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# Berries in Prevention of Metabolic Disease

## – focus on obesity, diabetes and gut microbiota

Lovisa Heyman-Lindén


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DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
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Berries in prevention of metabolic disease – focus on obesity, diabetes and gut microbiota


Abstract

The increasing prevalence of obesity is a worldwide health problem closely linked to diet and lifestyle factors. Obesity is associated with increased risk of several metabolic disorders including insulin resistance, nonalcoholic fatty liver disease and type 2 diabetes. Hence, there is a great need to identify dietary strategies for the prevention of obesity and related diseases. This thesis investigates the potential of different berries to mediate beneficial health effects in a mouse model of diet-induced obesity and prediabetes.

We found that supplementation with lingonberries, blackcurrants and bilberries reduced body weight gain, insulin resistance, low-grade inflammation and hepatic lipid accumulation in C57BL/6J mice fed a high-fat diet. Supplementation with raspberries, crowberries, blackberries or prunes had no or small effects, whereas açai berries promoted development of obesity and fatty liver compared to the control group receiving high-fat diet without berries.

Global hepatic gene expression analysis revealed that the phenotype in the lingonberry and bilberry groups was coupled to an anti-inflammatory effect, including downregulation of acute-phase proteins and inflammatory mediators. Mice receiving açai displayed an upregulation of steatosis markers and genes related to lipid synthesis, in line with the exacerbation of high-fat-induced fatty liver in these mice. The HELP-tagging assay was used to identify differentially methylated CpG sites in the lingonberry group compared to the high-fat control group. Lingonberries induced genome-wide and specific alterations of DNA methylation, however the significance of these findings remains to be established.

Furthermore, different batches of lingonberries were found to have different capacity to prevent obesity. However lingonberries prevented low-grade inflammation, metabolic endotoxemia and modified the gut microbiota of high-fat fed mice, including increasing the *Firmicutes/Bacteroidetes* ratio. These findings were independent of effects on body weight gain and achieved regardless of the source of berries.

The capacity of lingonberries to counteract negative outcomes of an unhealthy diet should be further evaluated in humans, including assessment of anti-inflammation and microbiota modulation. The generated knowledge about berries and their effects on metabolism may be useful in designing future dietary strategies aimed at preventing metabolic disease.



Key words: obesity, type 2 diabetes, berries, low-grade inflammation, gut microbiota, hepatic steatosis, gene expression, DNA methylation, lingonberry, blackcurrant, bilberry, açai

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# Berries in Prevention of Metabolic Disease

## – focus on obesity, diabetes and gut microbiota

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*To Olof, Bob and Frans*

*"Let your food be your medicine, and your medicine be your food"*

Hippocrates

*“The more I learn, the more I realize how much I don’t know”*

Albert Einstein

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*Under review in Food & Nutrition Research.*

* Current surname is Heyman-Lindén.

# Abbreviations

| | |
|---|---|
| AMP | Adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| BMI | Body mass index |
| CpG | Cytosine-phosphate-guanine |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| HC | High-cholesterol |
| HDL | High-density lipoprotein |
| HF | High-fat |
| IKK-β | IκB kinase catalytic subunit-β |
| IL-6 | Interleukin 6 |
| LDL | Low-density lipoprotein |
| LF | Low-fat |
| LBP | Lipopolysaccharide-binding protein |
| LPS | Lipopolysaccharide |
| MHO | Metabolically healthy obese |
| mTOR | Mammalian target of rapamycin |
| NAFLD | Nonalcoholic fatty liver disease |
| NFκB | Nuclear factor kappa B |
| ORAC | Oxygen radical absorbance capacity |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PPARγ | Peroxisome proliferator-activated receptor γ |
| PCR | Polymerase chain reaction |
| SAA | Serum amyloid A |
| STAT3 | Signal transducer and activator of transcription 3 |
| STZ | Streptozotocin |
| T2D | Type 2 diabetes |
| TLR4 | Toll-like receptor 4 |
| TNF-α | Tumor necrosis factor-α |

# 1. Introduction

## 1.1 A general introduction: obesity, prevention and diet

There are now over 1.9 billion adult humans in the world who are overweight and over 600 million of these are obese[1]. This epidemic affects all parts of the world and is accompanied by an increase in obesity-related disorders including type 2 diabetes (T2D), cardiovascular disease (CVD), nonalcoholic fatty liver disease (NAFLD) and cancers.

In our society, there is no doubt that the interplay between genetics and environmental factors strongly favors excessive body weight gain. As obesity has more than doubled during the past decades[1] (exemplified by obesity rates in the US in **Figure 1**), it seems clear that the root of the problem lies in a changed environment rather than in our genes. It also seems clear that reduced energy intake and increased physical activity is an effective strategy to maintain a healthy body weight. However, implementing the lifelong dietary changes needed for weight management has proven to be challenging. Therefore, there is a need to identify new food concepts that may contribute to dietary strategies useful in preventing obesity and metabolic disease.

Dietary patterns rich in fruits and vegetables are generally associated with decreased risk for developing chronic diseases[2-4], and the positive effects have sometimes been attributed to micronutrients, fiber and various polyphenolic compounds[5]. Berries contain high amounts of polyphenols and other components that show potentially interesting activities in terms of health benefits and diabetes prevention[5-9]. The aim of this thesis has been to investigate if weight gain and related effects of an unhealthy diet may be prevented by intake of berries, and if some berries are more effective than others. Furthermore, there is a need to increase our understanding of the complex interactions between the food we eat and how it affects us. In addition to providing calories, the diverse components of the human diet are likely capable of both fueling and counteracting different aspects of metabolic disease. Thus, the projects included in this thesis have also aimed at investigating how different mechanisms and tissues are affected by berry intake. This has been facilitated by the use of a mouse model to study the metabolic effects of berries.

## 1.2 Obesity and related metabolic disorders

Overweight is characterized by excessive body fat accumulation leading to an increased body weight. Body mass index (BMI, $kg/m^2$) is commonly used to quantify overweight (BMI ≥ 25) and obesity (BMI ≥ 30) of individuals and populations. According to this definition, estimates show that 39% of the world's adult population is overweight, and 13% are obese[1]. These figures are alarming, as obesity associates with a decreased life expectancy by one to eight years, with the effect on the number of healthy life-years being even greater[10]. Obesity often occurs together with hypertension, dyslipidemia, insulin resistance and hyperglycemia, which are characteristic components of the so called metabolic syndrome, and associate with several-fold increased risk of developing CVD and T2D[11]. However, excessive body fat accumulation does not necessarily lead to obesity-related cardiovascular and metabolic complications, and around 10 to 30% of obese individuals belong to this subgroup of 'metabolically healthy obese' (MHO). The MHO phenotype is characterized by low visceral and ectopic fat accumulation (including low liver and skeletal muscle fat storage) compared with obese individuals at higher metabolic risk.[12]

The mechanisms linking obesity to disease development are not fully elucidated, but the whole body is affected and several organs are likely important contributors. A dysfunctional adipose tissue, ectopic fat accumulation, insulin resistance and inflammation in liver, muscle and other tissues contribute to the pathogenesis, however the sequence of events is uncertain. This thesis aims to assess several metabolic changes in response to diet-induced obesity, and the following sections will introduce obesity-related disorders of special interest to this work.

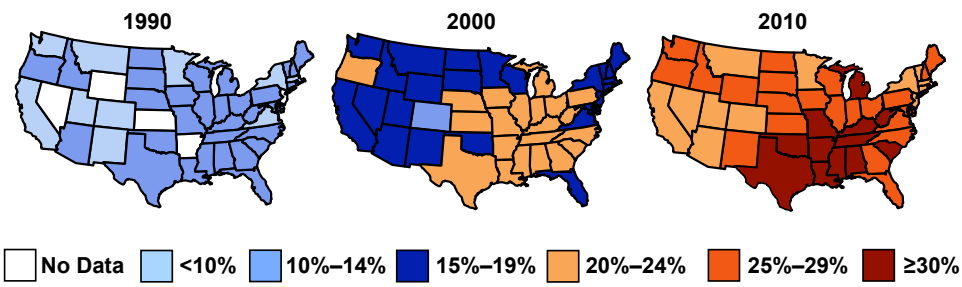



**Figure 1. Illustration of the increasing prevalence of obesity.** The maps show the percentage of obese adults (BMI ≥ 30) in the US from 1990 to 2010. Source: 'Obesity Trends Among U.S. Adults Between 1985 and 2010' by Behavioral Risk Factor Surveillance System, Centers for Disease Control and Prevention [Public domain] http://www.cdc.gov/obesity/data/prevalence-maps.html.

### *1.2.1 Low-grade inflammation*

The obese state is characterized by increased levels of circulating cytokines and acute-phase proteins, such as tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor-1 (PAI-1), C-reactive protein (CRP) and serum-amyloid A (SAA). In contrast, weight loss and healthy eating patterns are associated with lower concentrations of inflammatory markers.[13-15] The upregulation of inflammatory mediators in the obese state is modest, often less than twofold, whereas during infection it is tenfold or more[16]. This life-style-induced chronic inflammation is referred to as 'low-grade inflammation' and seems to play an important role in the development of metabolic disorders. As an example, the MHO subgroup of obese subjects at low risk of developing CVD and T2D, are characterized by having a more favorable inflammatory status[17] and adipose tissue inflammation is the strongest predictor of insulin-sensitive MHO[12].

The first mechanistic link between obesity and low-grade inflammation was the finding that white adipose tissue synthesizes and releases TNF-α[18], and it was later shown that the obese adipose tissue is infiltrated by macrophages which are an important source of the inflammation in this tissue[19 20]. While a majority of evidence has focused on the adipose tissue, the origin of obesity-related inflammation is a complex network of signals probably involving interconnections of several organs, which will be discussed in later sections.

### *1.2.2 Insulin resistance and type 2 diabetes*

Obesity is strongly correlated with development of insulin resistance, a condition in which cells fail to adequately respond to the hormone insulin. In the normal state, glucose homeostasis is precisely controlled by target tissues such as fat, liver and muscle adjusting metabolic flux to maintain plasma glucose levels within an appropriate range. In insulin resistance, the pancreatic β-cells have to secrete more insulin to achieve the same glucose lowering effect. If or when the β-cells no longer are able to produce the required amounts of insulin, blood glucose levels rise and overt type 2 diabetes has developed[21]. The strong link between obesity, insulin resistance and T2D is reflected by the estimation that 90% of subjects diagnosed with T2D are overweight or obese[22]. It should however be noted that a majority of obese individuals do not suffer from T2D. The defective insulin action and secretion leads to hyperglycemia, due to impaired glucose uptake and uncontrolled hepatic glucose release, and also dyslipidemia with perturbations in the homeostasis of fatty acids, triacylglycerol and lipoproteins.

The mechanisms linking overnutrition and obesity to insulin resistance are not fully understood, however several aspects leading to impaired insulin signaling have been explored, including elevated circulating non-esterified fatty acids, ectopic fat accumulation, intracellular lipid metabolites and activation of intracellular stress response pathways. On the molecular level, insulin

desensitizing events may result in increased serine phosphorylation of insulin receptor substrate (IRS), which attenuates the insulin signaling cascade[23]. Interestingly, nutrient-sensing and pathogen-sensing systems are highly integrated, with metabolic and immune response pathways converging on similar targets[24]. The nuclear factor kappa B (NFκB) pathway is a major regulator of inflammation that activates a host of proinflammatory markers and mediators.[23 25] NFκB has been shown to be activated in obesity, and overexpression of the NFκB-activating kinase IκB kinase catalytic subunit-β (IKK-β) results in insulin resistance and diabetes[26]. Both fatty acids and bacterial endotoxin (LPS) can induce Toll-like receptor 4 (TLR4) signaling, and TLR4-mediated activation of NFκB has been proposed to be pivotal for cytokine expression and impairment of insulin sensitivity[27 28]. Further supporting the link between inflammation and insulin resistance are observations that treatment with proinflammatory cytokines can cause insulin resistance, whereas anti-inflammatory agents may reverse it[18 26 29].

### *1.2.3 Hepatic steatosis – the obese liver*

Hepatic steatosis refers to excessive lipid accumulation in the liver, and is the hallmark of nonalcoholic fatty liver disease (NAFLD). NAFLD is the most common liver disorder in the western world and affects 20% to 40% of the population, however among obese or diabetics the prevalence is estimated to 75%[30]. Hepatic steatosis occurs when lipid influx and *de novo* lipogenesis exceed lipid export or oxidation, thus resulting in lipid accumulation. The liver is a highly metabolically active tissue, and processes compounds in the 1.5 liters of blood reaching the liver every minute. Importantly, the liver is a critical organ for maintaining whole body homeostasis due to its regulatory role in glucose, triacylglycerol, cholesterol and bile acid metabolism. Hepatic steatosis is associated with alterations in these processes with adverse consequences on health. As an example, HF feeding induces NAFLD in rats after 3 days, which is accompanied by development of hepatic insulin resistance[31]. In humans, increased liver fat content has been shown to predict the risk of T2D independently of obesity[32]. The link between NAFLD and insulin resistance is strong, however it is not fully elucidated whether NAFLD causes or is a consequence of insulin resistance, or possibly both. Proposed mechanisms implicate that the accumulation of lipids eventually impairs insulin signaling due to actions of generated lipid intermediates[33]. There is also evidence suggesting involvement of an inflammatory component in the pathogenesis of lipid-induced hepatic insulin resistance[26]. Several acute-phase proteins and inflammatory mediators are synthesized in the liver, such as SAA, CRP, PAI-1 and interleukin 6 (IL-6), and diet and genetically induced obesity cause steatosis, insulin resistance and activation of hepatic NFκB. In addition, selective activation of hepatic NFκB causes liver inflammation without steatosis, and results in hepatic as well as systemic insulin resistance[26]. Currently, the most effective intervention for improving NAFLD is weight loss.

# 1.3 The gastrointestinal tract – a novel player in metabolic disease

The gastrointestinal tract represents the initial interface where ingested food and nutrients interact with the body **(Figure 2)**. The digestion and absorption of food mainly take place in the small intestine, whereas the large intestine is the main site harboring the trillions of microbes resident in the human gut. The gut has extensive nervous and endocrine systems and encompass 70-80% of the body's immune cells[34], and is subsequently involved in controlling motility, secretions, feeding behavior and protection against bacteria and toxins. In recent years, there has been a considerable increase in research investigating the role of the gut in development of obesity and associated metabolic disorders.

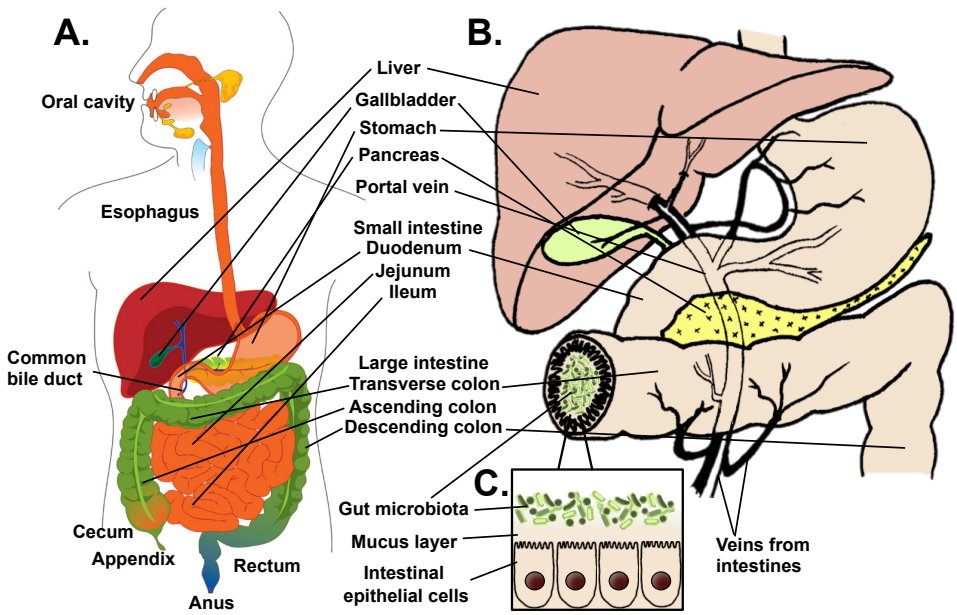



**Figure 2. The human gastrointestinal tract.** A) The illustration depicts the organization and localization of the organs in the gastrointestinal tract. B) Nutrients, metabolites and other compounds absorbed from the intestines are transported via the portal venous system, directing blood from the intestines to the liver via the hepatic portal vein. C) The adult gut contains tens of trillions of bacteria, the gut microbiota, with the highest concentrations of bacteria being found in the large intestine (colon). In the healthy gut, the intestinal epithelial cells constitute a barrier preventing translocation of bacteria and entry of potentially toxic compounds into the circulation. Source of illustration in the left panel: 'Digestive system' by Mariana Ruiz Villarreal [Public domain] via Wikimedia Commons.

### *1.3.1 Gut microbiota and obesity*

The amount of microbial cells living in the human gut outnumbers our own cells by 10 times[35], and the genomes of these approximately 1.5 kg of microbial residents contain 100 times more genes than the human genome. The relationships these microbes form with our bodies are diverse, e.g. the gut microbiota breaks down complex dietary polysaccharides, competes with pathogens and modulates the mucosal immune system. The gut microbiota of humans and mice are dominated by two major phyla, *Bacteroidetes* and *Firmicutes*[36], however the composition of the microbiota varies depending on several factors. With the introduction of high-throughput sequencing techniques, there has been a large increase in our understanding of how the bacterial community of the microbiota changes in response to obesity and dietary modifications.

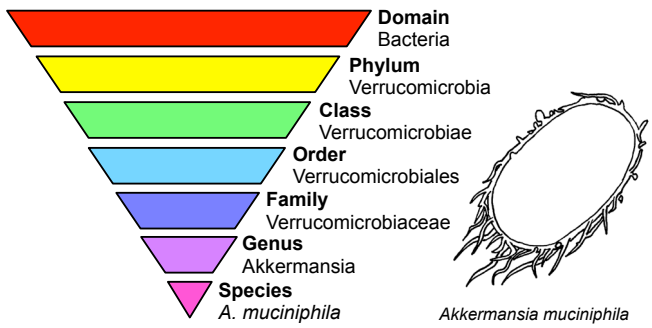



**Figure 3. Hierarchy of biological classification exemplified by the seven major taxonomic ranks of *Akkermansia muciniphila*.**

The earliest finding describing the gut microbiota as an environmental factor regulating fat storage showed that germ-free mice raised in absence of microorganisms are leaner than mice raised under normal conditions. Importantly, conventionalization of germ-free mice with a gut microbiota produced a 60% increase in body fat and development of insulin resistance within 14 weeks.[37] Additional work revealed a role for the microbiota in diet-induced obesity, as germ-free mice receiving HF or Western diets are protected against obesity[38 39], and it has become clear that there are differences in the composition of the microbial community in obese subjects compared to lean. However, the differences and shifts among hundreds of species are challenging to assess. The relative abundance of different bacteria at the phylum level **(Figure 3)** is commonly used to describe general shifts in microbiota composition in disease states or in response to diet modifications. Obesity in humans and mouse models has generally been associated with an increase of bacteria belonging to the *Firmicutes* phylum and a decrease of bacteria belonging to the *Bacteroidetes*

phylum, whereas the opposite is seen after weight loss[36 40-42]. Environmental exposures are important in determining microbiota composition[41], and especially diet as humanized gnotobiotic mice shift to an 'obesity-associated' microbiota composition less than a day after switching to a Western diet. Furthermore, the increased adiposity seen in Western diet-fed humanized mice is transferrable through microbiota transplantation[42]. There is also ongoing work to identify specific bacteria with beneficial impact on health. Two species evoking interest in terms of anti-obesity and antidiabetic effects are *Akkermansia muciniphila* (**Figure 3**) and *Faecalibacterium prausnitzii*[43-45].

### *1.3.2 Metabolic endotoxemia*

One hypothesis connecting changes in gut microbiota and metabolic disease is the concept of metabolic endotoxemia. The term was introduced by Cani et al. to describe the chronic increase in plasma concentrations of lipopolysaccharide (LPS) that is induced by HF feeding, and proposed to dysregulate inflammatory tone and trigger body weight gain and diabetes[46]. LPS, a large molecule formed by lipid and polysaccharide, is a component of the outer membrane of gram-negative bacteria and elicits a strong immune response promoting inflammation to protect the organism from bacterial infection, mainly via TLR4[47]. Similar to low-grade inflammation, the increased concentration of plasma LPS in metabolic endotoxemia is modest compared to the levels attained in infections, and is closely associated with glycemic control and obesity[48]. A hypothesis is that the gut microbiota is involved in controlling intestinal permeability **(Figure 2C)**, and that a leaky gut barrier enables translocation of LPS, with detrimental effects on inflammation and associated metabolic disorders[46 49]. LPS from the gut will reach the liver via the portal system **(Figure 2B)**, and the liver plays a major role in LPS clearance. Indeed, elevated LPS levels in response to HF feeding as well as LPS infusion induce increased expression of proinflammatory cytokines in the liver, and this precedes inflammatory responses in adipose tissue[46].

As the gastrointestinal tract is first exposed to the food we ingest, it has been hypothesized that intestinal perturbations and inflammation represent early events in development of obesity-associated low-grade inflammation and insulin resistance[50 51]. These thoughts are partially based on studies in mice revealing that microbiota and HF diet interact to promote intestinal inflammation, which precedes development of obesity and insulin resistance[50 52]. HF feeding itself may mediate increased metabolic endotoxemia due to co-transportation of LPS with dietary fat over the gut wall[53]. More research is needed to investigate the role of intestinal dysregulation as a trigger for metabolic disease, but nonetheless, the diet we eat may drive or prevent metabolic endotoxemia and low-grade inflammation. Thus, it is of interest to identify foods and food components which may limit intestinal inflammation induced by HF diet and dysbiosis.

