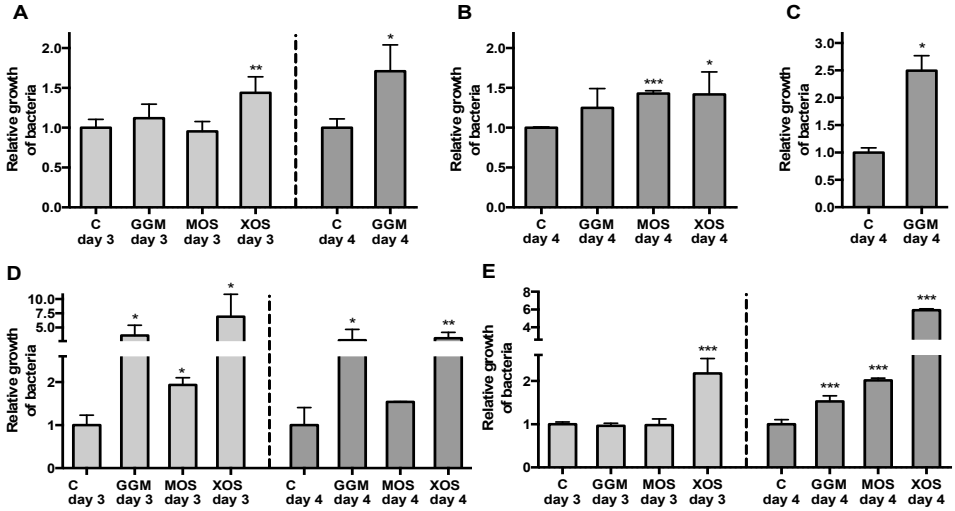

Figure 9. Optically measured (λ=600 nm) relative growth rates of bifidobacteria after the 3-4 days of anaerobic incubation in the presence of hemicellulose-derived saccharides (1 %, w/v). ***A.*** *Bifidobacterium bifidum* JCM 1254*,* ***B.*** *B. infantis* DSM 20088*,* ***C.*** *B. breve* JCM 1192*,* ***D.*** *B. pseudocatenulatum* JCM 1200*,* ***E.*** *B. adolescentis* JCM 1275*. The control medium in all assays is a carbohydrate free basal medium with 0,2 % saccharide content. GGM=galactoglucomannan, MOS=mannan oligosaccharides, XOS=xylo-oligosaccharides. Data is expressed as mean+SD. * P < 0.05, ** P < 0.01, *** P < 0.001, Mann-Whitney test.*

5.1.2 Bifidobacterium lactis Bb12 adapts to galactoglucomannan in growth medium (I)

We measured whether the prolonged exposure of *B. animalis* to GGM could enhance the ability of bacteria to utilize hemicellulose-derived saccharides. Culturing *B. animalis* for two weeks with GGM as the only carbohydrate source (1 % w/v) in the growth medium improved the growth rate of the bacteria. In addition to the growth of the bacteria in the presence of GGM, the GGM-adapted strain of *B. animalis* had a higher growth response to GGM-derived MOS as well.

Change in growth rates after the adaptation process was significant, especially in the early growth phase, 24-48 h after inoculation. The number of viable GGM-adapted *B. animalis* was five times higher in the GGM containing medium compared with the control after a three-day incubation period. In accordance with these results, the number of non-adapted *B. animalis* in the

medium was only slightly higher than the control after a three days incubation period in a GGM containing medium.

5.1.3 Hemicellulose as a prebiotic compound

These results demonstrate that the growth of the bacteria was faster and the number of viable microbes in the medium was higher when GGM was present, indicating that *Bifidobacterium* spp. is able to utilize softwood GGM-derived saccharides. Similarly, a stimulatory effect on the growth rate was found when XOS and MOS were present in the growth medium.

The prebiotic effects of various xylan-derived preparations have been previously reported and the present results are in accordance with these studies (Campbell et al., 1997; Mäkeläinen et al., 2010; Okazaki et al., 1990). GGM-derived oligosaccharides have been subject to less examination than xylan, and a few existing studies support the idea that these compounds stimulate the growth bifidobacteria (Faber et al., 2011a, 2011b). In these studies, it was suggested that diet supplemented with GGM-oligomers increases the number of fecal bifidobacteria. The same compounds stimulate the growth *in vitro* in fecal cultures. The results here show the direct growth stimulatory effect of GGM saccharides on bifidobacteria and *L. rhamnosus*. Moreover, these results show for the first time that the unhydrolyzed, high molecular weight GGM could stimulate the growth of probiotic bacteria. In previous studies, prebiotic candidates have typically had the polymerization rate of one tenth of that of the GGM substrate used in the present study. This GGM preparation requires less processing and it also has a practically unlimited, ecological source due to the fact that GGM is one of the main components of coniferous trees.