## 1.4 Berries

The botanical definition of a berry is "a fleshy fruit produced from a single ovary"[54]. In common terms and in this thesis "berries" are used to describe small, edible, soft-fleshed and often colorful fruits. Berries have been consumed by humans for thousands of years[55] and have attracted increasing scientific attention. Berries contain high amounts of several dietary components regarded as valuable to human health, including fiber, vitamins and minerals. In addition, berry fruits are rich in phytochemicals (the largest group being the *polyphenols*), bioactive plant compounds that may provide health benefits beyond basic nutrition. The diversity of the tens of thousands of phytochemical compounds is complex and different berries have different polyphenol composition profiles (**Table 1**).[56] Due to their nutritional properties, content of putatively health-promoting compounds, palatability and being well-accepted food items throughout history, an increased consumption of berries may constitute an attractive strategy for improving diet and preventing metabolic disease.

### *1.4.1 Berries and their role in health and disease*

Berries have been used in traditional medicine to manage various diseases, and an increasing amount of predominantly *in vitro* studies supports the concept that many berries possess anti-inflammatory, antioxidative and antimicrobial properties. The limited clinical evidence indicates a potential role for berries in controlling the risk of developing allergy, cognitive dysfunction, metabolic syndrome and cardiovascular and inflammatory disease[57]. However, further *in vivo* research is warranted, and the mechanisms at play are not fully understood. The bulk of the scientific interest in berries has emerged from the "antioxidant hypothesis" – essentially proposing that intake of molecules with putative antioxidant properties may protect against oxidative stress and lower the risk of chronic disease[58]. Due to the high content of phytochemicals, many berries have high *in vitro* antioxidant capacity (i.e. oxygen radical absorbance capacity, ORAC) which has added to the notion of berry consumption as being beneficial to health. In recent years, the commonly studied berries (e.g. blueberries and strawberries) have been accompanied by more "exotic" berries (e.g. goji and açai berries), which often have very high ORAC values and are marketed as 'superfruits' or 'superberries' with various health claims. However, the antioxidant capacity of a compound may be changed during metabolism in the gastrointestinal tract, and the concentration of the compound or its metabolites reaching the plasma is often very low compared to endogenous antioxidants[58]. In fact, recent literature suggests that compounds having an antioxidant-structure may perform bioactivities independent of their own putative antioxidant capacitity[58], for example by binding receptors and up/downregulating pathways, interacting with enzymes or the gut microbiota.

Since it may be the quality and composition of different nutrients and compounds, rather than quantity and total antioxidative capacity, that are of relevance for health effects it is likely that certain berries have especially useful properties in preventing disease. The work in this thesis aims to compare the ability of different berries to prevent the metabolic disorders that arise in response to an unhealthy diet in conjunction with obesity, predisposing to development of T2D. Since the diet-health relationship is very complex, we investigated how berries affect several aspects and mechanisms, including microbiota composition and gene expression patterns related to hepatic steatosis. The list of nutritionally interesting berries is long, but this thesis will focus on a selection of berry fruits **(Table 1)** of which several are commonly consumed and studied, such as blackcurrants, raspberries, blackberries and prunes. A few less studied "Nordic" berries are also included, i.e. lingonberries, crowberries and bilberries (European blueberry). Finally, the South American açai berry was investigated as a representative of the so-called superberries.

### *1.4.2 Polyphenols – bioactive compounds in berries*

Berries contain several interesting and potentially bioactive components, including fiber, essential fatty acids in berry seed oils, organic acids and polyphenols. Berries are among the foods richest in polyphenols[59], which may be the major reason for the interest in berries in providing health benefits. The work presented in this thesis does not allow identification of causal relationships between specific bioactive compounds present in berries and health effects, but given the large amount of research focusing on berry compounds and extracts, the phenolic phytochemicals characteristic for the studied berries will be briefly introduced (**Table 1**).

Phenolic compounds are secondary plant metabolites consisting of one or more aromatic rings with variable degrees of hydroxylation, methoxylation and glycosylation. There are different classification systems, but based on the overall structure, phenolic compounds are classified into the groups phenolic acids, lignans, stilbenes and flavonoids, which may be further divided into subclasses as a function of minor structural differences[60]. There are several thousands of phenolic compounds and derivatives, and polymerization and glycosylation further adds to the complex diversity of these compounds, which are often referred to as polyphenols. Polyphenols are produced by all plants to defend against ultraviolet radiation and microorganisms, and polyphenols also provide color, astringency and bitterness. The health benefits of consuming fruit and vegetables are sometimes attributed to these bioactive compounds, and several studies have focused on the flavonoids[5 61 62], a very large and diverse group of polyphenols ubiquitous in edible plants including berries. Flavonoids are divided into the subclasses anthocyanidins, flavonols, flavanols, flavones, flavanones and isoflavones[60].

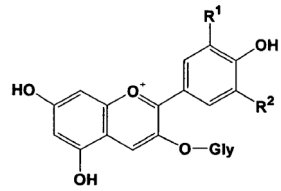
**Table 1**. Overview and composition of the berries investigated in this thesis.

| Name | Polyphenolic profile[a] | Other info[b] | Health effects *in vivo*[c] |
|---|---|---|---|
| **Lingonberry (Lingon)** ***Vaccinium vitis-idaea***  | **Proanthocyanidins, lignans, stilbenes (resveratrol),** flavonols (**quercetin),** anthocyanins (cyanidin), phenolic acids (**benzoic acid**) | Contains proanthocyanidins with A-type linkages and are rich in vitamin E. Grow in the Northern hemisphere. | Lingonberries modify postprandial glucose, insulin and fatty acid responses to sucrose[63] and glucose/berry sugars[64]. Lingonberries are studied in preventing urinary tract infection[65]. |
| **Blackcurrant (Svarta vinbär)** ***Ribes nigrum***  | Proanthocyanidins, **anthocyanins** (delphinidin, cyanidin), phenolic acids | Very high vitamin C content and rich in fiber. Cultivated throughout the world. | Intake of a blackcurrant juice improved vascular function in healthy subjects with low fruit intake[66]. Blackcurrants modifiy postprandial glucose, insulin and fatty acid levels in response to sucrose in healthy women[63]. |
| **Bilberry (Blåbär)** ***Vaccinium myrtillus***  | **Anthocyanins (delphinidin, cyanidin,** malvinidin), proanthocyanidins, phenolic acids (hydroxycinnamic and benzoic acids), flavonols (quercetin, myricetin), stilbenes (resveratrol) | Rich in vitamin E. Grow in the Northern hemisphere. The content of anthocyanins in bilberries (also called "European blueberries") is severalfold higher compared to American blueberries *V.corymbosum*. | Bilberries have anti-inflammatory effects[67 68] and slightly reduced body weight and waist circumference[69] in subjects with features of metabolic syndrome. A pilot study show improvement of ulcerative colitis in response to bilberry intake[70]. |
| **Raspberry (Hallon)** ***Rubus idaeus***  | **Ellagitannins,** anthocyanins (cyanidin) | High in folate. Contains the aromatic compound raspberry ketone. The cultivation of raspberries is widely distributed. | Intake of ellagitannin-rich berries (raspberries, strawberries and cloudberries) had little or no effect on clinical parameters in subjects with metabolic syndrome[71]. |
| **Blackberry (Björnbär)** ***Rubus fruticosus***  | **Ellagitannins, anthocyanins** (cyanidin)**,** lignans | High in fiber. Blackberries are grown globally. | Blackberry extracts are implied to decrease plasma glucose levels in diabetic rats.[72] |
| **Prune (Plommon)** ***Prunus domestica***  | **Phenolic acids (neochlorogenic acid, chlorogenic acid),** flavonols | Contains sorbitol. Widely distributed, most prunes originate from California or France. | Human studies imply laxative action and potential to reduce insulin secretion. Prevention of elevated LDL-cholesterol and bone loss in ovariectomized rats.[73] |
| **Crowberry (Kråkbär)** ***Empetrum nigrum***  | **Anthocyanins** (delphinidin, malvidin, cyanidin), proanthocyanidins, phenolic acids, flavonols | High in fiber and anthocyanins. Occurs widely in the Northern hemisphere. | Not previously studied *in vivo.* |
| **Açai (Kålpalm)** ***Euterpe oleracea***  | **Anthocyanins (cyanidin, peonidin,** pelargonidin**), phenolic acids (ferulic acid,** benzoic acid, gallic acid**),** flavanols (epicatechin) | High lipid content (oleic acid). The açai palm grows in Central and South America (Amazon region).The pulp of the fruit is commonly consumed as açai beverages. | A pilot study in healthy overweight subjects showed a reduction in plasma glucose and insulin after one month's supplementation with an açai product, compared to baseline[74]. One month of açai intake inreased BMI, truncal fat and inflammatory marker PAI-1 in women compared to baseline[75]. |

[a]Major phenolic compounds found in each berry. Phenols regarded as characteristic for the berry type are written in bold. Flavonoids typically occur as sugar conjugates *in planta* (e.g. anthocyanins = anthocyanidin glycosides).
[b]The macronutrient composition of berries is in general characterized by high water content (80-90%) and low energy content (30-80 kcal/100g) mostly derived from carbohydrates (4-11 g/100g) whereas the contribution from protein (<2 g/100g) and fat (<1g/100g, mostly polyunsatuated seed oils) is low. Berries are also good sources of vitamin C, E and folate. References: [72 73 76-88]
[c]The health effects are derived from human studies. If human data is lacking, the health effects refer to *in vivo* studies in the specified models.
Source of pictures: 'Blackcurrants' by Karen Jackson http://bit.do/blackcu and 'Crowberries' by Tere Tolvanen http://bit.do/crowb [CC BY-NC 2.0 (https://creativecommons.org/licenses/by-nd/2.0/)] via Flickr (cropped)
'Prune' by eLaboureur http://bit.do/prune [CC BY 2.0 (https://creativecommons.org/licenses/by/2.0/)] via Flickr (cropped)
'Açai' by P. S. Sena [CC BY-SA 4.0 (http://creativecommons.org/licenses/by-sa/4.0)] via Wikimedia Commons (cropped)

Berries are particularly rich in anthocyanins (glycosides of anthocyanidins, such as cyanidin, pelargonidin and delphinidin) which are responsible for their vibrant colors[8] **(Figure 4)**. Tannins are oligomeric flavonoids and are abundant in *Rubus* berries (hydrolysable ellagitannins) and *Vaccinium* berries (condensed proanthocyanidines) **(Table 1)**. The concentration of polyphenols in berries is influenced by many factors including environmental conditions, cultivar and degree of ripeness[60 89 90]. Although epidemiological studies implicate polyphenols as candidates for explaining protective effects on CVD, cancer and other outcomes[91], there are several factors that need to be taken into account before attributing the positive effect of fruit and vegetable consumption to specific compounds. Drawing conclusions from studies investigating health effects of administration of polyphenols is challenging due to many question marks regarding the influence of the food matrix, synergistic effects, dose and metabolizing enzymes of the host as well as the gut microbiota.[89 90]

During absorption, polyphenolic compounds present in plant food are generally hydrolyzed by intestinal enzymes followed by conjugation in intestinal and liver cells by methylation, sulfation or glucuronidation. Compounds may also undergo biotransformation by the gut microbiota into absorbable metabolites.[89] Even if the bioavailability of polyphenols has been considered low, it is evident that even though the plasma concentration of the parent compound is modest after ingestion of polyphenol-rich food, the metabolite concentration may be higher[92]. Furthermore, polyphenolic compounds and metabolites do not necessarily need to be absorbed to mediate effects, for example on the gut microbiota composition. A large proportion of polyphenols in berries reach the colon upon ingestion[93], and berry extracts as well as berry-derived phenolics can selectively inhibit pathogenic bacteria[94]. Hence, an increased focus on the gut is warranted in order to understand mechanisms of action of polyphenol-rich foods and berries.




| | $R^1$ | $R^2$ |
|---|---|---|
| Delphinidin | OH | OH |
| Cyanidin | OH | H |
| Malvidin | $OCH_3$ | $OCH_3$ |
| Pelargonidin | H | H |
| Peonidin | $OCH_3$ | H |
| Petunidin | OH | $OCH_3$ |

**Figure 4. Berries are a good source of polyphenols which provide color, bitterness and astringency to berries.** Anthocyanins, which are glycosides (Gly) of their respective anthocyanidin, are water soluble pigments (blue, red, purple, pink) that are highly abundant in berries. The right panel displays the common anthocyanin structure, which depending on the substitutions forms the most common anthocyanins i.e. delphinidin, cyanidin, malvidin, pelargonidin, peonidin and petunidin.

### *1.4.3 Berries and metabolic disease – potential targets affected by berry consumption*

Although the full picture is not understood, several mechanisms related to obesity and diabetes development are implicated to be modified by berries and berry-derived compounds[95]. In addition to the previously discussed aspects, protective effects may be mediated by interaction with various transcription factors and regulators. For example, bilberry extracts have been proposed to improve hyperglycemia and insulin sensitivity in diabetic mice by activation of AMP-activated protein kinase (AMPK).[96] In addition, berry extracts and polyphenols display inhibition of digestive enzymes and intestinal glucose transporters *in vitro*[97]. Blackcurrant and raspberry extracts inhibit α-glucosidase and pancreatic lipase activity, respectively, however the effect on starch and lipid digestion *in vivo* needs further evaluation.[97] Effects on these and other targets may aid in preventing weight gain, reducing postprandial glucose levels and improving insulin sensitivity, which could support utilization of berries in management of metabolic disease.

Further investigation of the effect of berry consumption on gene expression in different tissues is of value to specify the affected metabolic pathways. Moreover, in recent years the field of *epigenetics* (= over genetics) has emerged as a regulatory mechanism linking environment and genetics by suppression or activation of gene transcription. Epigenetics can be defined as reversible modifications of the DNA (i.e. not involving changes in nucleotide sequence) that may be transferred to the next generation.[98] Epigenetics, and notably DNA methylation, may constitute a factor being modified by environmental exposures, including nutritional status and diet, potentially predisposing to increased risk of

development of metabolic disease[98-100]. Interestingly, polyphenols have been shown to mediate effects on gene expression through alteration of DNA methylation and the epigenetic machinery[100 101]. The contribution of epigenetic mechanisms to development of metabolic disease remains to be defined, but represents one putative novel target affected by berry consumption.

# 2. Aims

The overall aim of this thesis was to identify berries with beneficial effects in relation to prevention of obesity, T2D and related metabolic disease, and to contribute to the understanding of how such effects are mediated.

Specific aims of the included papers were:

**I.** To screen different berries for their ability to prevent development of diet-induced obesity, insulin resistance, hepatic steatosis and related metabolic parameters.

**II.** To investigate pathways and mechanisms altered by consumption of berries capable of preventing (lingonberries, blackcurrants, bilberries) or promoting (açai) obesity and related metabolic parameters, with focus on hepatic steatosis and liver function.

**III.** To evaluate if the beneficial effects of lingonberries are associated with alterations of the gut microbiota, and if different batches of lingonberries mediate similar protective effects on adiposity and low-grade inflammation.

# 3. Methods and Strategies

This section provides an overview and discussion of the methods used. For further details please refer to the respective papers (*I-III*).

## 3.1 Animal model

The C57BL/6J mouse strain was used in all papers included in the thesis. This mouse strain is lean and insulin sensitive when fed a standard diet. However, when put on a HF diet, C57BL/6J mice develop obesity and glucose intolerance[102]. This mouse strain was chosen since it is a model for the human state of dietary intake leading to obesity and prediabetes. The HF fed C57BL/6J model was introduced by Surwit et al. in 1988[102], and is widely used in research related to diet-induced obesity and development of T2D. Animal models provide several advantages when performing nutritional studies. For example, it is possible to evaluate the metabolic effect of long-term intake of certain foods in a controlled manner, and it is easier to evaluate molecular mechanisms in internal tissues relevant for metabolic health. Moreover, the mouse and human genomes are similar as approximately 99% of mouse genes have a homologue in the human genome[103]. That being said, mice are not men. For example, rodents have a different plasma lipid profile, and the C57BL/6J model is not optimal for investigating HF-induced effects on dyslipidemia and cardiovascular effects[104]. All findings in animal models need to be confirmed in human subjects before health claims can be made about specific foods or food components.

**Figure 5. Lingonberries incorporated into feed pellets.** Mice were fed high-fat (HF) diets supplemented with 20% (w/w) of freeze-dried berries. The control group received HF diet without berry supplements.

## 3.2 Diets and experimental design

Mice were fed either a low-fat (LF) diet with 10% of energy coming from fat, or a high-fat (HF) diet (45 energy % from fat) to induce obesity and insulin resistance. To investigate which berries are most effective in preventing the detrimental effects of the HF diet (*Paper I*), experimental diets were designed to contain a 20% (w/w) supplement of berries, but otherwise consist of the same nutrient composition and energy content as the HF control diet. Freeze-dried berries were used to enable incorporation of the berries into feed pellets **(Figure 5)**. All freeze-dried berries were subjected to nutrient analyses and sent to Research Diets (New Brunswick, NJ, USA) for manufacturing. In *Paper III*, a second batch of lingonberries was used to manufacture a new lingonberry diet (”Lingon2”), which was compared to the HF control diet and the lingonberry diet (”Lingon1”) utilized in *Paper I*. All diet formulation was designed to give the berry diets and the control diets the same amount of calories coming from fat, protein, carbohydrate and the simple sugars glucose, sucrose and fructose. The aim of this compensation is to optimize the likelihood that observed metabolic effects are a due to intake of the food of interest, and not a consequence of differences in caloric or macronutrient intake. Body weights and food intake were monitored on a weekly basis, and body composition was measured at the end of each study using dual-energy X-ray absorptiometry (DEXA) **(Figure 6)**.

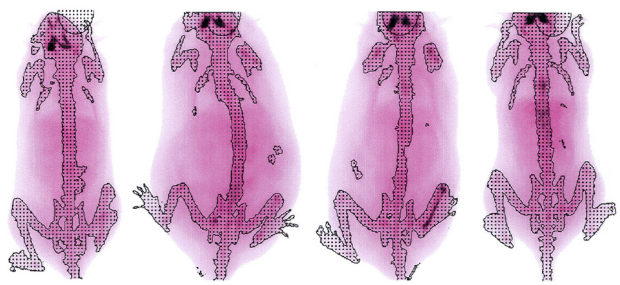


**Figure 6. Dual-energy X-ray absorptiometry (DEXA) is a noninvasive technique for measuring body composition in live mice.** Left to right: pictures depict mice from the groups receiving HF diet+lingonberries, HF diet+açai, HF diet and LF diet.

## 3.3 *In vivo* and *in vitro* analyses

Plasma samples, collected by orbital puncture from anesthetized mice, were subjected to analysis of various metabolic parameters including glucose, insulin, lipids and inflammatory markers. To investigate the effects of berry intake on different parts of the body, various tissues were collected for further analysis.

## 3.4 Gene and protein expression analysis

Global gene expression analysis is a valuable tool for generating hypotheses and was used in *Paper II* to investigate how the most interesting berries identified in *Paper I* mediate their effect on metabolism. Liver gene expression was analyzed using the Illumina Mouse WG-6 v2.0 platform. This microarray method is based on hybridization and enables profiling of over 45,000 transcripts (www.illumina.com). The generated data was further analyzed by online software and tools providing information about overall patterns and trends in the gene expression. For the study of specific genes of interest, quantitative real-time PCR (qPCR) was used. This approach was applied to validate the microarray data, and to study expression of genes in intestinal tissue in *Paper III*. Gene expression is a useful tool for mechanistic investigations, and global techniques provide large data sets suitable for hypothesis generation. However, the transcriptome provides a snapshot of information at a specific time under specific conditions, thus important variations in response to nutritional state and other factors are not accounted for. Moreover, the mRNA level of a gene does not necessarily predict the protein level. Protein expression was assessed by western blot analysis.

## 3.5 DNA methylation

Epigenetic mechanisms of transcriptional regulation are increasingly studied for their potential to link environmental cues, including diet, to disease pathogenesis. In *Paper II*, we sought to investigate if lingonberry intake alters DNA methylation, and whether this could be linked to changes in gene expression. In general, addition of methyl groups occurs on cytosine nucleotides that are followed by a guanine nucleotide (CpG sites). HpaII tiny fragment Enriched by Ligation PCR (HELP)-tagging[105] was used to identify differentially methylated CpG sites (differentially methylated regions, DMRs) in livers from mice receiving HF diet with or without lingonberry supplementation. HELP-tagging is a relatively inexpensive method that allows quantification of methylation status of individual CpG sites throughout the genome (>1.8 million loci) in both CpG dense and depleted regions. HELP-tagging is a restriction enzyme based method in which high-molecular weight DNA in the sample is subjected to HpaII cleavage. HpaII is methylation sensitive, and will not cleave its recognition site CCGG if the CpG site is methylated **(Figure 7)**. Its methylation-insensitive isoschizomer MspI cleaves independently of methylation status, and a reference library can thus be generated to compare methylation at each CpG site within the context of CCGG **(Figure 7)**. The obtained methylation scores at certain loci were validated using pyrosequencing and bisulphite MassArray.