The properties of probiotic strains *B. animalis* Bb-12 and *L. rhamnosus* GG are well characterized and they are widely used commercially (Doron et al., 2005) (Garrigues et al., 2010). Therefore, the combination of GGM and these probiotics could be further developed into novel functional foods components or nutraceuticals. However, prior to this, an assessment of the hemicellulose-derived components as potential ingredients in foods needs to be completed. For the first assessment of safety, information on the history of the use of GGM and similar components is needed. For the most part, our results suggest a significant stimulatory effect on the proliferation and concentration of several human-derived *Bifidobacterium* species and *L. rhamnosus* GG when GGM is present in the growth medium.

5.2 Anticancer properties of pine knot extract

A wide variety of polyphenols and plant extracts have been proposed as being able to modulate the growth of cancer cells and affect different phases of tumor development (Quideau et al., 2011). The role of softwood-derived extract and polyphenols in the survival of various cancer cells was assessed by measuring the effect of these compounds on the proliferation rate, apoptosis, and viability of cancer cells, with prostate cancer being of special interest. Moreover, the effect of dietary pine knot extract (PKE) on the growth of an orthotopic prostate cancer xenograft was determined, and the metabolism of this extract was studied in mice. PKE, the main components of which are lignans and stilbenoids, was found to have anticancer properties, which inhibit the growth of cancer cells *in vitro,* and promote apoptosis by sensitizing cells to TRAIL-mediated cell death. The anticancer properties of PKE were further verified by showing that dietary PKE inhibits the growth of a prostate cancer xenograft.

5.2.1 Composition and antioxidative properties of PKE (II & III)

PKE was chemically characterized by GC-FID, GC-MS and high-performance SEC methods (Smeds et al., 2012). The individual compounds found were identified by mass spectrometry using the commercial spectrums and spectral libraries of the laboratory of wood and paper chemistry at Åbo Akademi University. The major compound groups in the PKE (w/w) were: lignans (16%), stilbenoids (17%), oxidized resin acids (20%), resin acids (24%), and higher-molecular weight compounds (550–4000 g/mol, 18%). The content of the main GC-detectable free phenolics in PKE were pinosylvin monomethyl ether 10,2%, nortrachelogenin 7,0%, pinosylvin (PS) 4.0%, matairesinol 1,5%, and pinostilbene 0,4%. Minor amounts (0,1–0,5%) of lignans 7-hydroxymatairesinol, conidendric acid, secoisolariciresinol and todolactol A were also found in PKE. In addition, the high molecular weight fraction in PKE included resin acid oligomers and di- and oligomeric lignans. Parts of the phenolic compounds were also bound with high molecular weight compounds, and were therefore undetectable by GC analysis.

According to three separate assays, PKE was an efficient antioxidant *in vitro*. Compared with the known antioxidant Vitamin E, PKE's peroxyl radical scavenging capacity was better, (114 g/mol for Vitamin E and 89 g/mol for PKE). Of the individual compounds in PKE, lignans nortrachelogenin and matairesinol were the most potent peroxyl radical scavengers. PKE inhibited *tert*-butylhydroperoxide-induced lipid peroxidation in rat liver microsomes (IC50 concentration was 16 ng/ml) and oxidation of human LDL (IC50 3,8 µg/mg of LDL). These IC50 concentrations of PKE were comparable, although somewhat lower, to those of Vitamin E. Pinosylvin was the most active

compound in PKE to inhibit the oxidation of LDL (IC50 was 2.5 μg/mg of LDL) and nortrachelogenin and matairesinol to prevent *tert*-butylhydroperoxide-induced lipid peroxidation (IC50 values were 26 and 17 ng/ml). Our results are also in line with the previous estimations about the antioxidative properties of stilbenoids and lignans (Willför et al., 2003e). In conclusion, we present PKE as an efficient antioxidant *in vitro* and its polyphenol content contributes the most to the antioxidative efficiency of PKE.

5.2.2 Effect of PKE and its compounds on survival of cancer cells (II and unpublished data)

PKE significantly attenuated PC-3M-luc2 cell proliferation at 40 μg/ml (Figure 10), demonstrated by the reduced incorporation of BrDU *in vitro*. In line with this, the main constituents of PKE, stilbenoids (pinosylvin and its monomethyl ether), as well as lignans (matairesinol and nortrachelogenin) each significantly inhibited cell proliferation at 40–100 μM concentration. Furthermore, a lignan and stilbenoid (LS) mixture, the combination of these four main constituents of PKE: pinosylvin monomethylether, pinosylvin, matairesinol, and nortrachelogenin in a mass ratio of 10:4:7:1,5; similar to PKE, reduced cell proliferation in a manner equivalent to the original extract.

Both PKE (40 μg/ml) and LS mixture (40 μM) similarly increased the apoptosis rate of PC-3M-luc2 cells, as demonstrated by the flow cytometric analysis. PKE induced cell cycle arrest, i.e. increased the fraction of non-apoptotic cells in G0/G1 and, respectively, decreased the fraction of cells in the S and G2/M phases. Pinosylvin and its monomethyl ether, and their metabolite resveratrol modulated the cell cycle. In addition, matairesinol strongly induced cell cycle arrest. PKE (10 and 40 μg/ml) sensitized PC-3M-luc2 cells to TRAIL-induced apoptosis. The LS mixture and all the tested PKE-derived polyphenols and resveratrol significantly enhanced TRAIL-mediated apoptosis. However, enterolactone and enterodiol did not have any effect on the apoptosis rate or cell cycle.