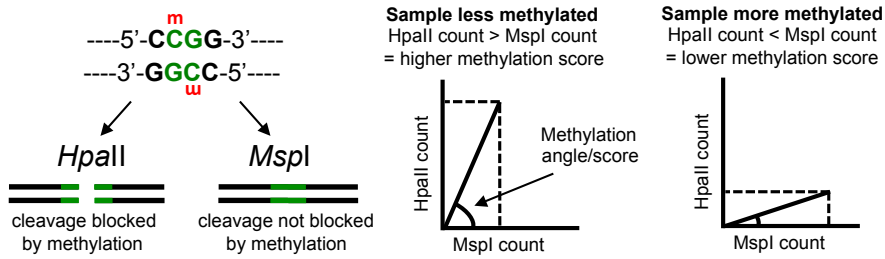



**Figure 7. Illustration of principles for HELP-tagging.** HELP-tagging is based on restriction enzyme digestion using HpaII (methylation sensitive) together with its isoschizomer MspI (methylation insensitive). These enzymes recognize and cleave the same DNA sequence (CCGG), however if the cytosine in the internal CpG-site (marked green) is methylated, HpaII will not cleave. Briefly, the DNA samples of interest (in this thesis from lingonberry and control mouse livers) are subjected to HpaII digestion, which generates HELP-tagging libraries which are sequenced. The HpaII count profile for each sample is compared to a MspI standard reference library. If the sample of interest has a lower degree of methylation (hypomethylated) at a specific CpG-site, then the sample will be associated with relatively greater HpaII counts and a larger methylation angle (aka methylation score). The developers of the HELP-tagging technique have shown that the methylation score negatively correlates with cytosine methylation, hence the higher and lower methylation scores define less (hypomethylated) and more (hypermethylated) methylated CpG-sites.[105]

## 3.6 Gut microbiota

In *Paper III*, a new set of mice were fed HF diets with or without lingonberries, with the purpose to collect gastrointestinal tissues and investigate if lingonberry intake modifies the gut microbiota. DNA was extracted from the content of the cecum and subjected to 16S rRNA gene sequencing. The cecum (first part of the colon, **Figure 2**) is large and has high fermentative activity in mice. 16S amplicon sequencing is commonly used for surveying which bacteria are present in a sample due to its universal conservation among bacteria. The 16S rRNA gene also contains highly variable regions which are targeted to enable classification to a specific area of phylogeny, e.g. identifying the relative abundance of specific bacterial taxa. The technique has some limitations, including not taking into account horizontal gene transfer and neglecting viral and eukaryotic communities in the gut. In *Paper III*, PICRUSt was used to infer microbiota functionality from the taxonomic profile derived from the 16S sequencing. Even if the mammalian digestive tract is considered to be conserved, there are discrepancies in the human and mouse gut function and gut microbiota which should be taken into account when interpreting the results.

# 4. Main Findings

## Paper I

- Lingonberries prevent diet-induced obesity in C57BL/6J mice. Supplementation with blackcurrants, bilberries or raspberries has similar but not as pronounced effects.
- Lingonberries in particular, but also blackcurrants and bilberries, prevent several negative effects associated with diet-induced obesity including insulin resistance, adiposity, liver lipid accumulation and low-grade inflammation.
- Intake of açai berries promotes weight gain and leads to development of large, steatotic livers.
- Analysis of polyphenols detected quercetin-3-O-glucoside and quercetin-3-O-galactoside in the lingonberry and the blackcurrant diets.

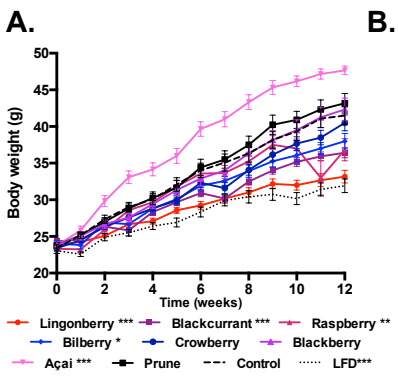
## Paper II

- Genome-wide hepatic gene expression profiling revealed that supplementation of HF diets with lingonberries, blackcurrants, bilberries or açai alters pathways related to inflammation, lipid- and cholesterol metabolism and redox processes.
- The protection against hepatic steatosis of lingonberries and bilberries is associated with several-fold downregulation of genes involved in acute-phase and inflammatory pathways, e.g. *Saa1*, *Cxcl1* and *Lcn2*.
- The effects of lingonberries are associated with reduced STAT3 and NFκB signaling.
- Anti-inflammatory effects of bilberries are associated with decreased NFκB translocation to the nucleus.

- Açai-fed mice exhibit marked upregulation of genes associated with steatosis, lipid and cholesterol synthesis, which is in line with the exacerbation of HF-induced hepatic steatosis in these mice.

# Paper III

- Lingonberries prevent obesity and associated complications of a HF diet, but the magnitude of the effect varies depending on the batch of berries.
- Regardless of the berry source, lingonberries prevent low-grade inflammation, metabolic endotoxemia and mediate positive effects on liver function.
- Lingonberry supplementation has large effects on cecal microbiota composition, including a decrease in relative abundance of *Firmicutes* and an increase of *Bacteroidetes*.
- Lingonberries increase the relative abundance of the genus *Akkermansia*, independently of effects on body weight.




B.

| Berry | Effect on HF-induced steatosis and obesity | Proposed regulators and mechanisms |
|---|---|---|
| **Lingonberry** | Prevention | **Anti-inflammation:** reduced STAT3 and NFκB signaling. |
| **Blackcurrant** | Prevention | **Decreased lipid synthesis:** likely involving inhibition of SREBP1c. **Anti-inflammation:** reduced NFκB translocation to nucleus. |
| **Bilberry** | Prevention | **Anti-inflammation:** reduced NFκB translocation to nucleus. **Reduced intestinal lipid absorption:** compensatory upregulation of lipid synthesis likely mediated by activation of SREBP1c. |
| **Açai** | Aggravates hepatic steatosis and obesity | **Increased lipid synthesis:** likely involving activation of SREBP1c. |

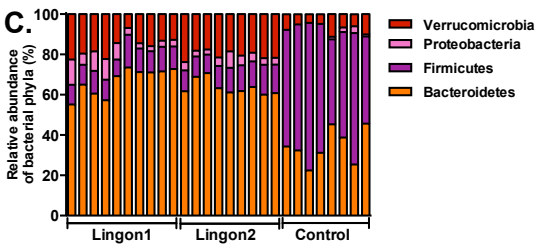



**Figure 8. Main findings from the papers of this thesis.** A) Lingonberries, blackcurrants, raspberries and bilberries reduce, whereas açai promotes, body weight gain in C57BL/6J mice compared to control (*Figure 1a, Paper I*). B) Summary of effects of different berries on HF-induced hepatic steatosis and obesity (*Figure 10, Paper II*). C) Lingonberry supplementation modifies the gut microbiota composition *(Figure 4A, Paper III)*.

# 5. Results and Discussion

## 5.1 Characterization of how different berries influence weight gain and metabolic parameters (*Paper I*)

The screening of eight species of berries *(Paper I)* showed promising results in relation to prevention of obesity and related abnormalities induced by HF diet. Moreover, we found that different berries have different metabolic effects – certain berries had little or no effect, whereas others prevented or even promoted diet-induced obesity (summarized in **Figure 9**).

### *5.1.1 Lingonberries, blackcurrants and bilberries prevent diet-induced obesity*

Perhaps the most striking finding in the *Paper I* study was the almost complete prevention of body weight gain seen in mice receiving HF diet supplemented with lingonberries, compared to the control mice receiving HF diet without berries. The mice in the lingonberry group followed the same weight gain curve *(Figure 1a, Paper I)* as the LF control group, and ended up weighing 21% less than the HF control mice. The decrease in weight was explained by lower body fat content and smaller visceral fat depots, whereas the lean body mass was unaltered. Lingonberries also stood out in terms of improving almost all of the obesity-associated parameters that were measured, including reduction of hepatic steatosis, insulin resistance, plasma total cholesterol levels and plasma levels of the inflammatory marker PAI-1. These findings led to a strong focus on lingonberries throughout the projects and thesis, and the outcomes of this work will be detailed in following *section 5.3*.

The berry with the second best capacity to prevent obesity and associated parameters was blackcurrant. At the end of the study period, mice receiving diet supplemented with blackcurrants weighed 14% less compared to the control mice. Blackcurrant supplementation improved several of the metabolic parameters that were impaired by HF feeding, but in general not as efficiently as lingonberries. The same was seen in mice receiving bilberries, this group weighed 10% less than the control group and had similar but less pronounced effects on adiposity, steatosis and insulin resistance in comparison to lingonberries and blackcurrants. To the best of our knowledge, at the time of the publication of *Paper I* the anti-

obesity effects of whole lingonberries and blackcurrants had not been previously described. Lingonberries, blackcurrants and bilberries were the only berries that improved insulin resistance, reduced hepatic lipid accumulation and lowered the marker of liver dysfunction (ALT) as well as the inflammatory marker PAI-1. All these parameters may relate to protection against HF-induced liver dysfunction. Hence, the effect of lingonberries, blackcurrants and bilberries on liver metabolism was further evaluated and will be discussed in *section 5.3.1*, *section 5.2.1* and *section 5.2.2*, respectively.

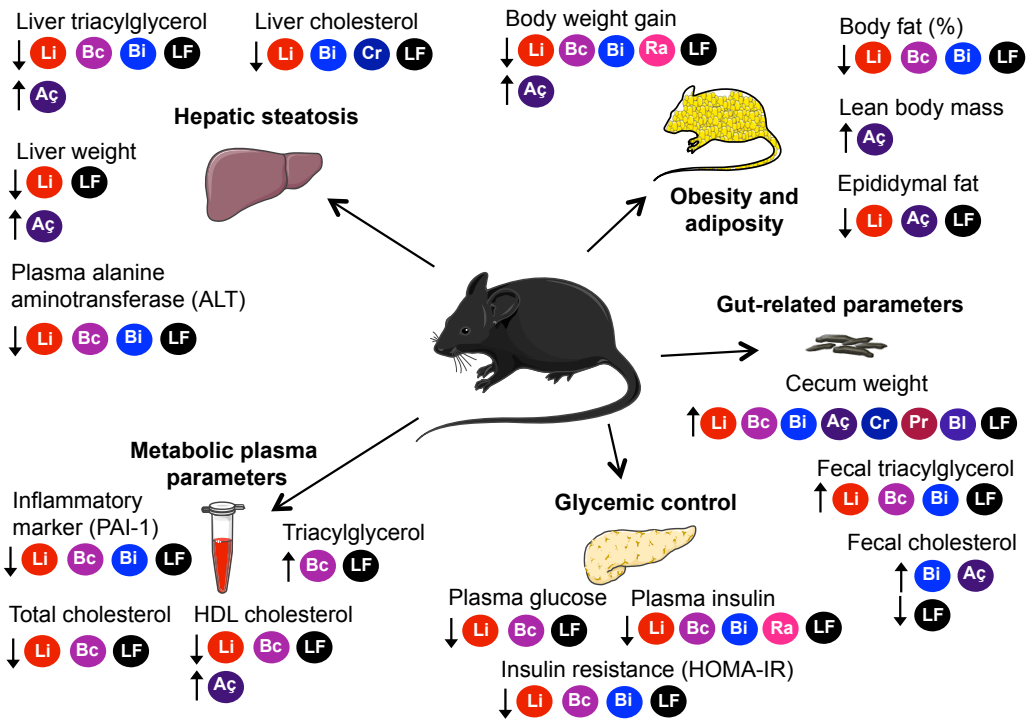



**Figure 9. Overview of metabolic effects of berry consumption observed in *Paper I*.** The picture depicts major findings in *Paper I*, showing how supplementation with different berries prevents or aggravates various effects induced by HF-diet, including hepatic steatosis and obesity. Arrows (up or down) indicate statistically significant effects ($p < 0.05$) compared to the control mice receiving HF diet without berries. Abbreviations: (Li) lingonberry, (Bc) blackcurrants, (Bi) bilberry, (Aç) açai, (Ra) raspberry, (Cr) crowberry, (Pr) prune, (Bl) blackberry and (LF) low-fat diet. PAI-1 and ALT are markers for inflammation and liver dysfunction, respectively. Homeostatic model assessment (HOMA) index reflects the level of insulin resistance. Illustrations were obtained and modified from Servier Medical Art by Servier (http://bit.do/servppt) under the license [CC BY 3.0 (http://creativecommons.org/licenses/by/3.0)].

### *5.1.2 Berries with little or no metabolic effect*

Raspberry intake decreased body weight gain with 12% compared to the control group, and tended to have beneficial effects on obesity-associated parameters, however the only significant effect was lowering of fasting insulin levels. The transient drop in weight at week 11 of the study *(Figure 1a, Paper I)* has been tracked in the records to a sudden loss of approximately 10 grams in all mice in one of the cages in the raspberry group, which was restored at the next measuring time point one week later. There was no corresponding reduction in food consumption, and one might strongly suspect that this "drop" is explained by an error in weight registration. Both human and *in vivo* studies specifically investigating health effects of consuming whole red raspberries are lacking. However, a mixture of ellagitannin-rich berries (raspberries, strawberries and cloudberries) has been found to have little or no beneficial effects in subjects with symptoms of metabolic syndrome[71]. Raspberries have gained some attention in the field of nutritional supplements for weight loss through the marketing of "raspberry ketone" (4-(4-hydroxyphenyl-)-2-butanone). This aroma compound is present in raspberries and other berries, but the raspberry ketone that is sold as supplements is mainly synthesized rather than extracted from berries[106]. To date, the scientific support for a slimming effect of raspberry ketone is very limited, notably human data appears to be lacking.

Supplementation with blackberries or with prunes did not significantly alter weight gain or any other effects induced by HF feeding. There is one study where blackberry extracts in drinking water were suggested to have a glucose lowering effect in rats made diabetic by streptozotocin (STZ) injection[107]. However, the present setting and utilization of whole blackberries have not been investigated previously, and do not support a role for blackberries in prevention of prediabetes and obesity. To the best of our knowledge, this is the first report investigating effects of crowberries *in vivo*. Compared to the control group, crowberries significantly reduced the liver cholesterol content. Moreover, mice consuming crowberries experienced some prevention of body weight gain, but this effect was not statistically significant. Based on these findings, blackberries, prunes and crowberries appear as being less attractive candidates in terms of dietary strategies for obesity-prevention. Naturally, further investigations are warranted as there are very few *in vivo* studies evaluating the health effects of these berries.

### *5.1.3 Açai aggravates diet-induced obesity and promotes hepatic steatosis*

In contrast to the other investigated berries, consumption of açai berries led to increased body weight gain (+14%). The most notable result was the development of large, steatotic livers; mice receiving açai supplementation had livers with 33% higher mass (relative to lean body mass) and 73% higher triacylglycerol content compared to the control mice receiving only HF diet. This phenomenon was

further investigated in *Paper II* and will be addressed in detail in *section 5.2.3* together with a discussion of relevant mechanisms and literature.

The finding that açai induces weight gain contrasts with the marketing of açai berries as a "superberry" supplement for weight loss. The interest in açai may be partly due to its very high *in vitro* antioxidant capacity reported in the US Department of Agriculture (USDA) ORAC Database, and was popularized on the Internet by incorrect claims of açai products as being recommended by well-known TV personalities[108]. *Note:* The USDA ORAC database has now been removed because of misuse for marketing purposes and due to *"mounting evidence that the values indicating antioxidant capacity have no relevance to the effects of specific bioactive compounds, including polyphenols on human health"*[109].

### *5.1.4 Polyphenolic profiles of the berry diets*

The characterization of the berry diets revealed differences in polyphenolic profile, and a few quercetin glycosides where only present in the diets with the most pronounced capacity to prevent weigh gain, i.e. the lingonberry and blackcurrant diets. As discussed in *Paper I*, quercetin has several interesting bioactivities and has even been investigated in the context of antidiabetic effects of lingonberries[110], but the design of our study does not allow the establishment of cause-relationships between health effects and specific compounds. However, some polyphenols characteristic for the different berries were not detected in the diets, for example in the prune diet. This may be due to methodological limitations or processing, as it has been reported that commercial drying of plums to prunes induces large losses of polyphenols[111]. The content of polyphenols and other micronutrients may be altered by storage conditions and processing, but the freeze-drying method used for preparing the berries is considered to be a relatively gentle drying process[112]. There was no measurement of polyphenols in the berries before they were processed into diets, however we conclude that berry polyphenols were present in the diets, and that the profile did vary. The polyphenolic profiles were hypothesized to be one factor relevant for health effects of different berries, however due to the inherent complexity of foods and synergistic effects there are numerous other constituents and factors that could be playing a role. Moreover, the health effects of consuming berries in other forms (fresh, frozen, jams, extracts) may differ from what is observed in the *Paper I* study and could be a subject for future investigations.

### *5.1.5 Further aspects to consider – interpreting the results (Paper I)*

The *Paper I* study characterizes berries and their metabolic effects, however mechanistic explanations were not elucidated. Altered food intake, absorption as well as excretion were assessed, but did not provide a full explanation for the

different effects on weights. Energy intake did not appear to be altered, with the exception of an *increase* in consumption of the blackcurrant and bilberry diets. There were no significant changes in the total excretion of feces, but several of the berry groups had higher concentrations of triacylglycerol in the feces, compared to the HF control group. An interpretation may be that lingonberries, blackcurrants, bilberries, raspberries, açai, crowberries and blackberries caused reduced digestion and/or absorption of the lipids in the diets. This is in line with studies describing inhibitory effects of polyphenols and berry extracts on pancreatic lipases[97]. However, judging from our data, reduced lipid uptake was not the only determinant of body weight gain prevention. For example, the crowberry and raspberry groups appeared to have higher excretion of triacylglycerol compared to the lingonberry group. That being said, the implied effects on food intake and excretion need to be interpreted with caution as mice were co-caged. Studies using single-caged animals, preferably in metabolic cages, would be a valuable addition to fully establish the effect on these parameters induced by berry supplementation. A common feature in all berry groups (except raspberry) was the enlargement of the cecum. This could be due to increased amounts of indigested material or alterations of the gut microbiota. As discussed in *section 3.2*, the diets were quantitatively balanced for fat, protein, carbohydrates, glucose, sucrose and fructose. However, we chose not to balance the diets for fiber content. Even if the fiber content did not correlate with body weight gain, it is possible that the quality of fiber present in the different berries may play a role for the observed metabolic effects.

## 5.2 Metabolic consequences and mechanisms affected by blackcurrants, bilberries and açai (*Paper II*)

Lingonberries, blackcurrants and bilberries prevented, whereas açai promoted, HF-induced hepatic steatosis and obesity. The global gene expression analysis of livers from these mice revealed that several pathways of relevance to the phenotype were affected by berry consumption, most notably inflammatory response and lipid/cholesterol metabolism. The effect on hepatic gene expression patterns was different for different berries, suggesting that the studied berries mediate their effects on steatosis and obesity via different mechanisms.

### *5.2.1 Blackcurrants – metabolic effects and potential mechanisms*

In our study, supplementation with blackcurrants had positive effects on adiposity (reduction of body weight gain, body fat accumulation and epididymal fat mass) and insulin resistance (reduced plasma glucose, insulin and HOMA) compared to mice receiving HF diet. Furthermore, a hepatoprotective effect was inferred as

mice consuming blackcurrant diet had lower concentrations of triacylglycerol in the liver and lower plasma levels of the liver dysfunction marker ALT. The significantly increased food intake is probably partly a consequence of the crumbly texture of the blackcurrant diet, since an increased spillage was observed for this diet. It is also possible that food intake increased in order to compensate for decreased nutrient uptake, as the mice receiving blackcurrants appeared to have an increased excretion of triacylglycerol into feces. The gene expression analysis of the livers implied that mice receiving blackcurrant had decreased expression of genes involved in fatty acid biosynthesis, and the genes encoding SREBP1c and PPARγ (*Supplementary Table S4, Paper II*) were predicted to be inactivated upstream regulators. Taken together, blackcurrant supplementation affects fatty acid/triacylglycerol metabolism, potentially by interfering with digestion and/or absorption of dietary fat. Higher excretion in combination with lower synthesis of fat may partly explain the ability of blackcurrants to counteract hepatic lipid overload and metabolic derangements induced by a HF diet.

Since the publication of the anti-obesity effect of blackcurrants observed in *Paper I,* additional papers have been published investigating the effect of blackcurrant intake in HF-fed C57BL/6J mice. Benn et al. 2014[113] fed a HF (35E%)/ high cholesterol (HC) diet supplemented with 0.1% of blackcurrant extract powder to mice for 12 weeks. Compared to mice receiving only HF/HC diet, there was a non-significant reduction in body weight gain (-23%) with blackcurrant supplementation, however similar to our study, the epididymal fat weight was significantly reduced. Results from the same study concerning effects on liver and plasma parameters were published separately (Benn et al. 2015)[114], and describe considerable but non-significant reductions in plasma ALT, liver weight and liver cholesterol content in the group receiving blackcurrants. Interestingly, the hepatic steatosis score was lower and liver triacylglycerol content tended ($p=0.07$) to be reduced compared to the control. Moreover, plasma cholesterol levels and glucose levels were reduced. Taken together, it is interesting to observe that Benn et al. see very similar effects in response to blackcurrant as described in our study, even if for example the preventive effect on body weight, liver triacylglycerol and plasma ALT did not reach significance. Differences between this study and our *Paper I* study include the utilization of blackcurrant extract as opposed to freeze-dried whole berries, and the dose as well as the different composition of the HF-diet (HF 35%/HC as opposed to HF 45%). Regardless of these differences, a glucose-lowering effect in response to intake of blackcurrants was obtained in both studies.

Similarly, Esposito et al. have provided additional evidence for the ability of blackcurrants to improve glucose homeostasis. Esposito et al. investigated metabolic effects of 1% blackcurrant powder extract supplementation in C57BL/6J mice consuming HF (60E%) or LF (10E%) diets during 8 weeks[115]. In this study, blackcurrant supplementation attenuated weight gain and improved insulin sensitivity in mice consuming both HF and LF diet. However, the prevention of

weight gain was not seen in mice that had an antibiotic disrupted microbiota. The authors observed an increased concentration of blackcurrant anthocyanins in feces in response to administration of an antibiotic cocktail in the drinking water, which implies reduced biotransformation and absorption of blackcurrant anthocyanins. The authors concluded that blackcurrant anthocyanins are susceptible to high microbial transformation in the gut (jejunum), and that these metabolites are partly responsible for the metabolic health outcomes of blackcurrants, at least when it comes to prevention of weight gain. What about the effect on glucose homeostasis? In the LF diet background, blackcurrant supplementation had no effect on glucose tolerance, but improved insulin sensitivity, independently of the presence of an intact or disrupted gut microbiota. However, blackcurrant supplementation prevented HF-induced impairments of glucose tolerance and insulin resistance, but these effects were not observed in mice with disrupted gut microbiota.