We also studied the effect of pine-derived polyphenols and PKE on the cell proliferation of other cancer cells. While there were some variations in the extent of response, the growth of all the studied cancer cell lines was inhibited by PKE and its phenolic components. The proliferation rates of PC-3 and VCaP prostate cancer cells, HepG2 hepatocellular carcinoma cells and U2 OS osteosarcoma cells were inhibited with 10-100 μg/ml PKE (Figure 10). PC-3 cells were more sensitive to PKE than PC-3M-Luc2 cells, which are the more metastatic variant of PC-3 cells. However, cells may respond differently to specific treatments and PC-3M cells (as PC-3M-Luc2 cells without Luc construct) are suggested as being more aggressive than parental PC-3 cells

(Kim et al., 2004; de Souza et al., 1997). Interestingly, the inhibitory effect of PKE on the proliferation of HepG2 and U2 OS cells was more potent than the effect of any single component of the extract (unpublished data). This suggests that extract with several bioactive components may possibly target multiple pathways that modulate cell survival. Of the individual compounds, pinosylvin monomethylether was the most potent growth inhibitor of cancer cells (II).

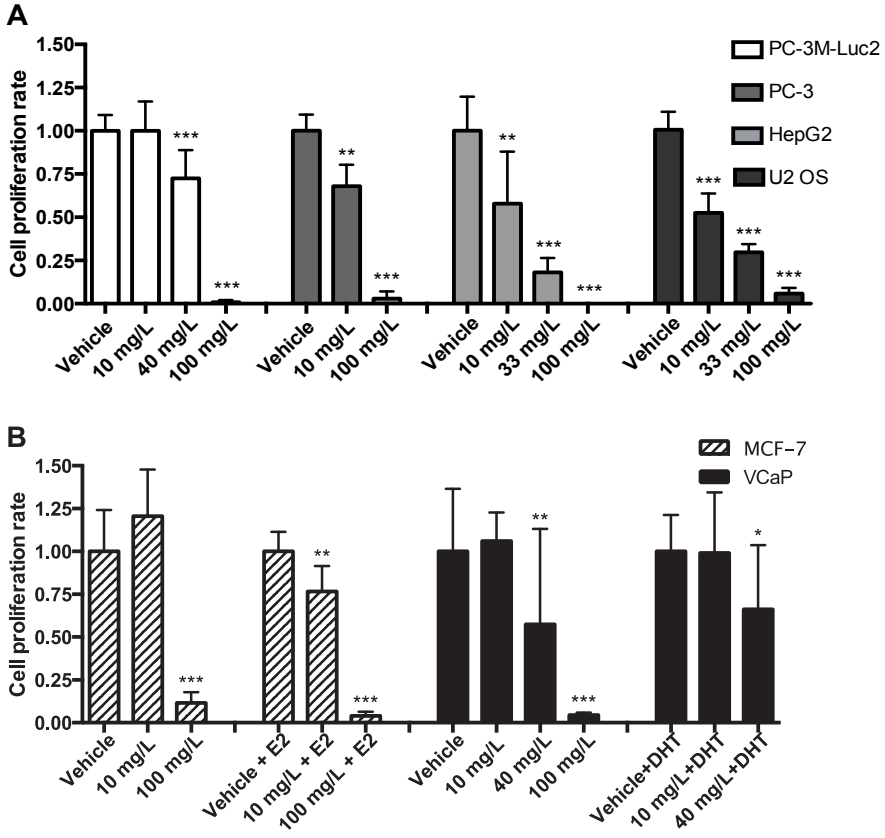

Figure 10. Pine knot extract induced a concentration-dependent reduction in cell proliferation, measured with a BrDU-based assay. ***A****. Effect of 10-100* µg/ml *pine knot extract (mean+SD) concentrations on the relative proliferation rate of PC-3M-Luc2, parental PC-3, HepG2 and U2 OS cancer cells after 48 h incubation with treatments.* ***B****. Effect of PKE on relative proliferation of estrogen-responsive MCF-7 breast cancer cells in serum-free medium with and without 1 nM 17β-estradiol (E2), and effect of PKE on the proliferation of androgen-responsive VCaP prostate cancer cells in serum-free medium with and without 0,1 nM dihydrotestosterone (DHT). Assays measured after 96 h incubation and results expressed as a mean+SD. DMSO (0,1 %) was used as a vehicle in all assays. * P < 0.05, ** P < 0.01, *** P < 0.001, one-way ANOVA and Dunnett's multiple comparison test.*

We used the androgen-responsive VCaP cells to study the effects of pine polyphenols on AR-mediated cell proliferation. However, the relative growth responses of VCaP cells to PKE were similar both in an androgen-depleted medium and in the presence of a strong androgen (dihydrotestosterone) (Figure 10). This suggests that PKE does not target AR-mediated cell proliferation in prostate cancer cells, but instead modulates other pathways that affect cell proliferation. In addition, we did not detect that any of the individual polyphenols in PKE would modulate the proliferation of VCaP cells differently in a presence of androgens (unpublished data).