In humans, intake of whole blackcurrants as well as blackcurrant nectar with added sucrose reduced postprandial glucose and insulin concentrations compared to ingestion of sucrose alone[63]. Thus, blackcurrants seem to mediate a delay of sucrose digestion and absorption which is interesting and in line with blackcurrants' *in vitro* inhibition of carbohydrate digestive enzymes[97]. This mechanism may partly explain the observed improvement of insulin sensitivity shown by us[116] and others[114 115]. Putting the pieces of information together, it seems that intake of blackcurrants (whole berries, extracts or nectar) improves glucose homeostasis in both lean (LF diet fed mice[115] and non-obese human subjects[63]) and obese (HF-fed mice[113 115 116]), and this effect is not dependent on effects on body weight gain[113 115]. The capacity of blackcurrants to reduce body weight gain have only been observed in mice provoked with HF diets[115 116]. In terms of blackcurrant extracts, the prevention of obesity seems to be connected to effects on the gut microbiota[115].

Finally, blackcurrants have also attracted interest in relation to vascular function and potential lowering of CVD risk. In our study, consumption of blackcurrants decreased hepatic expression of genes involved in redox and glutathione metabolism compared to the HF control. Interestingly, in a trial where subjects consumed flavored water (placebo) or blackcurrant juices daily during 6 weeks, blackcurrant juice significantly decreased a serum marker for oxidative stress, and improved endothelial function[66]. However, no significant effects were observed on body weight and plasma cholesterol levels (glucose levels were not assessed). In our study, we observed that blackcurrant supplementation reduced plasma total cholesterol levels, but also increased the plasma concentration of triacylglycerol and reduced plasma high-density lipoprotein (HDL)-cholesterol compared to the HF control group. One may discuss whether these results provide support for a role of blackcurrants in vascular health and reduction of CVD risk. Considering the prevention of HF-induced obesity in response to blackcurrants, the effect on

the plasma lipid profile might seem counterintuitive since low HDL-cholesterol is generally regarded as being a feature of obesity-associated dyslipidemia. However, there are differences in plasma lipid profile between mice and men, including the lack of cholesteryl ester transfer protein (CETP) causing mice to carry a majority of the plasma cholesterol in the HDL fraction[117]. Hence one may speculate that a lowering of total cholesterol in plasma is difficult to achieve in mice without also reducing plasma HDL. As an illustration, blackcurrant extracts given to rabbits, being animals with high CETP activity, ameliorated HF-induced hyperlipidemia by decreasing triacylglycerol, total and non-HDL-cholesterol concentration in serum[118].

Taken together, several lines of evidence indicate a potential for blackcurrants as having beneficial health effects in relation to insulin sensitivity, but also body weight and liver function. To the best of our knowledge, there is no human intervention study conducted to investigate the specific effects of blackcurrants on glucose homeostasis, body weight, lipid metabolism and liver function. Such studies would be valuable, as consumption of blackcurrants could be a successful strategy for preventing or delaying development of insulin resistance and hence T2D.

### *5.2.2 Bilberries – metabolic effects and potential mechanisms*

The positive effect of bilberries on development of weight gain and hepatic steatosis was pronounced in the *Paper I* study, however unlike lingonberries and blackcurrants, there was no significant effect on plasma lipid profile. The mice receiving bilberries had an increased food intake and were the only group with both increased triacylglycerol and cholesterol content in feces. In addition, hepatic triacylglycerol and cholesterol concentrations were reduced in the bilberry mice compared to the HF control. Nonetheless, the hepatic gene expression analysis revealed an enrichment of genes involved in lipid and triacylglycerol synthesis, and a pronounced upregulation of genes involved in cholesterol biosynthesis (e.g. *Hmgcr*) and bile acid synthesis (*Cyp7a1*). Moreover, SREBP1 (regulating fat synthesis), and especially SREBP2 (regulating cholesterol levels), were predicted to be strongly activated upstream regulators (*Supplementary Table S4*). Since cholesterol levels are tightly regulated, and there was no buildup of cholesterol or lipid in liver or plasma, one may reason that the upregulation is a compensatory mechanism in response to increased lipid and cholesterol excretion. Bile acids are excreted from the liver into the duodenum and recirculate via the enterohepatic circulation. The upregulation of *Cyp7a1*, the rate-limiting enzyme in the synthesis of bile acids from cholesterol, may indicate an impaired reuptake of bile acids, explaining the increased demand for cholesterol and bile acid synthesis. Interestingly, Kolehmainen et al. found that subjects consuming a dried bilberry/bilberry purée product for 8 weeks had increased plasma levels of a cholesterol synthesis marker, compared to the control group not receiving

bilberries. Similar to our observations, the subjects had no change in serum cholesterol, but there was a tendency to reduced cholesterol absorption. The preventive effect of bilberries on weight gain in C57BL/6J mice was also confirmed in a study by Mykkänen et al. 2014[119] where HF diet was supplemented with 5% or 10% (w/w) whole freeze-dried bilberries. The weight gain during the three months long study was significantly reduced in the group receiving 10% of bilberries, compared to HF control. However, data on liver and fecal parameters were not reported. Interestingly, a human dietary intervention study in overweight and obese subjects reports small but significant reductions in waist circumference and body weight compared to baseline after daily consumption of 100 g of frozen, whole bilberries for a month[69]. Based on our findings, one may speculate that increased excretion of lipids and cholesterol is one mechanism by which bilberries mediate metabolic effects.

The analysis of hepatic gene expression patterns highlighted an anti-inflammatory effect of bilberries. To summarize, pathways and genes related to inflammation and acute-phase response were downregulated in mice receiving bilberries compared to the HF control group. This was also reflected by a downregulation in the bilberry group of plasma markers of inflammation, including PAI-1 and SAA. These results are very interesting in relation to several previous bilberry studies where a reduction of low-grade inflammation has been observed[67-69 119]. In addition, we showed for the first time that there is a reduced translocation of NFκB in the liver of mice receiving bilberries. This is a putatively important finding to increase mechanistic understanding as NFκB is a major regulator of inflammatory responses. Our results are in line with a randomized controlled trial studying the effect of 4 weeks of bilberry juice consumption in subjects at increased risk of CVD[68]. Supplementation with bilberry juice decreased levels of several targets of NFκB, including CRP and other inflammatory markers. In addition, the bilberry polyphenols quercetin, epicatechin and resveratrol inhibited LPS-stimulated NFκB activation in a monocytic cell line. This further strengthens the finding that hepatic NFκB is inhibited *in vivo* by bilberry supplementation compared to HF control, and is of interest since the liver is an important source of inflammatory mediators. Furthermore, Kolehmainen et al. described that intake of the bilberry purée product reduced low-grade inflammation in subjects with features of metabolic syndrome[67]. During the study, subjects were successfully instructed to maintain their body weight, and no significant changes were observed in plasma glucose or lipid parameters, indicating that the anti-inflammatory effect of bilberries is more than a consequence of reduced obesity.

Based on current evidence, bilberry intake appears promising in terms of controlling body weight and reducing low-grade inflammation. This conclusion is drawn based on effects of consuming whole bilberries in HF-induced obese mice described by us and others[116 119] (prevention), and in overweight and obese subjects[69] (reduction). Moreover, several rodent and human studies report positive

effects on reducing or preventing development of low-grade inflammation, with or without effects on body weight[67-69 116 119] but likely involving NFκB. The reason for this effect is not clear, but upstream analysis of our expression data predicted LPS as an upstream regulator being significantly inactivated compared to the HF control group (*Supplementary Table S4, Paper II*). Neither LPS concentrations in plasma nor gut microbiota composition was assessed in the study, but one may speculate that mice receiving bilberries had less circulating LPS compared to the HF control group. The altered cholesterol/lipid/bile acid metabolism in response to bilberry intake could associate with modifications of the gut microbiota and reduced circulating LPS, however this remains to be established. Nonetheless, it has been shown that a considerable fraction of ingested bilberry anthocyanins reach the colon[120] and bilberry consumption has been shown to improve inflammatory disease in the gastrointestinal tract[70]. In addition, results from ileostomy probands suggest that colonic metabolism is important for the antioxidative activity of ingesting bilberry extracts[120]. Hence, additional studies investigating the relationship between inflammation, obesity and gut microbiota would be of interest to further elucidate the beneficial effects of bilberry consumption on metabolic disease.

### *5.2.3 Açai – metabolic effects and potential mechanisms*

In contrast to the other studied berries, intake of açai was found to promote weight gain and drastically increase liver lipid accumulation. The evaluation of liver metabolism in *Paper II* revealed a strong upregulation of fatty acid and lipid biosynthesis pathways, which was likely contributing to the development of fatty liver. In addition, the lipogenic transcription factors SREPB1, SREBP2 and PPARγ (*Supplementary Table S4, Paper II*) were predicted to be strongly activated by açai intake compared to intake of HF diet without açai. Even if cholesterol synthesis pathways were upregulated in response to açai, supplementation with this berry neither prevented nor aggravated HF-induced cholesterol accumulation into the liver. Moreover, the mice receiving açai supplementation had increased fecal excretion of cholesterol compared to the HF control, which suggests that the upregulation of cholesterol synthesis was a response to compensate for decreased cholesterol absorption. Conversely, our results suggest that the upregulated lipid synthesis in response to açai supplementation contributed to fat accumulation and development of hepatic steatosis. However, as the epididymal fat pads were significantly smaller in the açai group compared to the control, it appears that hepatic lipid storage was favored over uptake and storage of fat into visceral adipose tissue. The amount of subcutaneous adipose tissue was not recorded in our study, but visual examination suggested that subcutaneous adipose tissue depots were enlarged compared to the control group. Regardless of the molecular mechanisms behind the increased hepatic fat accumulation, it seems that the increased expression of lipid synthesizing pathways was maladaptive. On the other

hand, fat accumulation in the form of triacylglycerol may not necessarily be damaging, but rather depends on the quality of the fat, formation of reactive lipid species etc. As discussed in *Paper II*, the açai diet induced an increase in gene expression of several proteins belonging to the aldehyde dehydrogenase family, which potentially could be a response to counteract an increased formation of reactive lipid metabolites. Overall, even if the values did not reach statistical significance, there is a trend towards impaired insulin sensitivity, liver function (ALT) and low-grade inflammation (PAI-1) compared to the HF control, and certainly compared to intake of HF diet supplemented with some of the other berries. Hence, our study strongly suggests that açai berry supplementation may not be the most promising strategy for prevention of obesity and metabolic disease.

There are only a few other studies investigating the effects of açai consumption. In line with our findings, rodent studies on the effect of açai supplementation on oxidative stress and atherosclerosis report co-findings of significantly increased body weight gain in response to açai[121 122]. In a pilot study by Udani et al., daily intake of açai smoothies during a one month period was shown to improve fasting glucose and insulin levels compared to baseline in 10 overweight subjects[74]. Body weight data was not presented, but was reported to not have changed significantly throughout the study. However, the findings may have been a result of confounding factors as there was no control group and since the subjects were instructed to avoid certain foods during the study (e.g. hot dogs and bacon). After the publication of the *Paper I* study, de Sousa Pereira et al. published an intervention study where healthy and overweight women (n=40) consumed 200 g of açai pulp during one month[75]. Compared to baseline, açai supplementation increased body weight, BMI and truncal fat % in healthy women, and increased plasma concentration of the inflammatory marker PAI-1 in overweight women. This is the most extensive study on açai consumption to date, and the results by de Sousa Pereira et al. are well in line with our findings. In our mouse study, the mice receiving açai had elevated plasma levels of the inflammatory marker SAA and PAI-1 compared to the HF control group, however the increase in PAI-1 was not statistically significant. Taken together, further studies are warranted to evaluate which health effects can be obtained by açai berries, however it seems likely that promotion of weight loss is not one of them.

The Brazilian açai berry has traditionally been cultivated and consumed in the Amazonian region. In Brazil, it is consumed in a context of energizing beverages, juices and smoothies **(Figure 10)**.[108 123] In our study, the increased body weight in response to açai intake tended to be associated with obesity-associated metabolic impairments. However, it is interesting to note that despite the weight gain, açai significantly increased lean body mass, reduced epididymal fat pad size and did not significantly alter body fat percentage compared to the control group. Similarly, de Sousa Pereira et al. did not observe an increase in body fat

percentage in their study, however they described a redefinition of body fat from peripheral areas to the trunk[75]. Açai berries have a high lipid content compared to other berries and fruits, however due to the diet formulation (*section 3.2*), the difference in fat content should not be causing the effects in our study. That being said, the diets were only balanced for fat quantity, not quality. The components responsible for the actions of açai remains to be established, and the effects of açai supplementation needs further evaluation, potentially with a focus on anabolic effects and enhancing nutritional status.

**Figure 10. Açai is the fruit of the *Euterpe oleracea* Martius palm tree, indigenous to South America.** The pulp of the açai berry (surrounding a large seed) is often used to make a purée which may be consumed in the form of frozen smoothies and beverages.

## 5.3 Lingonberries – a potential functional food (*Paper I, II* and *III*)

The results from the *Paper I* study identified lingonberry supplementation as an effective strategy for preventing obesity and other metabolic aggravations induced by a HF diet. Notably, lingonberries were the only berries that significantly reduced the liver mass compared to HF control, which together with previously

discussed results (*section 5.1.1*) imply an effective prevention of development of fatty liver. To date, there is no dietary intervention study investigating the short or long-term effects of lingonberry consumption in humans. However, acute meal studies have shown that intake of lingonberries can reduce postprandial glucose and insulin levels in response to sucrose[63], but the implication of this finding on metabolic health is not known. Intake of berry meals, consisting of lingonberries but also three other berries (blackcurrant, bilberry and sea buckthorn) for 20 weeks has been shown to reduce the levels of the liver dysfunction marker ALT compared to a control group not receiving berries[124]. At the time of the publication of *Paper I*, lingonberries had not been investigated *in vivo* as a potential food capable of preventing obesity and obesity-related disorders. However our findings are in agreement with a study in rats reporting that lingonberry extracts favorably affected antioxidant defense enzymes, but also reduced body weight gain, compared to the control receiving HF/HC diet without lingonberry extracts.[125] Shortly after the publication of *Paper I* there was a report by Haddad et al. describing that HF-induced alterations in C57BL/6J mice tended to be reversed by supplementation with lingonberry extracts[126]. The results presented in this thesis add significantly to the knowledge about the *in vivo* bioactivities of lingonberries, and strongly suggest that lingonberries are interesting to further evaluate as a food with potential beneficial health effects.

### *5.3.1 Mechanisms and pathways affected by lingonberry intake in relation to prevention of hepatic steatosis*

The global gene expression analysis revealed that pathways related to inflammation and lipid metabolic processes were downregulated in livers from mice fed lingonberries. Several of the SAA acute-phase proteins were downregulated, and the plasma concentration of SAA and other inflammatory markers were confirmed to be reduced in plasma from mice receiving HF diet supplemented with lingonberries compared to the HF control. Several of the downregulated genes (*Saa, Cxcls, Ccl4* and *Socs3)* are targets of signal transducer and activator of transcription 3 (STAT3) (*Supplementary Table S3, Paper II)*. Interestingly, STAT3 was identified as being inhibited by lingonberries, and western blot analysis confirmed that the level of activated, phosphorylated STAT3 protein was reduced in livers from mice receiving lingonberries compared to the HF control group. Hence, we propose that lingonberries reduce hepatic inflammation and acute-phase response at least partly via suppressing phosphorylation and activation of STAT3. Interestingly, the mRNA level of the direct STAT3 target 'suppressor of cytokine signaling 3' *(Socs3)* was decreased twofold compared to the HF control (data not shown). SOCS3 proteins may inhibit insulin signaling by degradation of IRS, and inhibition of SOCS3 in obese diabetic mice has been shown to improve insulin sensitivity and hepatic steatosis by normalizing increased expression of SREBP-1c[127 128]. As inflammatory and insulin

desensitizing pathways are connected, one may speculate that reduced STAT3/SOCS3 signaling may be important for the improved insulin resistance, low-grade inflammation and hepatic steatosis observed in response to lingonberries.

Similarly, the finding that lingonberry supplementation inhibited the master regulator of inflammation NFκB is likely involved in the anti-inflammatory properties of lingonberries. Lingonberry extracts have been shown by Wang et al. to produce a dose-dependent inhibition of NFκB induction *in vitro* in mouse epidermal cells exposed to radiation or chemical stress[129]. In our study, mammalian target of rapamycin (mTOR) was also identified as being inactivated by lingonberries in the upstream regulator analysis, however only a tendency to reduced protein expression was observed compared to the HF control. As discussed in *Paper II,* NFκB and mTOR are implicated in pathways and processes involved in inflammation, insulin resistance and hepatic steatosis, and constitute interesting regulators targeted by lingonberry supplementation. Several of the above mentioned regulators interact with each other and are involved in pathways related to insulin resistance and lipid metabolism, for example by acting on SREBP1c. Even if pathways related to lipid biosynthetic processes tended to be downregulated and there were differences in SREBP activity amongst the groups, the presence of SREBP1c in the nucleus was not significantly reduced in the lingonberry group compared to the control group. As nuclear SREBP1c is measured to assess the level of transcriptionally active SREBP1c, our results imply that SREBP1c activity was not significantly reduced by lingonberries, but we cannot rule out that additional factors could be influencing the actual SREBP1c activity. Taken together, the activity of SREBP1c and other regulators and aspects involved in lipid homeostasis need further evaluation to precisely pinpoint the mechanisms at play. The underlying changes are likely the result of a complex interaction of multiple hepatic regulatory networks acting in the context of the metabolic state of the whole body.

The pathogenesis of NAFLD is a multifactorial event, and the proposed "two-hit" hypothesis postulates that accumulation of fat in hepatocytes makes the steatotic liver susceptible to secondary insults including oxidative stress, reactive lipid species, endotoxins and adipocytokines. Furthermore, the causality and relationship between hepatic steatosis and insulin resistance is not fully established[33], and may be argued to be dissociated from obesity[130]. Due to the different views in this field, a limitation of our study is that we do not know how important our observed effects on steatosis, gene expression, DNA methylation and different mechanisms are in contributing to the beneficial health outcomes. Given the vastly complicated interplay between nutrition and metabolism, it is reasonable to assume that the contribution of the different pathways by which NAFLD links to insulin resistance may vary depending on several factors and that one does not exclude the other. As an example, both inflammatory and lipid

mediators may induce signaling converging on serine phosphorylation of IRS and thereby reduce insulin sensitivity[24]. Nonetheless, the finding of the anti-inflammatory effect and inhibition of hepatic NFκB that we observe in response to lingonberries (and bilberries) may be of particular interest for understanding their ability to improve HF-induced metabolic impairments. Studies have shown that infusion with proinflammatory cytokines induces insulin resistance in humans[131] and anti-inflammatory agents reverse insulin resistance in obese mice via inhibition of NFκB[132]. Moreover, a study exploring time and tissue-specific responses to HF-induced obesity in mice has shown that insulin resistance first developed in the liver, and was concomitant with activation of hepatic NFκB[133]. The *Paper II* study shows an anti-inflammatory effect of lingonberries, but does not define *why* the inflammatory stimuli were lower in the lingonberry group compared to the HF group. One possibility is that the reduced obesity and fat mass led to decreased levels of adipose tissue-derived cytokines. However, even though the lingonberry group displayed similar or slightly higher amounts of body fat and epididymal fat compared to the LF group, the anti-inflammatory effect tended to be more prominent in the lingonberry group, exemplified by NFκB being inhibited in the lingonberry group, but not the LF group, compared to HF control. Another alternative trigger for HF and obesity-associated inflammation is gut-derived products such as endotoxin (LPS). Interestingly, the analysis of our expression data predicted LPS as an upstream regulator (*Supplementary Table S4, Paper II*) that was strongly inactivated in the lingonberry group compared to the HF control group, indicating a role for the gut microbiota.

### *5.3.2 Lingonberries modify gut microbiota composition (Paper III)*

Results from the third study included in this thesis (*Paper III*) revealed that consumption of lingonberries resulted in a drastic change in the composition of the gut microbiota. Interestingly, mice receiving HF diet supplemented with lingonberries had a decreased ratio of *Firmicutes* to *Bacteroidetes* compared to the HF control. A high *Firmicutes/Bacteroidetes* ratio is associated with obesity and HF diet consumption and the reduction of *Firmicutes/Bacteroidetes* ratio observed in response to lingonberries is similar to what has been reported previously in lean mice and upon dietary modifications including intake of LF diets and polyphenol supplementation [36 42 134]. As discussed in *Paper III*, several bacterial taxa reported to decrease in response to dietary treatments preventing HF-induced metabolic syndrome were also found to be decreased by lingonberry intake [134 135], e.g. members of the *Lachnospiraceae* family and *Bacteroides* genus. The abundance of *Akkermansia* species has been reported to correlate inversely with body weight in both rodents and humans [45 136 137], and in our study the relative abundance of the genus *Akkermansia* was increased by lingonberry consumption compared to mice receiving HF diet. Overall, our results are similar to studies in the same mouse

model where supplementation with polyphenols [134] or cranberry extracts[135] prevented negative metabolic effects of HF diet and increased abundance of *A. muciniphila*. However, in the present study the shift in *Firmicutes/Bacteroidetes* ratio and increase of *Akkermansia* in response to lingonberries were independent of body weight.