The estrogenic activity of compounds can be evaluated according to their ability to induce the cell growth of estrogen-responsive MCF-7 cells. Here, we measured the ability of PKE to stimulate the growth of estrogen-responsive cells in the absence of estradiol. Correspondingly, we observed the possible antagonistic effects of PKE by co-treating cells with PKE and 1 nM 17β-estradiol (E_2) (Figure 10). Similar assays were also carried out with 0,001-100 μM pinosylvin and resveratrol. Cell proliferation rates were fourfold in the presence of E_2, but otherwise the relative growth inhibitory effect of stilbenoids was not affected by the presence of E_2. Previous data about the estrogenic properties of resveratrol (chapter 2.3.5.6) are inconsistent and our results did not present any estrogenic properties of stilbenoids.

The high concentration of PKE (100 μg/ml) inhibited approximately 90 % of MCF-7 cell proliferation, independent of the presence of E_2. At the concentration of 10 μg/ml PKE in an E_2-containing medium, proliferation was significantly inhibited, but in an estrogen-depleted medium the proliferation rate was unchanged (Figure 10). Treatments at lower PKE concentration than 10 μg/ml did not affect the proliferation rate of MCF-7 cells (unpublished data).

5.2.3 Effect of PKE on orthotopic PC-3M-luc2 xenograft growth in mice (II)

The daily administration of 160 mg PKE/kg bw dose for three weeks, starting one week after the orthotopic PC-3M-luc2 tumor inoculation, significantly reduced the proliferation index and the total length of blood vessels in tumors compared to the vehicle group. Moreover, the tumor volume at sacrifice and tumor growth, according to bioluminescence, was reduced. With the lower daily PKE dose (32 mg/kg bw), there were no significant changes observed in tumor growth, volume, vascularization, or proliferation index. The apoptosis index of tumors was also measured in TUNEL-stained tumor sections, but there were no increase in the number of apoptotic cells in either of the PKE treatment groups. No mice were lost during the three weeks administration.

It may be difficult to demonstrate the effects of dietary interventions due to the large variation in tumor growth in xenograft studies (Laajala et al., 2012). We processed the results of the xenograft study with the combined multivariate analysis by defining the effect of treatments using tumor volume, tumor bioluminescence, apoptosis index, proliferation index, and blood vessel density and length as covariates. This analysis of the combined effect of these six covariates revealed a significant difference between the vehicle and higher PKE dose groups (P=0,005). The lower PKE dose group did not differ from control mice. The most prominent effects were detected in a combination of tumor volume, proliferation index, and vessel length. However, no significant difference was found between the lower PKE dose and the vehicle group.

5.2.4 Metabolism of PKE in mice (II & III)

The daily ingestion of PKE was well tolerated by mice and we did not observe any adverse effects. There was no significant difference in body weight; total fat content or food consumption between the HFD and HFD-PKE fed mice. The dosage of PKE to mice was relevant to the polyphenol intake of humans and based on the assumption that a lower PKE dose (32 mg/kg bw) is equivalent to the mean consumption of polyphenols in the common diets (around one gram, see Table 2, p. xx). The higher PKE dose was equivalent to the daily consumption of 5 grams of polyphenols by humans, which is well tolerated and possible to receive from a polyphenol rich diet (Boocock et al., 2007; Manach et al., 2004). Similarly, PKE mixed with HFD (1600 mg of PKE in 1 kg of HFD) was equivalent to the daily consumption of 5 grams of polyphenols. No adverse effects or reduced food consumption were observed in mice fed with HFD-PKE.

Several pine-derived stilbenoids, lignans, and their metabolites were found in the serum of the PKE fed mice. Four different stilbenoids were detected in micromolar concentrations after the administration of PKE (160 mg/kg bw): pinosylvin monomethylether (mean serum total concentration 2-3 h after intake 9,7 μM), pinosylvin (2,2 μM), pinostilbene (1,2 μM) and resveratrol (5,8 μM). Interestingly, no detectable amount of resveratrol was found in PKE, and the amount of pinostilbene in PKE was very low in comparison with the amount found in serum. This suggests that dietary pinosylvins are metabolized to pinostilbene and resveratrol. This result supports an earlier study that suggested that pinosylvin is metabolized to resveratrol by the enzymatic activity in the liver (Roupe et al., 2005). Stilbenoids were detected in serum 15 minutes after oral administration and their concentrations peaked in two hours.

Increased concentrations of several lignans were also detected in serum after PKE treatment. Nortrachelogenin was the most abundant as its mean concentration in serum was 3,2 μM two hours after administration. In addition, a few derivatives of nortrachelogenin were observed. Other lignans found at 0,05-1,0 μM concentrations in serum include matairesinol, hydroxymatairesinol, conidendrin, pinoresinol, secoisolariciresinol, enterolignans, enterolactone, and enterodiol. Like stilbenoids, increased lignan concentrations were detected 15 minutes after administration and concentrations peaked in two hours. The lignan metabolite profile in urine (24 hour collection after a single administration with the higher PKE dose) was similar to that in serum, apart from some lignans found only in urine at low concentrations after PKE treatment, including lariciresinol, cyclolariciresinol, medioresinol, oxo-matairesinol, and 7-hydroxyenterolactone. In addition to the xenograft study, we found a similar polyphenol profile in the urine of hARO-Luc mice, fed with PKE premixed in a high fat diet. This suggests that lignans and stilbenoids in PKE are bioavailable in spite of the food matrix or administration method.

Our results suggest that the bioavailability of unconjugated polyphenols in PKE is good and these compounds are rapidly absorbed after intake. This result is consistent with the pharmacokinetic studies on free lignans and stilbenoids, suggesting fast and efficient absorption as compared with polyphenol glucosides which require an additional hydrolysis with β-glucosidase to be absorbed (Boocock et al., 2007; Udani et al., 2013). Thus dietary PKE is a novel source of bioactive polyphenols and their metabolites, the most prevalent metabolites being enterolactone and resveratrol. This study is the first where pinostilbene is documented as a metabolite of pinosylvins.

5.2.5 Anticarcinogenic potential of PKE and its compounds (II)

Plant lignans and stilbenoids and their metabolites have been proposed as preventing various cancers, or delaying their onset, in a variety of preclinical models of carcinogenesis. In addition to the preclinical studies, epidemiological studies also suggest that these compounds have a protective role against prostate cancer (Heald et al., 2007; Mccann et al., 2005b; Rybicki et al., 2011; Schoonen et al., 2005). We have demonstrated the anticarcinogenic effects of PKE *in vitro* and *in vivo* in an orthotopic prostate cancer xenograft model. According to our results, the lignan and stilbenoid components of PKE are the major contributors to the antiproliferative and proapoptotic properties of the extract.

The effects of the LS mixture, comprising of the main phenolic components of PKE in a molar ratio similar to extract, were almost identical to those of PKE, indicating that these four phenolics are, indeed, the main anticancer components of PKE. The same compounds also inhibited PC-3M-luc2 cell proliferation and induced apoptosis *in vitro*, when added alone. Individual PKE-derived phenolics showed significant effects on the proliferation and apoptosis at 10-40 μM, which are in the same range as has been reported before in *in vitro* studies. The total concentration range of PKE-derived phenolics in the serum of mice receiving the higher dose of PKE varied from 7 μM to 73 μM, indicating that the active concentrations *in vitro* and *in vivo* are of the same magnitude. Furthermore, PKE and PKE-derived phenolics chemosensitized the PC-3M-luc2 cells to TRAIL-mediated apoptosis. Resistance to TRAIL is common in cancers and considered a major obstacle for TRAIL-based clinical applications. It has been previously proposed that resveratrol and matairesinol sensitize prostate cancer cells to TRAIL- induced apoptosis (Fulda and Debatin, 2004; Peuhu et al., 2013). The present study is the first to show that TRAIL activity is enhanced with pinosylvins. These results also suggest that extract containing several phenolic compounds show improved efficiency when compared with chemosensitization with single compounds.

In addition to PC-3M-luc2 cells, PKE was shown to have a similar effect on the proliferation rate of different cancer cells. This indicates the anticancer properties of PKE are not specific to prostate cancer, but instead PKE may target mechanisms that are common in different types of cancer. These targets might include increased oxidative stress and increased inflammation in the tumor microenvironment, as well as other malignant pathways that support the growth of tumors. Both the present results and previously published data support the role of stilbenoids and lignans as effective antioxidants and anti-inflammatory compounds (Corsini et al., 2010; Park et al., 2012; Son et al., 2010; Willför et al., 2003e). Several scientific publications suggest that polyphenols have been shown to suppress tumor angiogenesis in (Cao et al., 2002; Garvin et al., 2006; Lee et al., 2012; Lindahl et al., 2011; Saarinen et al., 2008). Therefore, the observation that dietary PKE inhibited the neovascularization in tumors, can be associated with the phenolic components of the extract.

5.3 Effects of PKE on diet induced obesity, and human aromatase gene expression (III)

The aim of the present study was to investigate the impact of high fat diet (HFD) induced weight gain and white adipose tissue (WAT) inflammation on

the regulation of the human aromatase (*CYP19A1*) gene in male WAT. The aromatase enzyme converts androgens into estrogens and the upregulation of aromatase may be involved in e.g. obesity-related hypogonadism in obese men and development of estrogen-responsive breast cancer in postmenopausal women. The regulation of aromatase gene has been studied mostly in women and very little is known about the expression of the aromatase in male WAT. We used a transgenic hARO-Luc reporter mouse as a model (Strauss et al., 2013). hARO-Luc reporter mice express the full-length regulatory region of the human aromatase gene attached to a luciferase reporter gene (Strauss *et al.* 2013). The tissue distribution of Luc reporter in hARO-Luc mice closely resembles that described for *CYP19A1* gene in human tissues.

In this study, HFD increased body weight and adiposity, induced metabolic dysregulation, and WAT inflammation. The first direct evidences were obtained demonstrating that HFD-induced weight gain and inflammation upregulate the expression of a human aromatase gene in male WAT. Second, the effect of diet-derived antioxidative polyphenols on weight gain, obesity-induced inflammation, and regulation of a human aromatase gene were evaluated. Here it is proposed that a polyphenol-rich diet alleviates inflammation and modulates aromatase expression in male WAT.