### *5.3.3 Different batches of lingonberries prevent low-grade inflammation and modify gut microbiota, independently of obesity (Paper III)*

In *Paper III*, two lingonberry diets made from independent batches of lingonberries were compared side-by-side to examine if different lingonberries mediate similar prevention of HF diet-induced metabolic abnormalities. The lingonberry diet that was also used in the *Paper 1* study, referred to as Lingon1, was again shown to prevent HF diet-induced obesity (*Figure 1, Paper III)*. In addition, the reduction of body fat percentage, liver weight, plasma glucose and cholesterol levels observed in response to the Lingon1 diet in *Paper I* were replicated in *Paper III*. Liver histology was not performed in *Paper I,* but the assessment of steatotic features of liver sections in *Paper III* further confirms the ability of Lingon1 to prevent hepatic steatosis. However, the Lingon2 diet did not have the same capacity to prevent obesity, and in the end of the study the body weight of mice receiving Lingon2 was not significantly lower than the HF control group. Moreover, several of the obesity-associated parameters were not significantly reduced by the Lingon2 diet compared to the HF control group.

Interestingly, intake of both batches of lingonberries led to reduced plasma levels of LPS-binding protein (LBP), which indicates reduced LPS levels compared to mice receiving HF diet[138]. Furthermore, both lingonberry diets reduced plasma levels of SAA and ALT compared to the HF control. The histology assessment further indicated a reduced hepatic steatosis and inflammation in liver and adipose tissue in mice receiving both lingonberry diets as compared to the HF diet. As for the gut microbiota, there was a large difference between the composition of the microbiota in mice receiving HF diet compared to the mice receiving HF diet supplemented with lingonberries (*Figure I, Paper III*). There was also some variation in the microbiota composition between the two different lingonberry groups, but in general the different lingonberry diets promoted very similar modifications of the microbiota.

Some interesting points can be made from these results. First, the Lingon2 diet did not prevent obesity but still reversed diet-induced dysbiosis commonly associated with obesity. This suggests that the effects of lingonberries on the gut microbiota are independent of weight gain and adiposity. Previous investigations in mice have indeed indicated that HF diet, rather than the obese state, may be responsible for altered microbiota[139 140]. Our study shows that supplementation with lingonberries to a HF diet counteracts the changes in microbiota that are characteristic for HF

feeding, with or without effects on obesity. Secondly, supplementation with both lingonberry diets had preventive effects on inflammation, metabolic endotoxemia and liver dysfunction, and these effects were independent of weight modulation and adiposity. These results are of interest as one may speculate that lingonberries could provide health benefits that are more than a result of health improvements caused by weight loss. Obtaining a healthy body weight may be difficult for several reasons, and identifying foods that could improve metabolic status independent of effects on body weight is of interest to reduce the risk of developing metabolic disease.

### *5.3.4 The anti-inflammatory effect of lingonberries in relation to gut microbiota*

The results in *Paper III* highlight the fact that the gut dysbiosis is probably more related to inflammation and liver homeostasis than to obesity. *Akkermansia* is implicated in prevention of HF-induced obesity and endotoxemia[44 134 135] and was shown to increase in response to lingonberry. However, *Akkermansia* may also have an effect on preventing inflammation, exemplified by experiments showing that inoculation of germ-free mice with *A. muciniphila* alters the intestinal expression of genes involved in balanced immune response and immune tolerance [141] and increased numbers of *Akkermansia* have been associated with lower intestinal proinflammatory cytokine expression and less incidence of diabetes in non-obese diabetic (NOD) mice[142]. Moreover, the genus *Faecaliebacterium* was identified as a marker for Lingon2 supplementation. The species *F. Prausnitzii* is reported to be less abundant in subject with T2D and to have anti-inflammatory properties in both mice and humans[143 144]. For example, improvement of metabolic status after gastric bypass surgery is associated with an increased abundance of *F. Prausnitzii*, and correlates negatively with inflammatory markers[43]. However, further studies are needed to determine the importance of specific bacteria for the effects of lingonberries, and preferably also comparing effects on the microbiota to baseline. Shifts in microbiota composition may associate with positive health outcomes of various treatments and interventions, as in our study, but there is a need for complementary studies involving germ-free animals, antibiotic treatment and fecal transplants to establish the causality of such relationships.

The finding that both lingonberry batches reduced plasma levels of LBP indicates prevention against diet-induced metabolic endotoxemia, and we investigated the effect of lingonberry intake on inflammation and barrier function-related gene expression in jejunum. Gut permeability is controlled by tight-junction proteins such as occludin, which is a proposed key marker of tight-junction integrity[145]. Cani et al. have shown that the inflammatory phenotype of genetically obese *ob/ob* mice is coupled to disrupted barrier function, and treatment with prebiotics decreased inflammation and endotoxemia, correlating with improved barrier

function and increased jejunal mRNA levels of *occludin* [146]. In the *Paper III* study, *occludin* was significantly upregulated in jejunum in response to the Lingon2 diet compared to the HF control, which could imply an improved barrier integrity. Additionally, the genes encoding the LPS receptor TLR4 and the macrophage marker F4/80 (*Emr*) were significantly downregulated in the Lingon2 group. In contrast, the levels of these inflammation and barrier-related markers were not affected by supplementation with the Lingon1 diet. This discrepancy suggests that the capacity of lingonberries to reduce inflammation and metabolic endotoxemia was not dependent on gut barrier integrity. However, some limitations have to be considered before arriving at final conclusions regarding the potential protection against HF-induced intestinal inflammation and barrier dysfunction. For example, effects on different intestinal segments and the expression of markers on the protein level could be of interest.

Nonetheless, due to the anti-inflammatory effect (*Paper I, II* and *III*), prediction of lower LPS-activation (*Paper II* and *III*), reduced ALT and LBP levels (*Paper I* and *III)* together with distinctive changes in the gut microbiota (*Paper III*) in response to lingonberries, it is tempting to speculate that lingonberries mediate beneficial health effects via alterations in the gut and liver. The interplay between these organs, the *gut-liver-axis*[147], is of interest in relation to our findings since endotoxin and gut-related products may trigger inflammation as well as hepatic steatosis. Studies that support this hypothesis have shown interesting data where treatment with TNF-α antibodies or probiotics improved liver histology, reduced hepatic lipid content and decreased serum ALT levels in HF-fed *ob/ob* mice[148], and these changes were coupled to decreased hepatic NFκB activation. Moreover, administration of low levels of LPS to rodents have been shown to stimulate hepatic *de novo* lipogenesis[149], and HF-fed hepatocyte-specific TLR4-null mice exhibit improved insulin sensitivity, reduced hepatic steatosis, reduced low-grade inflammation and reduced numbers of crown-like structures in adipose tissue, despite the fact that loss of liver *Tlr4* did not prevent HF-induced obesity[150]. These findings are similar to what we observe in response to lingonberries, and it is possible that the modification of gut microbiota in response to lingonberries is coupled to reduced proinflammatory factors and LPS accessing the liver and influencing host metabolism, including triggering liver inflammation and steatosis.

### *5.3.5 Batch variations in lingonberries (Paper III)*

In *Paper III*, the Lingon1 diet prevented weight gain and adiposity compared to the HF control, whereas Lingon2 did not prevent HF-induced obesity. In contrast to *Paper I*, we observed a tendency to reduced energy intake per cage in mice supplemented with lingonberries, especially in the group receiving Lingon1. We also observed a tendency to increased energy content in feces from mice receiving lingonberries, potentially caused by fiber or due to less digestion and absorption of nutrients. As previously discussed, further investigations are needed to assess food

intake and excretion, especially since bomb calorimetric measurements do not discriminate between non-metabolizable energy and energy that can be utilized by the organism. Furthermore, modifications of the gut microbiota may also influence feed efficiency and fecal nutrient excretion[39]. It is not unlikely that lingonberries impair absorption and digestion since these processes may be influenced by phytochemical intake[97] and a study in humans indicates that lingonberries could delay the uptake of sucrose[63]. One may speculate that the microbiota functionality analysis in *Paper III* also point towards differences in absorption and digestion. The lingonberry-induced enrichment of functions related to metabolism in the microbiota could be an adaptation to utilize higher amounts of unabsorbed nutrients reaching the colon.

The reason for the difference in metabolic outcomes in response the Lingon1 and Lingon2 diet has not been identified. As the diets were formulated and prepared in the same fashion by the same manufacturer with freeze-dried lingonberries from the same company, one might suspect a batch effect in the lingonberries. This is not unlikely, as the quantity of nutrients and secondary metabolites in plants is highly variable and depends on growth conditions, time of harvest, environmental exposures, cultivar etc [60 151]. For example, there is a high variation in the content and distribution of anthocyanins in wild bilberries[90]. Stress from microorganisms may induce production of certain polyphenols, phenolic acids in particular, due to their protective antimicrobial properties. Time of harvest is another important factor as phenolic acids in general decrease, whereas anthocyanin concentrations increase, with ripeness[60 129]. It has been theorized that new plant tissues produce defense compounds that affect growth and metabolism of for example microorganisms (e.g. acids and flavonoids), whereas more ripe tissues produce digestibility reducers that act on general digestive processes [152 153]. However, extensive research is required to identify which lingonberries and compounds are most active. The presented results demonstrate that variability between different batches of berries, and most likely other plant products, is an important factor to take into consideration in nutritional research. Identification of bioactive compounds in berries responsible for certain health effects is further complicated by the interplay with the gut microbiota. Depending on the microbial enzymatic activities, there is individual variability in the efficiency of converting parent compounds to colonic metabolites, which may affect bioavailability. For example, upon ingestion of raspberries, there are qualitative and quantitative variations in the ability to degrade ellagitannins to urolithins[154]. As it is proposed that at least part of the biological activities ascribed to berry polyphenols are due to their colonic metabolites[92], this phenomenon is of relevance to studies investigating physiological outcomes of berry intake. Furthermore, non-extractable polyphenols (generally not included in polyphenol analysis of foods) should also be considered as they are subjected to extensive transformation by colonic microbiota[155].

# 6. Concluding remarks and future perspectives

Lingonberries, blackcurrants and bilberries show promising effects on the prevention of obesity and prediabetes in C56BL/6J mice fed HF diet. We found that supplementation with lingonberries in particular, but also blackcurrants and bilberries, reduced body weight gain, adiposity, low-grade inflammation, insulin resistance and hepatic steatosis compared to mice receiving HF diet without berries. These results suggest that intake of these berries could have beneficial effects in relation to T2D. Raspberries, crowberries, prunes and blackberries had little or no effects on obesity and related parameters in our study, and intake of açai berry even promoted body weight gain and development of fatty liver. Hence, our results show that certain berries show more potential for the prevention of metabolic disease than others, indicating that the specific composition and nutrient profile of the berry is of importance for the metabolic effects.

The ability of lingonberries and bilberries to prevent HF-induced metabolic disease is associated with a downregulation of inflammatory response. Supplementation with lingonberries, blackcurrants and bilberries reduced hepatic activation of the inflammatory transcription factor NFκB, suggesting that this is a potential target of health-promoting effects of berries. The capacity of bilberries to counteract obesity and hepatic steatosis may be coupled to impaired lipid absorption in the gut. Our results also show that lipid synthesis is affected by berry supplementation. Açai intake promotes lipid synthesis, which may explain the development of large, steatotic livers in mice receiving açai. It would be interesting to further assess pathways related to inflammation, insulin resistance and lipid metabolism in adipose tissue and muscle.

To the best of our knowledge, this is the first work describing the beneficial effects of lingonberries in terms of preventing metabolic disease in response to an unhealthy diet. In addition, our results indicate that interactions with the liver and gut microbiota may represent a significant aspect of the health effects of lingonberries. We show that supplementing HF diet with lingonberries prevents low-grade inflammation, metabolic endotoxemia and modifies the gut microbiota. Interestingly, these effects seem to be independent on changes in body weight, as we found that different batches of lingonberries have different capacity to prevent obesity, whereas effects on low-grade inflammation, liver function and gut

microbiota were obtained by both batches. It would be interesting to further define the importance of the gut microbiota for the effects of lingonberries by experiments in mice with disrupted microbiota or microbiota transplantation. Another interesting topic that has not been covered in this thesis but deserves further exploration is the impact of berry intake on short chain fatty acid production and the bile acid pool.

Berries are rich in polyphenols and other compounds that are attributed to the protective effects of diets rich in fruits and vegetables. Future studies using different fractions of lingonberries, and rigorous analysis of batches with different metabolic effects, could aid in identifying bioactive components with particular relevance for the effects of lingonberries. Such studies should also take into account that metabolites of phenolic compounds produced by the action of microbiota may be important. Even if such studies are of interest from a mechanistic and pharmacological perspective, there are likely synergistic effects between components that together with characteristics of food matrix and other factors contribute to the health effects of lingonberries and other berries. Given the mixed results from intervention trials administrating specific polyphenols, in terms of long-term prevention of metabolic disease it might be a favorable strategy to further investigate the potential of eating whole berries rather than supplements and extracts.

Our results highlight that various factors influence the quality and quantity of nutrients and secondary metabolites of berries, and this variation seems to cause differences in metabolic outcomes. It is likely a challenging task to evaluate the relationship between growth conditions of berries and metabolic effects. Nonetheless, it is important to acknowledge and further investigate this variation that could potentially explain some of the inconclusive results observed in research exploring health outcomes of different plant foods.

The strength of the work presented in this thesis lies in our characterization of how different berries affect major phenotypic features of metabolic disease, and the description of how the effects relate to alterations in pathways identified by global gene expression analysis. This allowed us to define an anti-inflammatory effect of lingonberries as important for the phenotype, and we suggest that modification of the gut microbiota may be involved. Interestingly, the anti-inflammatory effect of lingonberries seems to be more than a consequence of prevention of weight gain. Taken together, our results suggest that increased consumption of berries is a promising strategy to delay or prevent metabolic disorders that arise in response to an unhealthy diet which may predispose to development of T2D. Our findings, supported by previous literature[57] [156], identify berries as modulators of inflammation and that increased berry intake may be a dietary strategy to manage the inflammatory burden that has important implications chronic disease reduction.

There are certain aspects that deserve further evaluation, such as the effect on food intake and digestive enzymes, as well as gender and dose-effects. Most importantly, the potential benefits of lingonberries described in this thesis should be verified in human studies.

# 7. Summary in Swedish – Populärvetenskaplig sammanfattning

## Mindre fet med bärdiet – ny forskning om bärs hälsoeffekter.

***Att bär är nyttigt*** *är ett påstående som de flesta känner igen. På butikshyllorna trängs våra nordiska bär med exotiska ”superbär” som utlovar att göra oss friska. Men vilka hälsoeffekter kan egentligen uppnås genom att äta bär? Är vissa bär mer nyttiga än andra? Forskningsresultaten som presenteras i denna avhandling visar att lingon, svarta vinbär och blåbär kan motverka viktuppgång och fetmarelaterade sjukdomar, medan superbäret açai tvärtemot kan bidra till utveckling av fetma - i alla fall i möss.*

**Övervikt är en global folksjukdom** som ökat dramatiskt under de senaste decennierna. I nuläget beräknas 40% av världens befolkning vara överviktig, och 1 av 10 lider av kraftig övervikt, så kallad fetma. Denna utveckling är oroande då fetma är en starkt bidragande riskfaktor för utvecklandet av metabola sjukdomar, till exempel typ 2 diabetes.

**Kosten är en viktig faktor** för att upprätthålla en god hälsa. I våra försök har vi använt oss av möss som lätt lägger på sig fett när de äter en ”ohälsosam” kost, i detta fall en så kallad högfettskost. Denna musmodell ska motsvara människor som genom kosten har ökad risk för att utveckla fetma och förstadier till typ 2 diabetes. Genom att kombinera högfettskosten med olika bär och ge dessa dieter till möss under tre månader, har vi studerat om dessa bär kan motverka de negativa hälsoeffekterna av högfettskosten.

**Möss som fick lingon** i högfettskosten visade sig vara skyddade mot fetma jämfört med kontrollmössen som fick högfettskost utan bär. Dessutom hade de som fick lingon lägre blodsocker- och insulinvärden. Även kolesterolhalterna och fettinlagringen i levern var avsevärt lägre än hos kontrollmössen som inte fått bär. Svarta vinbär, blåbär och hallon gav likande positiva effekter men lingonen gav klart störst effekt. Kråkbär, björnbär och katrinplommon var också inkluderade i studien, men dessa bär hade inte så stor inverkan på de studerade parametrarna.

**Det kanske mest överraskande** fyndet var att açaibäret – som marknadsförts som ett superbär för viktminskning – gav motsatt effekt. Mössen som fick açai-tillskott gick upp ännu mer i vikt än kontrollmössen och utvecklade dessutom fettlever.

**Vad händer i kroppen** när vi äter dessa olika bär? För att besvara frågan fördjupade vi forskningen om hur de bär som hade störst effekter påverkar kroppen och ämnesomsättningen. Vi använde oss av så kallad *genuttrycksanalys*. Alla celler och vävnader hos en individ består av samma genetiska material, vilket kan liknas vid en bok full av ritningar. Men hur kommer det sig då att t ex levern har helt annorlunda form och funktion än ett öra? Utseendet och funktionen hos en levercell är beroende på vilka gener i det genetiska materialet som är aktiverade. Man kan säga att i levercellen är det leverritningarna som används, medan det i örat istället är öronritningarna som är aktiva. Genuttrycksanalys utnyttjas för att studera vilka gener som används. I vår studie undersökte vi vilka av alla tusentals gener i levrar från möss som var mer eller mindre aktiverade. Resultatet visade att mössen som fick açai hade fler aktiverade gener involverade i fettinlagring än vad kontrollmössen hade. Dessutom upptäcktes att lingon och blåbär inaktiverade gener involverade i inflammation. Det verkar alltså som om lingon och blåbär har en antiinflammatorisk effekt.

**Den antiinflammatoriska effekten av lingon** och blåbär är ett intresseväckande fynd eftersom inflammation är kopplad till fetma och andra metabola sjukdomar. Den inflammatoriska reaktionen är en naturlig del av kroppens försvar mot angrepp av mikroorganismer, men ihållande låggradig inflammation är skadlig och verkar kunna motverkas med kost och livsstilsförändringar. För att få ledtrådar om hur lingon minskar låggradig inflammation undersökte vi ytterligare en aspekt – hur påverkas tarmen av bärintag?

**Tarmfloran är en viktig faktor** som länkar kost och hälsotillstånd. Kroppen består av 10 gånger fler mikroorganismer än egna celler, och majoriteten av dessa bakterier återfinns i tarmarna. Tarmsystemet härbärgerar ca 1.5 kg bakterier, *tarmfloran*, som utgör ett ekosystem i symbios med kroppen. Dock har det visat sig att sammansättningen av tarmfloran kan rubbas – t ex genom kosten – vilket kan bidra till utveckling av låggradig inflammation, fetma och andra sjukdomar. I vår studie framkom att möss som fått högfettskost hade en tarmflorasammansättning som är kopplad till fetma och negativa hälsoeffekter. Dock hade mössen som fått tillskott av lingon en helt annan tarmflora, vars sammansättning är förknippad med positiva hälsoeffekter.

**I samma studie jämfördes hälsoeffekter av olika lingon.** Det visade sig att två olika satser lingon, införskaffade från samma leverantör, skiljde sig åt i kapaciteten att förebygga fetma. Dock hade båda satserna lingon samma effekt på tarmfloran och motverkande låggradig inflammation. Skillnaden i ursprung och innehåll mellan de två olika lingonsatserna har inte kunnat fastställas, men fyndet

är intressant då det antyder att lingon har positiva hälsoeffekter utöver de effekter som uppnås genom kroppsviktsminskning.

**Blir man smal av lingonsylt?** Vad kan vi dra för slutsatser av fyra års bärforskning? Våra studier i möss har för första gången identifierat lingon som ett bär med positiva hälsoeffekter på fetma och låggradig inflammation – effekter som bland annat kan vara kopplade till tarmfloran. Dock är möss inte människor, och det återstår att bevisa om lingon – eller lingonsylt – kan påverka kroppsvikt och inflammation även i människa.

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# Paper I

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*Research Article*

# Evaluation of Beneficial Metabolic Effects of Berries in High-Fat Fed C57BL/6J Mice

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*Objective*. The aim of the study was to screen eight species of berries for their ability to prevent obesity and metabolic abnormalities associated with type 2 diabetes. *Methods*. C57BL/6J mice were assigned the following diets for 13 weeks: low-fat diet, high-fat diet or high-fat diet supplemented (20%) with lingonberry, blackcurrant, bilberry, raspberry, açai, crowberry, prune or blackberry. *Results*. The groups receiving a high-fat diet supplemented with lingonberries, blackcurrants, raspberries or bilberries gained less weight and had lower fasting insulin levels than the control group receiving high-fat diet without berries. Lingonberries, and also blackcurrants and bilberries, significantly decreased body fat content, hepatic lipid accumulation, and plasma levels of the inflammatory marker PAI-1, as well as mediated positive effects on glucose homeostasis. The group receiving açai displayed increased weight gain and developed large, steatotic livers. Quercetin glycosides were detected in the lingonberry and the blackcurrant diets. *Conclusion*. Lingonberries were shown to fully or partially prevent the detrimental metabolic effects induced by high-fat diet. Blackcurrants and bilberries had similar properties, but to a lower degree. We propose that the beneficial metabolic effects of lingonberries could be useful in preventing obesity and related disorders.

## 1. Introduction

During the last decades, the prevalence of obesity and type 2 diabetes mellitus has increased dramatically. This epidemic of lifestyle-related disorders is affecting all parts of the world, and 439 million people are estimated to suffer from diabetes mellitus in 2030 [1]. Obesity is a strong risk factor for type 2 diabetes with 90% of affected patients being overweight or obese. Obesity is also associated with increased risk of various metabolic disorders including insulin resistance, chronic low-grade inflammation, dyslipidemia, nonalcoholic fatty liver disease (NAFLD), and cardiovascular disease. Oxidative stress, inflammatory response, and altered gut microbiota can play a significant role in the development of obesity-related disorders [2–4]. Type 2 diabetes is a multifactorial disease; however, it appears clear that prevention is possible by avoiding overeating and a sedentary lifestyle to maintain a healthy body weight [5]. The difficulty for many individuals to comply with dietary and lifestyle changes makes it of great interest to identify new foods with well-established effects on preventing the development of obesity and thereby type 2 diabetes and its associated metabolic complications.