5.3.1 PKE modulates metabolic parameters but does not inhibit HFD-induced weight gain (III)

The effect of an 8-week high-fat diet was investigated on the body weight and adiposity of hARO-Luc male mice. HFD-fed males surpassed the control mice as regards weight gain in one week from the beginning of the experiment, and they gained weight steadily throughout the study. As expected (Frederich et al., 1995), both the average body weight and adiposity were higher in the HFD group. Body weight and adiposity also had a linear correlation in all diet groups. Along with increased adiposity and body weight, the concentrations of insulin and leptin in serum and fasting glucose in blood were also increased in the HFD group. In clinical studies, increased serum glucose and leptin concentrations and insulin resistance are both strongly associated with obesity and metabolic syndrome (World Health Organization, 1999). Therefore, HFD-fed hARO-Luc mice are a human-relevant model to study obesity-induced metabolic dysregulations.

Mice received pine knot extract premixed in the HFD (HFD-PKE), and the daily dietary PKE intake (approximately 160 mg/kg bw) corresponded to the intake of five grams polyphenols by a 70 kg human (see also chapter 5.2.4). Dietary PKE did not significantly affect weight gain or fat content, and the average weight of mice after eight weeks dietary intervention was 29,5 , 37,5

and 35,5 g in LFD, HFD and HFD-PKE groups, respectively. However, dietary PKE decreased the serum leptin, insulin, and fasting blood glucose levels, which are among the hallmarks of metabolic disorders in humans (Barness et al., 2007).

5.3.2 PKE in diet alleviates adipose tissue inflammation in male mice (III)

Obesity induces chronic low-grade inflammation in adipose tissue (Sell et al., 2012). Low-grade inflammation in WAT is characterized by the increased production of chemokines and cytokines and infiltration of proinflammatory immune cells, forming crown-like structures (CLS) around adipocytes (Sell et al., 2012). The macrophage infiltration into WAT was quantified according to the density of CLS (Cinti et al., 2005). The results show that hARO-Luc mice on a HFD had significantly more CLS in WAT compared with control mice. In addition, CLS densities were much higher in gonadal fat tissue (GF) than in subcutaneous fat (SCF) suggesting that GF was more inflamed. It was also suggested in previous studies that visceral fat displays a more intense macrophage infiltration in both mice and men (Harman-Boehm et al., 2007; Murano et al., 2008). Along with adiposity and CLS density, obese mice had increased circulating cytokine concentrations. Independent of individual variations in cytokine levels, we showed that there was a significant correlation between the concentrations of pro-inflammatory cytokines IL-6, MCP-1 and TNFα, and adiposity. Cytokine concentration in serum also correlated with CLS density in GF. However, similar correlations were not found in SCF, possibly due to the lower CLS density in SCF in all diet groups.

The results suggest that dietary PKE alleviates the obesity-induced inflammation in WAT. The CLS densities in both GF and SCF were significantly lower in HFD-PKE –fed mice, compared with HFD group. However, the size of white adipocytes was similar in HFD and HFD-PKE-fed mice. Therefore, the adipocyte densities in samples do not bias the differences in the CLS density between HFD- and HFD-PKE groups. Previously, it has been suggested that resveratrol can inhibit macrophage infiltration and suppress inflammatory responses in adipocytes (Jeon et al., 2012; Zhu et al., 2008). The present results indicate that PKE, alleviating obesity-induced inflammation in WAT, also suppresses the development of inflammation-related pathologies in WAT.

5.3.3 Effects of obesity and PKE on the expression of the human aromatase reporter gene in adipose tissues (III)

The effects of HFD-induced adiposity and supplemental PKE were measured on the regulation of human aromatase gene expression in adipose tissue,

indicated by luciferase activity in hARO-Luc reporter mice. The results show that hARO-Luc activity in both GF and SCF was increased in parallel with the body weight of HFD-fed mice. The hARO-Luc activity in adipose tissue also correlated positively with the pro-inflammatory cytokine concentrations, secreted from adipose tissue. In addition, these results show that diet, supplemeted with PKE, downregulated the obesity-induced expression of human aromatase reporter gene in the SCF of HFD-fed mice.

Glucocorticoids have been suggested to activate aromatase expression involving the aromatase promoter I.4 (Chen et al., 2009a). Cortisol was suggested to increase aromatase activity in male omental preadipocytes and decrease in subcutaneous preadipocytes (McTernan et al., 2000, 2002). To study the effect of inflammation and glucocorticoids on the modulation of aromatase expression in WAT, adipose tissue explants *ex vivo* were treated with potent glucocorticoid (dexamethasone, DEX) and LPS. LPS is known to induce the activation of inflammatory cascade in cells, including the increased production of several cytokines and chemokines (Bonizzi and Karin, 2004). Therefore it can be used to induce an inflammatory response *ex vivo*. We observed that reporter activity in WAT was 5-8 fold in the presence of DEX in both SCF and GF-derived fat tissues. However, DEX concentration 250 nM was needed in SCF to activate hARO-Luc, compared only 10 nM in GF. A reason could be differences in glucocorticoid receptor expression (Joyner *et al*. 2000) or in tissue composition (Murano et al., 2008) between different adipose tissues. In addition to DEX, LPS treatment increased the luciferase activity in these conditions *ex vivo* 10-15 fold compared to the basal level. This suggests that also an inflammatory response upregulates aromatase expression in male WAT. In general, these results *in vivo* and *ex vivo* suggest that both obesity-induced inflammation and glucocorticoids stimulate the expression of a human aromatase gene in adipose tissue.