Dietary patterns with high consumption of polyphenol rich foods (fruits, vegetables, and berries) have been associated with reduced risk of type 2 diabetes. In general, berries are rich in polyphenols which are suggested to play a role in health benefits of plant-based diets [4, 6–8]. Plant phenolics are a large group of secondary metabolites which provide color and taste in fruits and berries and include flavonoids (anthocyanins, flavonols, and flavanols), tannins, stilbenoids, and phenolic acids [9]. The antioxidant effect of berry anthocyanins has been studied extensively, but still little is known about the biological activities linking berries and polyphenols to the prevention of type 2 diabetes [4, 10, 11].

The aim of the present study was to perform a screening to investigate and compare metabolic effects of different

berries in the C57BL/6J mouse. The C57BL/6J is a mouse strain that develops obesity and prediabetes when fed a diet rich in fat, thus mimicking detrimental effects of an energy dense western diet [12]. By supplementing high-fat diets with potentially health-promoting berries, we sought to identify berries capable of ameliorating risk factors from excessive energy intake. Here we report that different berries, possibly due to their polyphenol composition, have varying ability to prevent weight gain and metabolic disorders ultimately leading to diabetes.

## 2. Methods

*2.1. Animals and Study Design.* The study was approved by the Animal Ethics Committee in Lund, Sweden, (Permit Number: M185-11) and is in accordance with the Council of Europe Convention (ETS 123). Male C57BL/6JBomTac mice, 6 weeks old, 21.2 ± 1.1 g were obtained from Taconic (Skensved, Denmark). The animals were housed in a controlled environment (12 h light/dark cycle, light on 7 a.m.). After 9 days of acclimatization the mice were divided into 10 groups of 12 mice each housed in groups of 6 mice per cage. The mice were fed high-fat diets (45 kcal% fat) (Research diets, New Brunswick, NJ, USA) supplemented (20% w/w) with eight different freeze dried berries *ad libitum* for 13 weeks. A control group was fed a macronutrient-matched, isocaloric diet without supplements. One group received a low-fat diet (10 kcal% fat) as an internal control to the high-fat diet induced obesity. Body weight and food intake were monitored weekly throughout the study period. The energy intake was calculated based on registered food consumption. Mice were housed with minimal bedding material and feces was quantitatively collected for two consecutive days at the end of the study and stored at −20°C prior to lyophilization, weighing and powdering with a mortar. At the end of the study, 4 h-fasted animals were anesthetized with an intraperitoneal injection of midazolam (Midazolam Panpharma 5 mg/mL, Panpharma S.A., Luitré, France) and a mixture of fluanisone 10 mg/mL and fentanyl citrate 0.315 mg/mL (Hypnorm, VetaPharma, Leeds, UK). Body composition was determined with dual-energy X-ray absorptiometry (DEXA) technique using a Lunar PIXImus (GE Lunar, Madison, WI, USA). Blood samples were taken by intraorbital puncture. The animals were sacrificed by cervical dislocation and liver, cecum, spleen, and epididymal fat pads were excised, weighed, and snap frozen in liquid nitrogen.

*2.2. Preparation and Analysis of Diets.* The experimental diets were high-fat diets supplemented with one of eight freeze dried berries; Lingonberry (*Vaccinium vitis-idaea*), blackcurrant (*Ribes nigrum*), raspberry (*Rubus idaeus*), bilberry (*Vaccinium myrtillus*), and blackberry (*Rubus fruticosus*) were obtained from MOLDA AG (Dahlenburg, Germany). Crowberries (*Empetrum nigrum*) were from Olle Svenssons Partiaffär AB (Olofström, Sweden) and prunes (*Prunus domestica*) from Semper AB (Sundbyberg, Sweden). Freeze dried açai berry powder (*Euterpe oleracea*) was purchased from Superfruit Scandinavia AB (Sweden). Information about origin and processing of the berries can be found in Table S2 (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/403041). Based on data of macronutrient composition of the berries (obtained from supplier and/or Covance, Madison, WI, USA) the diets were designed to have identical macronutrient composition (Tables 1 and S1, Supporting information). After manufacturing, all diets were analyzed for soluble and insoluble dietary fiber content by Eurofins (Lidköping, Sweden) (Table 1).

*2.3. Analysis of Plasma Samples and Assessment of Insulin Resistance.* Plasma was prepared by immediate centrifugation of blood samples. Glucose, total cholesterol, triacylglycerol, high-density lipoprotein (HDL) cholesterol, alanine aminotransferase (ALT) (Infinity, Thermo Fisher Scientific, Waltham, MA, USA), and nonesterified fatty acid (NEFA) (NEFA-HR, Wako Chemicals, Neuss, Germany) concentrations in plasma were measured using kits. Low-density lipoprotein (LDL) cholesterol was estimated by the Friedewald formula [13]. Insulin was measured using an enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). Plasma levels of tumor necrosis factor-alpha (TNF-$\alpha$) and plasminogen activator inhibitor-1 (PAI-1) were analyzed using Luminex technology (LX200, Luminex Corporation, Austin, TX, USA). Insulin resistance was assessed by the homeostasis model assessment (HOMA), a mathematical model describing the degree of insulin resistance from fasting plasma glucose and insulin [14, 15]. Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated by multiplying fasting plasma insulin (mU/L) with fasting plasma glucose (mmol/L) divided by 22.5.

*2.4. Fecal Analyses.* Lipids were extracted from feces using a modified version of the protocol by Hara and Radin [16]. In short, around 100 mg lyophilized, grounded feces from each sample were extracted in hexane-isopropanol (3 : 2 v/v) with 0,005% 2,6-di-tert-butyl-4-methylphenol. Five mL of the extract was dried under $N_2$ after which the remaining residue was redissolved in 100 $\mu$L isopropanol containing 1% Triton X-100. The solution was analyzed in triplicates using the triacylglycerol and cholesterol kits used for plasma samples.

*2.5. Extraction and Quantification of Liver Lipids.* The frozen livers were grounded to a powder in a mortar under liquid nitrogen. Samples were prepared in duplicates from ten randomly selected livers from each group and subjected to lipid extraction as previously described. For extraction of hepatic lipids, 20 mg of frozen liver powder was used. The solution containing redissolved lipids was first analyzed in triplicates for cholesterol. After adding another 90 $\mu$L isopropanol 1% Triton X-100 the solution was analyzed for triacylglycerol.

*2.6. Extraction of Polyphenols from Diets.* Five g of each diet was grounded, weighed, and extracted using 25 mL of heptane, ethyl acetate, and methanol, respectively. Samples were stirred during 24 h at room temperature and the extracts were filtered, concentrated, and weighed. Finally, 25 mL of methanol : water : acetic acid (85 : 15 : 0.5; v/v) was used for

Table 1: Composition of diets[1].

| | Lingonberry | Blackcurrant | Bilberry | Raspberry | Açai | Crowberry | Prune | Blackberry | Control | LFD |
|---|---|---|---|---|---|---|---|---|---|---|
| *Calculated energy (kcal)* | | | | | | | | | | |
| Protein | 812.0 | 812.0 | 812.0 | 812.0 | 812.0 | 812.0 | 812.0 | 812.0 | 812.0 | 812.0 |
| Carbohydrate | 1422.4 | 1422.4 | 1422.4 | 1422.4 | 1422.4 | 1422.4 | 1422.4 | 1422.4 | 1422.4 | 2840.4 |
| Starch | 731.2 | 731.2 | 731.2 | 731.2 | 731.2 | 731.2 | 731.2 | 731.2 | 731.2 | 2149.2 |
| Sucrose | 347.2 | 347.2 | 347.2 | 347.2 | 347.2 | 347.2 | 347.2 | 347.2 | 347.2 | 347.2 |
| Fructose | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 |
| Glucose | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 | 172.0 |
| Fat | 1822.5 | 1822.5 | 1822.5 | 1822.5 | 1822.5 | 1822.5 | 1822.5 | 1822.5 | 1822.5 | 405.0 |
| Fiber | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Other | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Total kcals | 4057 | 4057 | 4057 | 4057 | 4057 | 4057 | 4057 | 4057 | 4057 | 4057 |
| *Calculated energy per gram diet (kcal/g)* | | | | | | | | | | |
| kcal/g | 4.2 | 4.1 | 4.3 | 4.3 | 4.2 | 4.2 | 4.6 | 4.5 | 4.5 | 3.7 |
| *Calculated energy (kcal%)* | | | | | | | | | | |
| Protein | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Carbohydrate | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 35 | 70 |
| Fat | 45 | 45 | 45 | 45 | 45 | 45 | 45 | 45 | 45 | 10 |
| Fiber | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Analyzed fiber*[2] *(g/100 g diet)* | | | | | | | | | | |
| Insoluble fiber | 7.4 | 8.2 | 9.3 | 10.8 | 8.8 | 12.5 | 6.3 | 11.9 | 10.1 | 8.5 |
| Soluble fiber | 1.8 | 2.5 | <1 | 1.2 | 1.6 | <1 | 1.1 | <1 | <1 | <1 |
| Total fiber | 9.2 | 10.7 | 10.0 | 12.0 | 10.4 | 13.0 | 7.4 | 12.6 | 10.5 | 9.1 |

[1]All diets were designed to have an equal caloric content of fat, protein, and carbohydrates (including glucose, fructose, and sucrose). LFD: low-fat diet.
[2]Fiber analyzed by Eurofins, Sweden.

polyphenol analysis. The extraction was carried out using 15 min of sonication. Ethyl acetate extracts were dissolved in 2 mL methanol and the soluble fraction was filtered through 0.2 $\mu$m GH polypro membrane to remove insoluble particles and kept at −18°C until analysis.

*2.7. LC-MS Analysis of Polar Polyphenols (Anthocyanins).* The liquid chromatography-tandem mass spectrometry (LC-MS) analyses were performed on a LC-MS Agilent technologies 1260 infinity equipped with an quaternary pump, autosampler, thermostatted column compartment, diode array detector tandem quadrupole LC/MS. A 250 × 4.6 mm i.d. Zorbax SB-$C_{18}$ column, 3.5 $\mu$m particle size, was used at 40°C. The method used is described by Wu et al. [17] using mobile phase A (5% formic acid in water) and B (methanol). The flow rate was 1 mL/min, the temperature used was 40°C, and UV detection was at 520 nm. The gradient used was 5% B, 0–2 min; 5–20% B, 2–10 min; 20% B, 10–15 min; 20–30% B, 15–30 min; 30% B, 30–35 min; 30–45% B, 35–50 min; 45% B, 50–55 min, 45–5% B 55–65 min, 5% B. The injection volume was 20 $\mu$L in all samples. Enhanced product ion mass spectrometry (EPI-MS) analysis was performed in positive mode using a capillary voltage of 3000 V, nebulizer pressure 40 psig, drying gas flow 12 L/min, and drying gas temperature 300°C. The UV-vis, reference times, and mass spectra were used for identification of the peaks and compared to anthocyanin data in the literature [17–19].

*2.8. LC-MS Analysis of Medium Polar Polyphenols.* The LC-MS system together with a 2.1 × 100 mm i.d. 3 $\mu$m Atlantis C18 column was used for the analysis of the mobile phase A (0.1% formic acid in water) and mobile phase B (methanol). The flow rate was 0.3 mL/min, the temperature used was 30°C, and the UV detector was fixed at 360 nm. The injection volume was 20 $\mu$L. The initial gradient elution was used at 6–12% B, 20 min; 12–55% B, 20–50 min. The conditions for MS were set in both positive and negative mode (method described in [20]). Qualitative standards (resveratrol, apigenin, and quercetin-3-O-glucoside) as well as comparison of MS data in the literature were used to enable identification of compounds.

*2.9. Statistical Analysis.* Data are presented as mean ± standard error of the mean (SEM). Unless stated otherwise, results were analyzed by one-way analysis of variance (ANOVA) in conjunction with Dunnett's multiple comparisons test. In cases where Gaussian distribution could not be assumed, groups were compared using Kruskal-Wallis and Dunn's post test. All results are compared to the high-fat control group. Differences with a $P$ value < 0.05 were considered significant. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistical analyses were performed using GraphPad Prism versions 5.0 and 6.0 (GraphPad Software, San Diego, CA, USA).

## 3. Results

*3.1. Energy Intake, Body Weight, and Body Fat Content.* The high-fat control diet induced obesity in mice was compared to the low-fat diet group (Figure 1(a)). In week 12 of the experiment, the mean body weight in the low-fat group was 32±0.9 g, whereas the mean weight of the high-fat fed control group was 42 ± 1.2 g. Body weight was significantly lower in groups receiving lingonberry (33 ± 0.9 g), blackcurrant (36 ± 0.7 g), raspberry (37 ± 1.5 g), and bilberry (38 ± 1.1 g) compared to the high-fat control. Consumption of the açai diet resulted in significantly increased body weight (48 ± 0.6 g). The DEXA scan showed significantly decreased body fat content in the groups receiving lingonberry, blackcurrant, and bilberry compared to the high-fat control. In fact, mice fed the lingonberry-supplemented diet had a body fat content as low as the low-fat diet group. The lean body mass was similar in all groups (Figure 1(c)), except in the group receiving açai where lean mass was increased (+15%) compared to the control. The mass of the epididymal fat pads was expressed per gram lean tissue to take into account differences in body size. The relative size of the fat pad was lower in groups receiving lingonberry and açai, compared to the high-fat control (Figure 1(d)). The accumulated mean energy intake per body weight was similar for all groups, except for mice receiving blackcurrant and bilberry supplementation where a higher food and energy intake was registered (Figure S1).

*3.2. Plasma Parameters and Insulin Resistance Index.* Four-hour fasting plasma glucose levels were significantly reduced in groups receiving lingonberry- and blackcurrant-supplemented diets compared to the high-fat control diet (Figure 2(a)). These groups together with the bilberry and raspberry groups had reduced fasting insulin levels (Figure 2(b)). In addition, the lingonberry, blackcurrant, and bilberry supplementation resulted in a lower HOMA index of insulin resistance (Figure 2(c)). The lingonberry and blackcurrant groups had glucose, insulin, and insulin resistance levels very similar to the group receiving a low-fat diet. A tendency of increased HOMA-IR, glucose, and insulin was observed in mice consuming açai-supplemented high-fat diet compared to control; however, the increase was not significant ($P$ value; 0.07, 0.25 and 0.55, resp.).

The plasma lipid profiles are shown in Table 2. Compared to control, the total plasma cholesterol was significantly lower in groups fed lingonberry, blackcurrant, and low-fat diet whereas it tended to be higher in the acai group ($P = 0.05$). The lingonberry, blackcurrant, and low-fat groups displayed decreased levels of LDL and HDL cholesterol whereas açai had increased HDL cholesterol. However, there were no significant changes regarding the calculated LDL/HDL ratio. Plasma triacylglycerol levels were significantly increased in the group receiving blackcurrant and in the low-fat control. There were no significant differences in circulating nonesterified fatty acids.

*3.3. Liver Lipid Accumulation and Liver Function.* The liver masses (relative to lean body mass) were significantly lower in mice fed a diet supplemented with lingonberries compared to control ($P = 0.009$), whereas açai supplementation led to significantly increased liver mass ($P < 0.0001$) (Figure 3(a)). The plasma levels of the enzyme ALT, a marker of liver

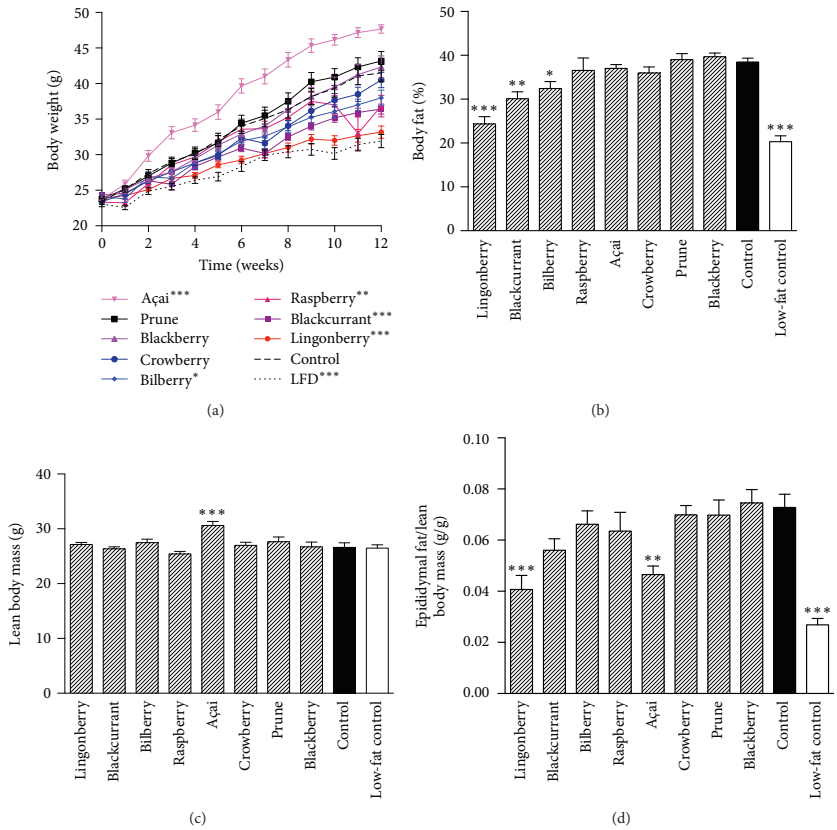


Figure 1: Body weight and composition after 13 weeks of high-fat diet and berry supplementation. (a) Weekly body weight registration. Statistical comparisons of body weight compared to control were made using a two-way ANOVA with Bonferroni post test. The stars represent significant differences in body weight at the last time point of weight registration before ending the study. Body fat (b) and lean body mass (c) were recorded using DEXAscan technique at week 13 of the study. (d) Epididymal white adipose tissue weight related to lean body mass after 13 weeks on the different diets ($n$ = 11-12). Values represent mean ± SEM, $n$ = 12 mice/group. Mean values significantly different from the control are denoted with $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

dysfunction, were significantly elevated in the açai group compared to all groups except the blackberry and control groups (Figure 3(b)). Compared to the high-fat control, ALT levels were significantly reduced in groups receiving lingonberry and blackcurrant as well as bilberry. The liver contents of triacylglycerol (Figure 3(c)) were markedly decreased in the mice receiving supplement of lingonberries, blackcurrant, and, to a lower degree, bilberries whereas açai induced an increase in liver triacylglycerol. Lingonberries, bilberries, and crowberries diets reduced liver cholesterol content compared to the high-fat control (Figure 3(d)). All the studied liver parameters were decreased in the low-fat diet compared to the high-fat control.

*3.4. Effect of Berry Supplementation on Fecal Excretion and Cecal Weight.* The total amount of feces collected over 24 h as well as fecal excretion of triacylglycerol and cholesterol is presented in Table 3. There were no significant differences in total amount of excreted feces, whereas fecal content of cholesterol was elevated in the bilberry, açai, crowberry, blackberry, and low-fat control group. Compared to control, the amount of excreted triacylglycerol was significantly higher in all groups except the low-fat control and the group receiving prune. The first part of the large intestine (cecum) is a site for bacterial fermentation. The mass of the cecum, including content, was increased in all groups compared to control except in the raspberry group (Table 3). In this study, bilberry

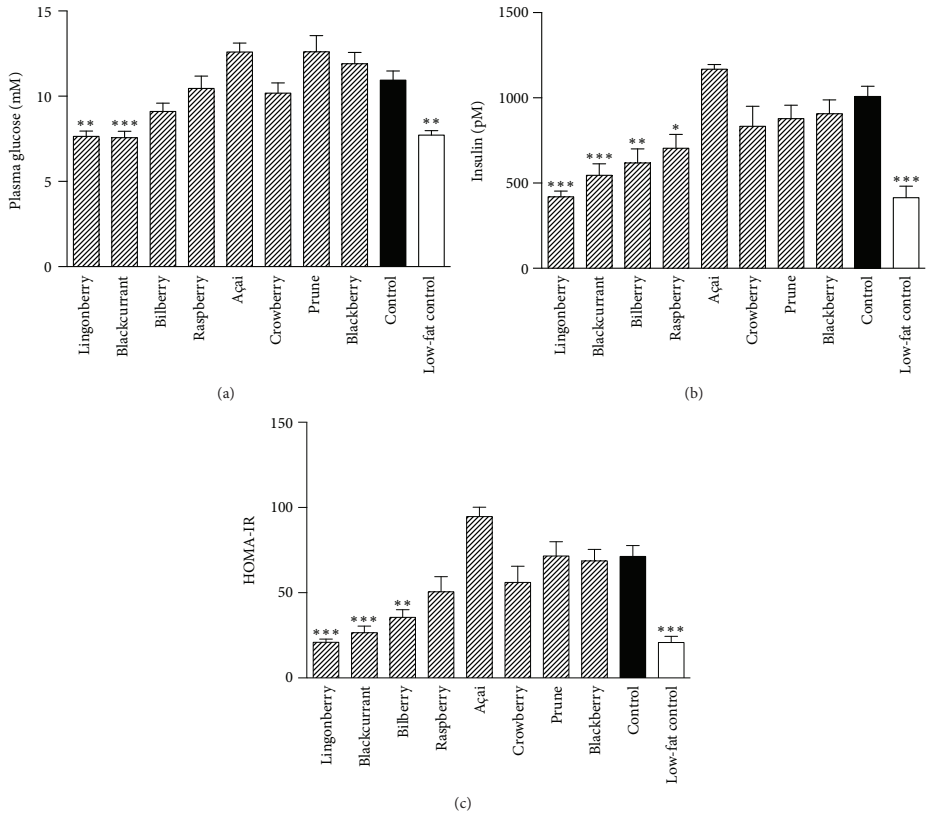


Figure 2: Glycemic control in mice after 13 weeks of high-fat diet and berry supplementation. Plasma glucose (a) and insulin (b) concentrations after 4-hour fasting were used to calculate HOMA-IR index (c). Results represent mean ± SEM, $n = 11$-12. Values significantly different from the control are denoted: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2: Plasma lipid profiles (mM) in mice after 13 weeks on high-fat diets supplemented with berries[1].

| | Triacylglycerol | | Total cholesterol | | HDL cholesterol | | LDL cholesterol | | LDL/HDL ratio | | NEFA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| Lingonberry | 0.65 | 0.06 | $2.6^{***}$ | 0.11 | $1.2^{***}$ | 0.04 | $1.0^{**}$ | 0.06 | 0.84 | 0.04 | 0.56 | 0.04 |
| Blackcurrant | $0.83^{*}$ | 0.04 | $2.9^{**}$ | 0.14 | $1.4^{**}$ | 0.06 | $1.1^{*}$ | 0.09 | 0.76 | 0.06 | 0.66 | 0.03 |
| Bilberry | 0.59 | 0.06 | 3.4 | 0.15 | 1.7 | 0.06 | 1.5 | 0.08 | 0.87 | 0.03 | 0.57 | 0.06 |
| Raspberry | 0.66 | 0.03 | 3.6 | 0.24 | 1.8 | 0.13 | 1.5 | 0.14 | 0.87 | 0.05 | 0.54 | 0.04 |
| Açai | 0.59 | 0.03 | 4.3 | 0.13 | $2.1^{*}$ | 0.08 | 1.9 | 0.07 | 0.90 | 0.04 | 0.49 | 0.03 |
| Crowberry | 0.56 | 0.04 | 4.1 | 0.11 | 2.0 | 0.05 | 1.8 | 0.08 | 0.91 | 0.04 | 0.54 | 0.05 |
| Prune | 0.57 | 0.03 | 4.1 | 0.15 | 2.0 | 0.06 | 1.8 | 0.15 | 0.94 | 0.08 | 0.45 | 0.02 |
| Blackberry | 0.67 | 0.02 | 4.0 | 0.17 | 1.9 | 0.08 | 1.8 | 0.13 | 0.92 | 0.06 | 0.60 | 0.04 |
| Control | 0.64 | 0.03 | 3.7 | 0.16 | 1.8 | 0.07 | 1.6 | 0.11 | 0.88 | 0.05 | 0.56 | 0.05 |
| Low-fat control | $0.84^{*}$ | 0.09 | $2.4^{***}$ | 0.15 | $1.1^{***}$ | 0.06 | $0.85^{***}$ | 0.13 | 0.76 | 0.12 | 0.67 | 0.05 |

[1]Mean values significantly different from control are depicted with $^{*}P < 0.05$, $^{**}P < 0.01$, respectively, $^{***}P < 0.001$. SEM: standard error of the mean. $n = 12$ mice from all groups except $n = 11$ in the lingonberry group in low density-lipoprotein LDL and low-/high-density lipoprotein LDL/HDL cholesterol. NEFA: nonesterified fatty acids.

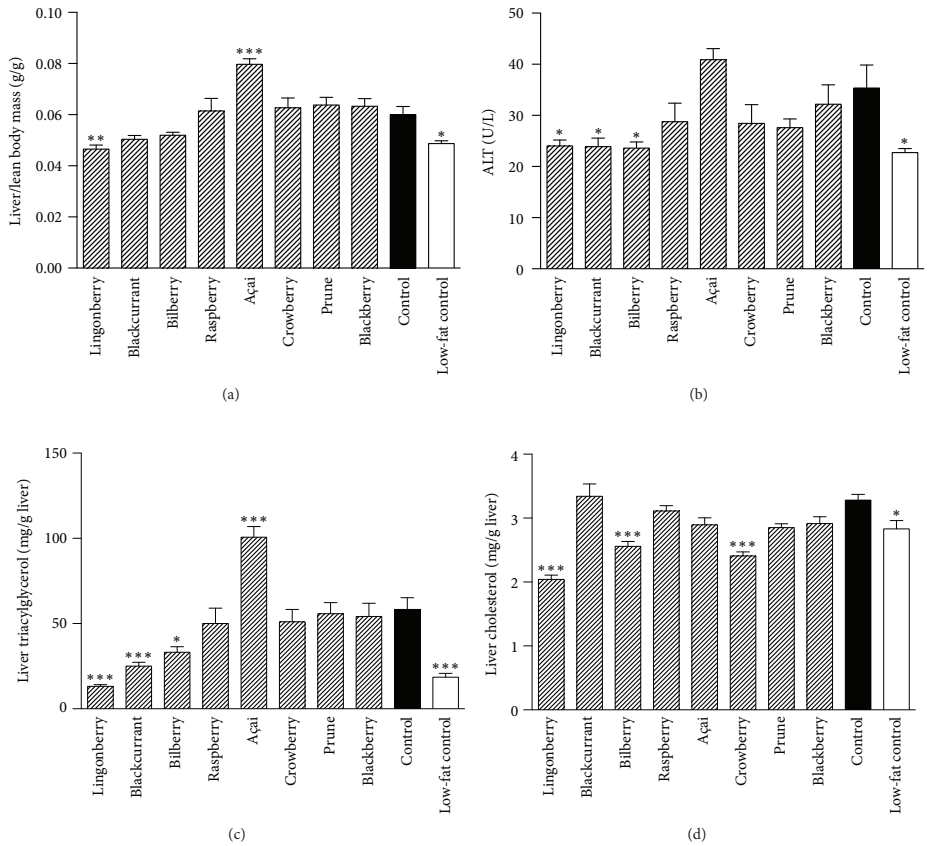


Figure 3: Effects of berry supplementation on liver size, function and lipid accumulation. (a) Liver weights, expressed as gram per gram lean body mass, after 13 weeks of high-fat diet supplemented with different berries. (b) Plasma alanine aminotransferase (ALT) concentration (units per liter). Liver triacylglycerol (c) and cholesterol (d) content, expressed as mg per g liver weight. Data are means ± SEM, $n = 10$–12. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and lingonberry-supplemented diets gave rise to the largest cecum masses.

*3.5. Effects of Berry Supplementation on Inflammation.* In order to assess the effects of berry supplementation on low-grade inflammation, plasma levels of the inflammation markers PAI-1 (Figure 4(a)) and TNF-$\alpha$ were measured. The PAI-1 concentration was lower in plasma from mice receiving lingonberries, blackcurrants, bilberries, and low-fat diet compared to control. The PAI-1 levels in mice receiving açai were elevated compared to other groups, although not significantly compared to control. Plasma TNF-$\alpha$ was below the detection limit in all samples. Spleen size is sometimes analyzed as a reflection of inflammatory activity [21]. In this study, the spleen mass relative to lean tissue mass was significantly lower in the lingonberry group compared to high-fat control (Figure 4(b)).

*3.6. Polyphenolic Compounds in Berry Diets.* The polyphenols detected in the different berry diets are displayed in Table 4. Anthocyanins were present in extracts from all experimental diets, except the prune diet. However, medium polar polyphenols were only detected in the lingonberry and blackcurrant diets. Quercetin-3-O-glucoside and quercetin-3-O-galactoside were identified in both lingonberry and blackcurrant diets and in agreement with the earlier

Table 3: Effect of berry supplementation on cecum weight, total fecal excretion, and lipid excretion[1].

| | Cecum weight (g) | | Dry feces (g/c/24 h) | | Fecal triacylglycerol (g/c/24h) | | Fecal cholesterol (g/c/24 h) | |
|---|---|---|---|---|---|---|---|---|
| | Mean ($n$ = 12) | SEM | Mean ($n = 4^{\dagger}$) | SD | Mean ($n = 4^{\dagger}$) | SD | Mean ($n = 4^{\dagger}$) | SD |
| Lingonberry | $0.64^{***}$ | 0.028 | 2.44 | 0.15 | $6.22^{***}$ | 1.22 | 3.74 | 0.93 |
| Blackcurrant | $0.35^{**}$ | 0.022 | 2.12 | 0.45 | $10.68^{***}$ | 3.45 | 2.80 | 0.39 |
| Bilberry | $0.78^{***}$ | 0.035 | 2.86 | 0.25 | $15.18^{***}$ | 1.61 | $5.88^{***}$ | 1.21 |
| Raspberry | 0.29 | 0.013 | 2.85 | 0.40 | $7.89^{***}$ | 1.36 | 3.74 | 0.34 |
| Açai | $0.50^{***}$ | 0.031 | 3.18 | 0.55 | $5.57^{**}$ | 1.64 | $5.75^{***}$ | 1.03 |
| Crowberry | $0.42^{***}$ | 0.030 | 3.41 | 0.21 | $9.50^{***}$ | 1.83 | $7.52^{***}$ | 0.93 |
| Prune | $0.38^{***}$ | 0.023 | 2.25 | 0.12 | 0.96 | 0.26 | 3.91 | 0.53 |
| Blackberry | $0.35^{**}$ | 0.019 | 3.15 | 0.34 | $4.73^{**}$ | 1.16 | $2.33^{*}$ | 0.41 |
| Control | 0.21 | 0.012 | 2.85 | 0.24 | 1.40 | 0.55 | 3.60 | 0.21 |
| Low-fat control | $0.32^{*}$ | 0.036 | 2.68 | 0.30 | 0.77 | 0.14 | $2.26^{*}$ | 0.15 |

[1]Statistical comparisons of cecum weight were made using ANOVA and Dunnett's posttest, all groups compared to control, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Remaining statistical comparisons to control were made using repeated-measures two-way ANOVA and Sidak's posttest. SEM: standard error of the mean; SD: standard deviation. $^{\dagger}$The number refers to the number of observations (two cages (c) per group analyzed over 24 hours, two days in a row). Six mice were housed in each cage.

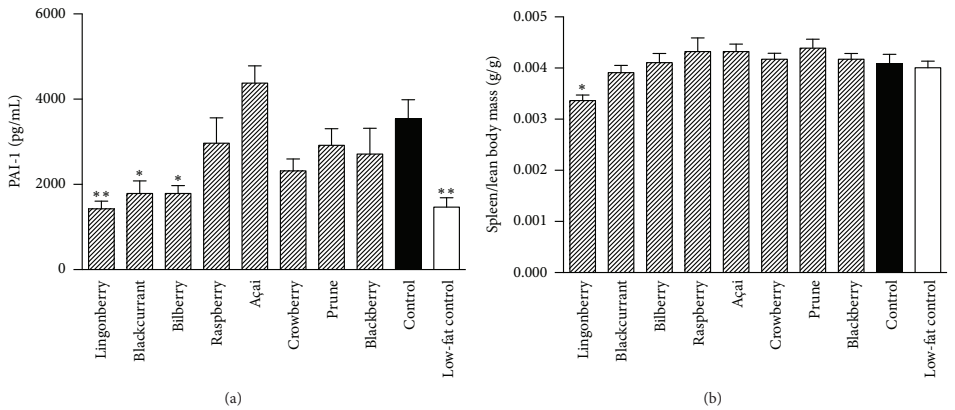


Figure 4: Effect of berry supplementation on inflammatory markers. (a) The plasma concentration of PAI-1 (plasminogen activator inhibitor 1), mirroring low-grade inflammation, was reduced in mice receiving lingonberries, blackcurrants, and bilberries compared to control. Spleen weight (b) related to lean body mass was reduced in the lingonberry group compared to the control. Data are means ± SEM, $n$ = 10–12. $^{*}P < 0.05$, $^{**}P < 0.01$.

literature [20] quercetin-3-O-$\alpha$-rhamnoside (quercitrin), kaempferol-deoxyhexoside, and quercetin-3-O-(4″-HMG)-$\alpha$-rhamnoside were identified in the lingonberry diet. Resveratrol and apigenin were not detected in our analysis. No polyphenol signal was detected in the control diets.

## 4. Discussion

In this study, we show that intake of certain species of berries can prevent weight gain and counteract the metabolic derangements induced by a high-fat diet. Dietary intake of lingonberries prevented adiposity, hepatic lipid accumulation, alleviated hyperglycemia, hyperinsulinemia, and dyslipidemia and decreased plasma PAI-1 and ALT levels in mice fed a high-fat diet. Blackcurrants and, to a lower extent, bilberries and raspberries had similar effects, whereas neither crowberries, prunes, nor blackberries caused any significant improvements of metabolic parameters in this study.

The almost complete prevention of body weight gain observed in the group receiving lingonberry-supplemented diet is an effect of reduced adiposity. Our finding is in agreement with a study in Wistar rats, in which it was shown that lingonberry extracts favorably affected antioxidant defense enzymes, but it was also apparent that the lingonberry extracts reduced weight gain compared to the control [22]. However, to the best of our knowledge, there

Table 4: Polyphenolic composition of berry diets.

| Anthocyanins | Lingonberry | Blackcurrant | Bilberry | Raspberry | Açai | Crowberry | Prune | Blackberry |
|---|---|---|---|---|---|---|---|---|
| Cyanidin-3-glucoside | x | x | x | x | x | x | | x |
| Cyanidin-3-galactoside | x | | x | | | x | | |
| Cyanidin-3-arabinoside | x | | x | | | x | | |
| Cyanidin-3-sophoroside | | | | x | | | | |
| Cyanidin-3-rutinoside | | | | x | x | | | |
| Cyanidin-3- (6″-malonyl)-glucoside | | | | | | x | | x |
| Delphinidin-3-rutinoside | | x | | x | | | | |
| Delphinidin-3-glucoside | | | x | | | x | | |
| Delphinidin-3-galactoside | | | x | | | x | | |
| Delphinidin-3-arabinoside | | | x | | | x | | |
| Petunidin-3-rutinoside | | | | x | | | | |
| Petunidin-3-galactoside | | | x | | | x | | |
| Petunidin-3-glucoside | | | x | | | | | |
| Petunidin-3-arabinoside | | | x | | | x | | |
| Peonidin-3-rutinoside | | | | x | x | | | |
| Peonidin-3-galactoside | | | x | | | x | | |
| Peonidin-3-glucoside | | | x | | | | | |
| Peonidin-3-arabinoside | | | | | | x | | |
| Malvidin-3-galactoside | | | x | | | x | | |
| Malvidin-3-glucoside | | | x | | | | | |
| Malvidin-3-arabinoside | | | x | | | x | | |
| *Medium polar polyphenols* | | | | | | | | |
| Quercetin-3-O-glucoside | x | x | | | | | | |
| Quercetin-3-O-galactoside | x | x | | | | | | |
| Quercetin-3-O-$\alpha$-rhamnoside | x | | | | | | | |
| Kaempferol-deoxyhexoside | x | | | | | | | |
| Quercetin-3-O-(4″-HMG)-$\alpha$-rhamnoside | x | | | | | | | |

are no other publications addressing the antiobesity effect of lingonberries. Increased fat excretion is unlikely to entirely explain the protection against adiposity since fecal excretion of triacylglycerol was not elevated in the lingonberry group compared to groups receiving some of the other berries. Also, there was no correlation between body weight and dietary intake of soluble, insoluble, or total fiber (data not shown). Interestingly, blackcurrant supplementation efficiently prevented weight gain and body fat accumulation despite an increased energy intake. However, the monitoring of caloric intake was based on registered food intake and due to texture, the blackcurrant diet gave rise to a higher spillage than other diets which could be incorrectly interpreted as a higher food intake. The bilberry group also had a higher food intake compared to control. The increased excretion of triacylglycerol in bilberries, blackcurrants, and raspberries could be caused by reduced energy absorption and explain some of the beneficial effects on adiposity. This potential mechanism should be further investigated since increased fat excretion also was observed by supplementation with crowberry, acai, and blackberries, without preventing weight gain. Polyphenol-rich extracts have been shown to inhibit pancreatic lipase [23, 24] although it remains to be established if this mechanism operates also in vivo.

Approximately 70–80% of patients with type 2 diabetes suffer from nonalcoholic fatty liver disease (NAFLD), which is linked to increased risk of cardiovascular disease [25, 26]. Fat accumulation in the liver is associated with impaired hepatic insulin sensitivity [27–29] and production of inflammatory markers [30, 31]. Interestingly, lingonberry supplementation significantly reduced liver mass and liver lipid accumulation. Elevated ALT levels in plasma correlate with reduced liver function and steatosis and predicts type 2 diabetes [32–34]. In this study, the significant reduction in liver triacylglycerol and ALT levels in the lingonberry, blackcurrant, and bilberry groups implies protection against liver steatosis and subsequent improved liver function. Our findings are supported by a 20-week dietary intervention study where a diet rich in berries (bilberries, sea buckthorns, blackcurrants, and lingonberries) reduced ALT in overweight women [35]. However, in a follow-up study using only bilberries and sea buckthorn no effects on ALT-levels were observed; thus, the authors concluded that either the lingonberries and/or blackcurrants were responsible for modulating hepatic lipid metabolism in a positive direction with lower ALT as a result [36]. In contrast to the beneficial effects observed following intake of lingonberry, blackcurrant, or bilberry, livers dissected from mice receiving supplementation with açai were

large and visibly more whitish than other groups, indicating liver steatosis. Analysis revealed that the livers weighed more and had higher triacylglycerol content, and mice receiving açai had significantly higher plasma ALT levels than the rest of the berry groups, but not compared to the control group. Taken together, this suggests that açai promotes rather than prevents fatty liver. However, lipid accumulation per se may not be causal for the insulin resistance associated with fatty liver [37].

Mice fed lingonberries, blackcurrants, and bilberries had lower fasting glucose and/or insulin, resulting in a lower HOMA-insulin resistance index (Figure 2). Thus our data indicate that these three berries protected against high-fat induced insulin resistance. The improved glucose control may be an effect of the reduced adiposity. However, in humans, ingestion of lingonberries has been shown to abolish and decrease, respectively, the postprandial hyperglycemic response of ingesting glucose or sucrose in normal-weight healthy subjects [38, 39]. A cell-assay based bioactivity screening of plants traditionally used to treat symptoms related to diabetes in a native Canadian population identified an extract of lingonberries as having the highest antidiabetic potential [40]. A follow-up study by Eid et al. revealed that a lingonberry extract stimulated glucose uptake into muscle cells by activating the AMP-activated protein kinase (AMPK) pathway [41]. Also, a bilberry extract fed to diabetic KK-$A^y$ mice ameliorated hyperglycemia and enhanced insulin sensitivity accompanied by AMPK activation [10].

In the polyphenolic characterization of the diets, quercetin-3-O-glucoside and quercetin-3-O-galactoside were found in the lingonberry and the blackcurrant diets. Lingonberries and bilberries belong to the *Vaccinium* genus and Eid et al. proposed that the traditional use of this genus to treat diabetes is due to the ability of quercetin and some of its glucosides to transiently inhibit ATP synthase and activate AMPK [41]. In a study by Kobori et al., supplementation with 0.05% quercetin alleviated hepatic fat accumulation and decreased visceral fat deposition, hyperglycemia, hyperinsulinemia, dyslipidemia, and inflammation in C57BL/6J mice fed a high-fat Western diet [42]. Since lingonberries and blackcurrants had the most beneficial effects in our study, the finding that quercetin glycosides were detected only in the lingonberry and blackcurrant diets is interesting and will be further evaluated. Moreover, Erlund et al. have shown that daily intake of lingonberries, blackcurrants, and bilberries significantly increase, serum quercetin levels in humans [43].

Berries are in general rich in anthocyanins and several anthocyanins were found in the experimental diets. Many of the detected anthocyanins and quercetin glycosides are associated with relevant bioactivities [11, 42, 44, 45], but our study design does not permit elaboration of causal relationships between specific compounds and health outcomes. The fact that resveratrol was not detected in the diets is somewhat unexpected since its presence in some of the studied berries has been demonstrated [46]. However, contents of polyphenols are known to vary considerably due to different varieties, cultivars, and growing conditions [47].

Obesity and type 2 diabetes are associated with a systemic low-grade inflammation, and it has been suggested that an altered gut microbiota could be the underlying cause of this inflammation [48–50]. Berries are high in fiber and phenolic compounds which could promote or inhibit growth of certain species of bacteria and thereby alter the microbiota. The finding that all berry diets, except raspberries, increased cecum mass suggests a change in microbial fermentative activity. Dietary administration of lingonberries, blackcurrants, and bilberries reduced plasma levels of PAI-1, which indicate an anti-inflammatory effect. In addition, the significantly lower spleen mass observed in the lingonberry-group may reflect a reduced systemic inflammation. Many previous studies on berries have focused on the capacity of antioxidative polyphenols to reduce oxidative stress and prevent cardiovascular disease. Increased PAI-1 concentrations result in reduced fibrinolytic activity and play a key role in atherothrombosis [51]. Elevated PAI-1 is also associated with NAFLD [52]. The reduction of PAI-1 in combination with lower plasma cholesterol levels by lingonberry and blackcurrant supplementation suggests that these berries may be useful in preventing cardiovascular events. However, there was no significant effect on LDL/HDL cholesterol ratio and in the blackcurrant group an increase in plasma triacylglycerol was observed. In contrast, mice receiving açai had increased total cholesterol and PAI-1 in plasma, further questioning the health aspects of this berry.