5.3.4 Stilbenoids and lignans downregulate the expression of a human aromatase gene in mesenchymal stromal cells (III)

We investigated the effect of PKE and PKE-derived polyphenols on the luciferase activity in mesenchymal stromal cells (MSC) from the bone marrow of HFD-fed hARO-Luc mice (Prockop, 1997). In humans, aromatase in WAT is mainly expressed in MSC (Price et al., 1992). Therefore, MSC from hARO-Luc mice are a relevant model to study the human aromatase gene expression in adipose tissue. Combination of DEX and TNFα stimulated hARO-Luc activity *in vitro*, possibly by increasing the expression of the aromatase promoter I.4 as indicated by earlier findings on the association of glucocorticoids and TNFα with the activation of promoter I.4 (Chen et al., 2009a; Samarajeewa et al.,

2011; Zhao et al., 1996). PKE treatment also modulated DEX-induced luciferase activity *in vitro*. Interestingly, a PKE concentration of 10 μg/ml had a further stimulatory effect on DEX+TNFα induced luciferase activity, while PKE 40 μg/ml inhibited the induction. However, PKE did not affect the basal luciferase activity of MSC. In addition, the effect of PKE-derived compounds on hARO-Luc activity was determined, and according to the results, nortrachelogenin at 10 μM concentration inhibits DEX+TNFα-induced luciferase activity. Pinosylvin at 10 μM had a similar, stimulatory effect as PKE 10 μg/ml, but the LS mixture, the combination of PKE-polyphenols in the same ratio as present in PKE, downregulated the DEX+TNFα induced hARO-Luc expression.

Previously, forskolin and phorbol 12-myristate 13-acetate (PMA) were shown to increase promoter I.3/II activity in human adipose stromal cells (Chen et al., 2009a; Kovacic et al., 2004; Samarajeewa et al., 2011). According to the present results, forskolin and PMA in combination increased luciferase activity in male MSC by two-fold. In this study 1-40 μg/ml PKE reduced the PMA+forskolin-induced activation of a human aromatase gene, 10 μg/ml being the most efficient PKE concentration. The impact of PKE-derived compounds and their metabolites was studied using the same assay. Nortrachelogenin and enterolactone were the most potent inhibitors of aromatase expression at 10 μM and they reduced PMA+forskolin-induced reporter activity to a basal level. Pinosylvin and LS mixture also reduced PMA+forskolin-induced hARO-Luc expression, but two other stilbenoids, resveratrol and pinosylvin monomethylether did not.

5.3.5 HFD-induced weight gain, inflammation and stilbenoids and lignans as modulators of aromatase gene expression

The present data, obtained from a transgenic humanized mouse model, is the first direct evidence that HFD-induced obesity and inflammation upregulate the expression of a human aromatase gene in male WAT. The results from mechanistic *ex vivo* and *in vitro* studies with MSC and fat explants suggest that the expression of the aromatase gene in male WAT is upregulated by glucocorticoids and obesity/inflammation-associated factors, such as LPS and TNFα. Significantly, it was demonstrated that dietary PKE downregulates the expression of a human aromatase reporter gene in the subcutaneous fat of HFD fed male mice. The *in vitro* results suggest that dietary PKE-derived lignans and pinosylvin contribute to the downregulation of aromatase gene in WAT.

It was found that PKE, mixed in diet, alleviated inflammation in SCF and GF. However, no reduction in luciferase activity was observed in GF. Therefore, it

is possible that the moderate anti-inflammatory effect of dietary PKE was not sufficient to inhibit obesity-induced aromatase expression in GF, which was more inflamed compared to SCF. The higher CLS density in GF is also described in previos study (Murano et al., 2008), but there is no previous literature comparing the effects of polyphenols on aromatase expression between various fat depots.

Our findings are in line with earlier clinical data on the correlation between BMI and serum E_2/T ratio in men, and support the idea that the increased aromatization of androgens in WAT, via suppression of the CNS-gonadal axis, contribute to hypoandrogenism in obese men (Dobs et al., 2001; Mogri et al., 2013; Wu et al., 2008; Yeap et al., 2014). Moreover, the present results demonstrate that anti-inflammatory and antioxidative pine polyphenols modulate hARO-Luc expression in male WAT, and thus phenolic compounds may inhibit obesity-induced aromatization in WAT. It has been suggested that resveratrol inhibit aromatase expression in various tissues (Chottanapund et al., 2014; Subbaramaiah et al., 2013; Wang and Leung, 2007; Wang et al., 2006, 2008b). Interestingly, the human-relevant mouse model used here shows that in comparison with resveratrol, other plant polyphenols, namely pinosylvin and nortrachelogenin, are more efficient inhibitors of aromatase expression in WAT. Previpusly, it has been suggested certain polyphenols possess anti-inflammatory properties (Chapters 2.3.3.2 and 2.3.5.4). There is still no previous data showing dietary lignans to downregulate aromatase gene and alleviate WAT inflammation. However, a recent study suggests that healthy diet, including also polyphenol-rich food, downregulated the inflammatory gene expression, independent of body weight changes, in adipose tissue of obese subjects (Kolehmainen et al., 2015). Taken together, these results suggest that dietary interventions might alleviate the adipose tissue inflammation and possibly also reduce the risk of developing other obesity-related diseases.

In summary, the present data suggests that dietary PKE inhibits aromatase expression and might decrease excessive aromatization in WAT of obese males. Therefore, other lignan and stilbenoid rich foods might also reduce E_2 production in obese men, and thus possibly attenuate obesity-associated androgen deficiency. Our results may warrant future studies focusing on the impact of stilbenoids and lignans on aromatase expression in the adipose tissue of women, and thus their contribution to breast cancer prevention. Also the alleviation of WAT inflammation with diet, supplemented with polyphenols might have additional health-promoting effects.

6. CONCLUSIONS

Diet, supplemented with health-promoting compounds may alleviate inflammation, improve obesity-associated dysregulation, promote the growth of beneficial bacteria, and reduce oxidative stress and cancer risk. Therefore such a diets might play a significant role in the prevention of various common diseases. Pine and spruce are excellent sources of bioactive compounds: especially polyphenols and hemicellulose-derived molecules that are also in bioavailable form in large quantities. Thus in the future, if the health-promoting efficiency of compounds, found in coniferous, will be shown also in clinical studies, some of these substances could be utilized as a part of functional food production and healthy diets.

More precisely, the conclusions of this thesis are that:

1. Softwood-derived hemicellulose exhibits prebiotic properties. It stimulated the growth of various bifidobacteria including the commercially most utilized probiotics *Bifidobacterium animalis* subsp. *lactis* Bb12 and *Lactobacillus rhamnosus* GG. This suggests that spruce hemicellulose or its components could be used for synbiotic formulations together with bifidobacteria and lactobacilli. Interesting targets for dietary softwood hemicelluloses to be addressed in future studies include modulation of microbiota *in vivo*, obesity-related disorders, and butyrate production.

2. Pine knot extract is a rich source of both stilbenoids and lignans. The components of pine knot extract and their metabolites include pinosylvin and its methyl ether, resveratrol, pinostilbene, matairesinol, nortrachelogenin and enterolactone. These polyphenols have a good bioavailability: mixed in diets they were rapidly absorbed, reached active concentrations *in vivo*, and were well tolerated. As a next step, a safety assessment of the pine knot extract and its components, especially pinosylvins and nortrachelogenin that are not found in common diets, are needed.

3. Pine knot extract and pine-derived stilbenoids and lignans reduced the proliferation rate of various cancer cells. Pinosylvins in particular inhibit the growth of prostate, bone, liver, and breast cancer cells. Orally administered pine knot extract inhibited the cell proliferation in orthotopic prostate cancer xenografts and reduced the tumor growth. This suggests that dietary pine-derived compounds reach the prostate in effective concentrations. Due to promising *in vitro* results, the ability of

pine polyphenols to inhibit the growth of other cancers *in vivo* should be assessed. In the future, the characterization of molecular targets of pine-derived polyphenols in cancer cells is required. Moreover, it should be further studied whether pine polyphenols can be utilized as new functional food components - the consumption of which might reduce the risk of cancer.

4. Several wood lignans and stilbenoids sensitized prostate cancer cells to TRAIL activated apoptosis *in vitro*. The effect of TRAIL-mediated cell death combined with the administration of pine-derived polyphenols needs to be investigated in appropriate animal models. Co-treatments based on TRAIL receptor agonist and polyphenols could be developed into effective cancer therapy in the future.

5. The expression of an aromatase gene in male adipose tissue was upregulated by glucocorticoids and obesity-induced inflammation. Dietary pine knot extract alleviated adipose tissue inflammation and downregulated the obesity-induced aromatase gene expression in male fat tissue. Of the individual compounds, nortrachelogenin, enterolactone, and pinosylvin contributed to the downregulation of the aromatase gene. Wood-derived polyphenols could be therefore used to target excessive aromatization and adipose tissue inflammation in humans. In addition, the role of a polyphenol rich diet in the alleviation of obesity-induced inflammation in WAT and in the E_2/T ratio of obese subjects should be studied further.

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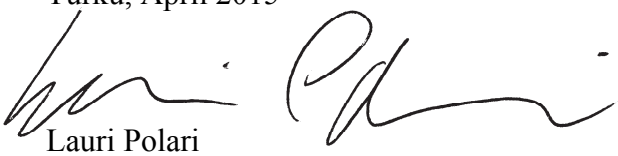
Turku, April 2015



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