In conclusion, this study demonstrated that daily supplementation with lingonberries and also blackcurrants and bilberries had pronounced antiobesity and beneficial metabolic effects in high-fat fed C57BL/6J mice. The mechanisms behind the effects should be further evaluated, taking into account lower doses and reproducibility in humans. The capacity of lingonberries to counteract the negative outcomes of an unhealthy diet could be useful in designing dietary intervention strategies aimed at preventing development of obesity and type 2 diabetes.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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# Paper II

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# Berry intake changes hepatic gene expression and DNA methylation patterns associated with high-fat diet☆

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**Abstract**

The liver is a critical organ for regulation of energy homeostasis and fatty liver disease is closely associated with obesity and insulin resistance. We have previously found that lingonberries, blackcurrants and bilberries prevent, whereas açai berries exacerbate, the development of hepatic steatosis and obesity in the high-fat (HF)-fed C57BL/6J mouse model. In this follow-up study, we investigated the mechanisms behind these effects. Genome-wide hepatic gene expression profiling indicates that the protective effects of lingonberries and bilberries are accounted for by several-fold downregulation of genes involved in acute-phase and inflammatory pathways (e.g. *Saa1*, *Cxcl1*, *Lcn2*). In contrast, açai-fed mice exhibit marked upregulation of genes associated with steatosis (e.g. *Cfd*, *Cidea*, *Crat*) and lipid and cholesterol biosynthesis, which is in line with the exacerbation of HF-induced hepatic steatosis in these mice. *In silico* transcription factor analysis together with immunoblot analysis identified NF-κB, STAT3 and mTOR as upstream regulators involved in mediating the observed transcriptional effects. To gain further insight into mechanisms involved in the gene expression changes, the HELP-tagging assay was used to identify differentially methylated CpG sites. Compared to the HF control group, lingonberries induced genome-wide hypermethylation and specific hypermethylation of *Ncor2*, encoding the corepressor NCoR/SMRT implicated in the regulation of pathways of metabolic homeostasis and inflammation. We conclude that the beneficial metabolic effects of lingonberries and bilberries are associated with downregulation of inflammatory pathways, whereas for blackcurrants, exerting similar metabolic effects, different mechanisms of action appear to dominate. NF-κB, STAT3 and mTOR are potential targets of the health-promoting effects of berries.
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*Keywords:* Berries; Inflammation; Liver steatosis; Gene expression; Methylation; High-fat diet

## 1. Introduction

Overweight and obesity are health issues that continue to increase and are now affecting almost 40% of the global adult population [1]. Obesity is associated with metabolic changes including insulin resistance, nonalcoholic fatty liver disease (NAFLD), low-grade inflammation and dyslipidemia, which may lead to development of type 2 diabetes (T2DM) [2,3]. The liver is a critical organ for the regulation of whole body energy homeostasis due to its central role in lipid and glucose metabolism, as well as its close connection via the portal vein to nutrient uptake in the intestine. The prevalence of NAFLD is increasing in parallel with the epidemic of obesity and insulin resistance that is coupled to the diet of the Western lifestyle [3,4]. High-fat (HF) diets have been shown to cause dysregulated hepatic gene expression with perturbations in lipid, cholesterol, inflammatory and oxidative pathways [5]. NAFLD is characterized by accumulation of lipids and lipid derivates sensitizing the liver for further damage by inflammation and fibrosis, potentially by a second hit involving oxidative stress [6,7]. The hepatic fat accumulation may derive from increased dietary lipid intake, increased lipid synthesis (*de novo* lipogenesis) and/or decreased oxidation. In addition, obesity and T2DM states may associate with increased uptake of free fatty acids from adipose tissues into nonadipose tissues such as the liver [7,8].

The types of foods we eat are important for maintenance of a healthy body weight. It is possible that increased consumption of vegetables and fruit protects against body weight gain [9], thereby

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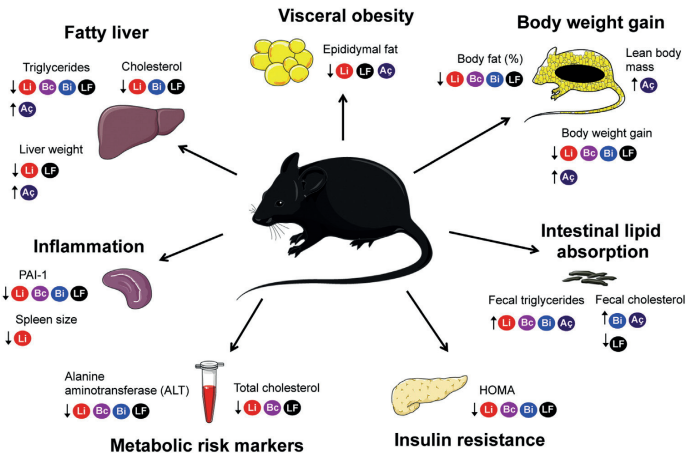



Fig. 1. **Overview of metabolic effects of supplementing HF diet with different berries.** Mice received HF diet without supplementation or HF diet supplemented with 20% lingonberry (Li), blackcurrant (Bc), bilberry (Bi) or açai (Aç) for 13 weeks [10]. One group received a low-fat (LF) diet. Arrows indicate the statistically significant effects ($P<.05$ or less) compared to the control mice receiving HF diet. PAI-1 and ALT are markers for inflammation and liver dysfunction, respectively. Homeostatic model assessment (HOMA) index reflects the level of insulin resistance. Illustrations were obtained and modified from Servier Medical Art by Servier, http://www.servier.com/Powerpoint-image-bank, licensed under Creative Commons Attribution 3.0 Unported License, http://creativecommons.org/licenses/by/3.0/.

preventing obesity and NAFLD. In a previous study [10], we found that addition of different berries to HF diets can prevent development of obesity and fatty liver in C57BL/6 mice, a mouse model used to study diet-induced obesity and prediabetes [11]. The previous findings are schematically described in Fig. 1. In brief, mice were protected against HF-induced weight gain when the diet was supplemented with lingonberries (−21%), blackcurrants (−14%) or bilberries (−10%), whereas açai promoted weight gain (+14%) [10]. Furthermore, mice fed lingonberries, blackcurrants and bilberries showed a drastic reduction of fat accumulation in liver (−77%, −57% and −43% mg/g liver), whereas the açai berry had the opposite effect (+73%) compared to mice receiving a HF diet without berries. In addition, plasma levels of the liver injury marker alanine-aminotransferase (ALT) were reduced by around 30% by lingonberries and blackcurrants. Characteristic of colorful berries is that they are rich in polyphenolic compounds, which are proposed to have a range of health properties [12]. Polyphenols are generally metabolized in the intestine and liver, they recirculate in the enterohepatic circulation and certain metabolites have been suggested to accumulate in association with hepatic fat droplets and immune cells [12,13]. Diets rich in natural antioxidants [14] as well as Nordic berries [15] have been shown to improve liver function in humans. However, very little is known about what pathways in the liver are affected by berries *in vivo*. Both the abovementioned human studies and our recent mouse study suggest that the liver is a major site of action for the beneficial health effects of berries. In order to get insight into the mechanisms underlying these effects, we sought to follow up the findings by in-depth characterization of livers from the same cohort of animals as in our previous study [10].

Here, expression microarrays were employed to analyze the hepatic transcriptome in mice that were protected against HF-induced obesity and liver steatosis (i.e. HF diets supplemented with lingonberries, blackcurrants or bilberries) and compared to mice that were not protected (HF control) or with even increased obesity and fatty liver (HF diet with açai). Furthermore, changes in DNA methylation of CpG sites, which represent a potential mechanism by which nutrients and natural compounds may regulate gene expression [16,17], were assessed as genome-wide DNA methylation of individual CpG sites in livers from mice receiving lingonberry — the berry with the most pronounced health-promoting effects according to our previous study [10].

## 2. Methods and materials

### 2.1. Animals and study design

The liver tissue used for gene expression analysis was taken from male C57BL/6JBomTac mice receiving HF diets (45 kcal% fat) supplemented with 20% (w/w) of different freeze-dried berries for 13 weeks [10]. The general composition (kcal%) of the diets was as follows: for the HF diets, 45% fat, 35% carbohydrate and 20% protein; for the low-fat (LF) diet, 10% fat, 70% carbohydrates and 20% protein. In addition, the diets were formulated to have the same glucose, fructose and sucrose content. The study was approved by the Animal Ethics Committee in Lund, Sweden, (Permit Number: M185-11) and was in accordance with the Council of Europe Convention (ETS 123). Dietary composition as well as phenotypical and metabolic characteristics of the mouse study is described in Ref. [10]. In the current study, liver tissue and plasma ($n$=6–12) were used from 4-h-fasted mice receiving the following diets: HF diet (control group), LF diet (10 kcal% fat) or HF diet supplemented with lingonberries, blackcurrants, bilberries or açai.

### 2.2. DNA and RNA isolation from liver

DNA and RNA were extracted with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) and purity and concentration were determined using spectrophotometry (NanoDrop). RNA integrity was evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Loveland, CO, USA).

### 2.3. Microarray processing

Global gene expression profiles in livers were determined by the Swegene Center for Integrative Biology Genomics DNA Microarray Resource Center (SCIBLU, Lund, Sweden) using MouseWG-6 v2.0 Whole-Genome Expression Beadchips (Illumina, San Diego, CA, USA). Expression analysis was conducted on 6 randomly selected mice (from a total of 12) per diet group, giving 36 microarrays in total. Images and raw signal intensities were acquired using the Illumina BeadArray Reader scanner. Data preprocessing and quantile normalization were performed using Illumina GenomeStudio software. The data have been deposited in NCBI's Gene Expression Omnibus (GEO) database (accession number GSE66711).

### 2.4. Microarray data analysis

Data analysis was done using the R software environment (version 3.0) and the 'limma' package [18]. Raw data together with negative control probes were imported and quantile normalized. All samples were thoroughly quality controlled by assessing signal-to-noise ratios and manual inspection of MA and NUSE plots and deemed to pass. Probes with a detection $P$ value <.05 in at least three samples were kept for further analysis. Differentially expressed probes were identified by fitting a linear regression model comparing each berry diet to the HF control diet. To illustrate group similarities, Venn diagrams were calculated and illustrated using the limma package. To interpret functional changes in the datasets, differentially expressed genes (compared to HF control) from each group were annotated with Gene Ontology (GO) biological process and analyzed with Kyoto encyclopedia of genes and genomes (KEGG) pathway using the Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (DAVID, http://david.abcc.ncifcrf.gov). Significantly upregulated or downregulated probes (FDR<.05) from each diet group were separately uploaded to DAVID. The following annotation categories were used: GO Biological Process_ALL (GOTERM_BP_ALL, includes all go-terms) and GOTERM_BP_FAT (mildly filtered list) and KEGG_PATHWAY to create functional annotation chart reports. Pathways with an EASE score (a modified Fisher's Exact $P$ value) less than <.05 were selected for further analysis. To illustrate trends in gene expression, genes were put into context with other genes involved in similar pathways based on the information from the GO processes, KEGG pathways as well as scientific literature. Descriptions of the encoded proteins were derived using the UniProt database and NCBI/PubMed literature search.

The 'upstream regulator' application in Ingenuity Pathway Analysis (IPA, www.ingenuity.com, Redwood City, CA, USA) was used to determine likely upstream regulators that are connected to dataset genes through a set of direct or indirect relationships. Lists with Illumina identifiers and associated log ratios of differentially regulated genes ($P$<.05) were used for IPA analysis. Due to the large metabolic effects observed by lingonberry supplementation, the analysis was focused on differences between the lingonberry and the HF control group. The computed $z$-score, a measure of significance as well as predictor for the activation state of each regulator, was used to identify activated ($z$-score of more than 2) and inactivated ($z$-score of less than −2) transcription regulators and kinases predicted to regulate gene expression. The detected regulators were ranked on predicted activation based on the $z$-score, number of target genes in the dataset and $P$ value and were validated on protein expression level (Section 2.9).

### 2.5. Validation of microarray data by real-time qPCR

Differentially expressed genes of interest from the microarray analysis were selected for validation by real-time quantitative PCR (qPCR) ($n$=6). Total RNA (1 μg) was treated with DNaseI amplification grade (Invitrogen, Carlsbad, CA, USA) and reversely transcribed using random hexamers (Amersham Biosciences, Piscataway, NJ, USA) and SuperScript II RNaseH reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. The cDNA was used in duplicates for qPCR using TaqMan chemistry (assays on demand; Applied Biosystems, Foster City, CA, USA) or SYBR green chemistry, with an ABI 7900 system (Applied Biosystems). Primers were used to quantify mRNA expression of *Saa1*, *Lcn2*, *Acacb*, *Pparg2*, *Cyp7a1*, *Hmgcr*, *Ncor2* and *Il16* (see Supplementary Table S1 for primer IDs and sequences). The relative quantification of mRNA was calculated using the ΔΔCt method with normalization by geometric average of the genes cyclophilin A (*Ppia*) and ribosomal protein S29 (*Rps29*) [19]. *Ppia* and *Rps29* were chosen as they are well-accepted reference genes and the microarray data indicated that these genes were highly expressed (top 5%) with a low variation between different animals (<1% coefficient of variation).

### 2.6. HELP tagging to test epigenome-wide DNA methylation

HpaII tiny fragment enriched by ligation-mediated PCR (HELP) tagging was used to identify differentially methylated CpG sites (DMRs) in livers from the lingonberry group compared to the HF control group ($n$=4 per group) as described by Suzuki *et al.* [20,21]. HELP tagging is a technique based on restriction enzyme digestion using HpaII (methylation sensitive) together with its isoschizomer MspI (methylation insensitive). Briefly, high-molecular-weight genomic DNA was extracted from livers using dialysis tubing preparation. Five micrograms of DNA was digested with HpaII and ligated to two custom adapters containing Illumina adapter sequences, an EcoP151 recognition site and the T7 promoter sequence. The libraries were sequenced using Illumina Hiseq 2500 sequencer following the manufacturer's instructions. HpaII profiles were obtained for each sample and the methylation scores were calculated using a previously generated MspI mouse reference library.

The data analysis was performed as described by Delahaye *et al.* [22]. Briefly, DNA methylation scores from 0 (fully methylated) to 100 (unmethylated) were filtered based on confidence scores. Confidence scores were determined for each sample by determining the total number of HpaII-generated reads as a function of the total number of MspI-generated reads, excluding loci for which the confidence score was lower than the expected mean by locus. After confidence score filtering, the number of testable loci decreased from >1.5 million to 788,076. The locus-specific angle was compared between the lingonberry and HF control groups and DMRs defined as the ones having an absolute difference in methylation angle value of >5 (which corresponds to ~5% difference in methylation [20]) and a $P$ value of <.05. We obtained 24,304 DMRs. The DMRs were defined in terms of genomic content such as promoter (−2 kb to +2 kb from the transcription start site), gene body and intergenic regions using an arbitrary cutoff. DAVID software was used to identify pathways enriched in genes containing DMRs in the promoter region. All HELP-tagging data were uploaded on the GEO database (GSE67277).

### 2.7. Bisulfite conversion, pyrosequencing and MassArray

All samples used in the genome-wide DNA methylation study ($n$=4 per group) were subjected to bisulfite conversion, PCR and pyrosequencing (Epitect Bisulfite Kit, PyroMark PCR Kit and PyroMark Q96 ID; Qiagen) according to the manufacturer's instructions at Beijing Genomics Institute, Shenzhen, China (pyrosequencing service provider). PCR and sequencing primers were designed using PyroMark Assay Design 2.0 and synthesized by Beijing Genomics Institute to assess percent methylation at 14 loci (Supplementary Table S1). To further validate HELP-tagging data at loci *Ncor2* and *Il16*, bisulfite MassArray was performed on $n$=6 samples per group [23]. Bisulfite conversion was performed using EZ DNA Methylation Gold kit (Zymo Research, California, USA) according to the manufacturer's instruction. Primers are listed in Supplementary Table S1. The Albert Einstein College of Medicine (AECOM) Genomics Shared Facility performed Sequenom MassArray assays according to the company's standard protocol and matched peak data were exported using EpiTYPER software and data were analyzed as described by Thompson *et al.* [24]. The same protocol was used to assess methylation of *Ncor2* and *Il16* ($n$=6 samples per group) in livers from mice in a separate study receiving the same diets for 11 weeks (biological replicate).

### 2.8. Analysis of plasma samples

Plasma previously collected from mice (following fasting for 4 h, $n$=12) receiving the different diets [10] was used for determining concentrations of proteins secreted by liver to validate findings from the microarray gene expression data. Serum amyloid A (SAA) and CXCL1 were measured using enzyme-linked immunosorbent assay kits (PHASE Murine Serum Amyloid A Assay, Tridelta Development, Kildare, Ireland; Mouse CXCL1 Elisa Kit, Nordic Biosite, Täby, Sweden).

### 2.9. Immunoblot analysis

The following groups with the strongest phenotypes were subjected to protein expression analysis: lingonberry, açai, HF and LF diet ($n$=6). In addition, nuclear content of proteins NF-κB subunit p65 and SREBP1c was measured in all groups ($n$=12). Total liver lysates were prepared by homogenization in lysis buffer [50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% (w/v) Nonidet P-40, 1 mM $Na_3VO_4$, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.25 M sucrose, 1 mM dithiothreitol and complete miniprotease inhibitor (1 tablet/10 ml)] followed by centrifugation at 13,000*g* for 10 min at 4°C. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Lysates and nuclear extracts were subjected to polyacrylamide gel electrophoresis on precast NuPAGE Novex gradient gels 4–12% (Invitrogen) and immunoblot analysis. Primary antibodies against the following proteins were used: signal transducer and activator of transcription 3 (STAT3) and phosphorylated STAT3 Tyr705 (pSTAT3), regulatory-associated protein of mTOR (Raptor), acetyl-CoA carboxylase (ACC), pACC Ser79, AMP-activated protein kinase (AMPK) subunit $\alpha_1/\alpha_2$, pAMPK $\alpha_1/\alpha_2$ Thr172, AMPK $\alpha_1$, AMPK $\beta_1/\beta_2$, AMPK $\beta_2$ and AMPK $\gamma_1$ (Cell Signaling Technology, Beverly, MA, USA); sterol regulatory element-binding protein 1c (SREBP1c) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA); and nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) p65 (Abcam, Cambridge, MA, USA). Antibodies against cyclophilin B (CypB) and TATA box-binding protein (TBP), used as loading control for total lysates and nuclear extracts, respectively, were from Abcam. Detection was performed with horseradish-peroxidase-conjugated secondary antibodies (antirabbit: Pierce, Rockford, IL, USA; antimouse: GE Healthcare, Buckinghamshire, UK) and chemiluminescent substrate (SuperSignal West Pico or Femto Maximum Sensitivity Substrate, Thermo Scientific). Images were acquired with a Bio-Rad Chemidoc XRS+ system and the band intensities were quantified using Image Lab 4.0 (Hercules, CA, USA).

### 2.10. Statistical analysis

Data are displayed as boxplots showing the median, quartiles and minimum and maximum values. Unless stated otherwise, results were analyzed by one-way ANOVA in conjunction with Dunnett's multiple comparisons test. In cases where Gaussian distribution could not be assumed, groups were compared using Kruskal–Wallis posttest. The ROUT test was performed to statistically reject outliers with 99% confidence level. The nonparametric Mann–Whitney $U$ test (two-tailed) was used for comparisons involving only two groups. Unless stated otherwise, all results are compared to the HF control group. Differences with a $P$ value <.05 were considered significant: *$P$<.05, **$P$<.01 and ***$P$<.001. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Statistical analysis of microarray and HELP assay and DNA methylation data have been indicated in Sections 2.4 and 2.6 mentioned above.

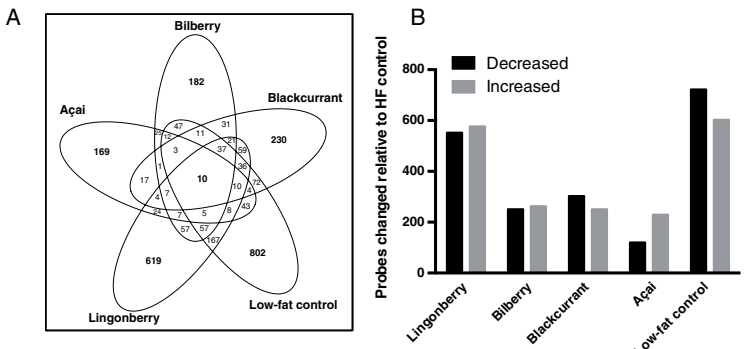
## 3. Results

### 3.1. The effect of berries on hepatic gene expression

Gene transcription profiles in livers from mice receiving different berries were characterized by microarray. Of 46,255 tested probe sets in the microarray, 20,861 had a detection *P* value <.05 in at least three animals and were carried forward for further analysis. When the expression of each gene probe was compared to the HF control group, 2777 unique probes were changed significantly by at least one of the diets (FDR corrected *P* value <.05), and 1975 probes were changed by supplementation with berries (gene expression trends compared to HF control are illustrated in Fig. 2A and B). The microarray results revealed that 10 genes (Fig. 2A and C) were regulated by all diets compared to the HF control. Out of these, the genes *Cidea*, *Anxa2* (markers for steatosis and oxidative stress, respectively) and *Tceal8* were downregulated by all diets except açai, where they were upregulated. The LF diet had the largest number of differentially expressed genes compared to the HF diet group. The number of changed genes was also large in the mice receiving HF diet supplemented with lingonberries, indicating a switch from HF-induced changes in gene expression. The mice in the açai group, which had an obese phenotype similar to the HF control group [10], had the smallest number of differentially expressed genes and thus the most similar expression profile to the HF control group.

### 3.2. General analysis of affected GO processes and pathways

Pathways involving inflammatory response, lipid and cholesterol biosynthesis, glutathione/drug/xenobiotic metabolism and oxidation/




C

| Gene | | Description |
|---|---|---|
| **Genes significantly changed by all diets compared to the HF control group** | | |
| Cidea | ↓Li Bc Bi LF ↑Ac | Cell death activator CIDE-A. Binds to lipid droplets and regulates their enlargement, thereby restricting lipolysis and favoring storage. |
| Anxa2 | ↓Li Bc Bi LF ↑Ac | Annexin A2 (Lipocortin 2). Involved in membrande-trafficking events such as transport of cholesterol ester from caveolae to internal membranes. Potential biomarker of oxidative stress. |
| Tceal8 | ↓Li Bc Bi LF ↑Ac | Transcription elongation factor A protein-like 8; Regulation of transcription. |
| Txnip | ↑Li Bc Bi LF Ac | Thioredoxin-interacting protein inhibits thioredoxin activity. |
| G6pc | ↑Li Bc Bi LF Ac | Glucose-6-phosphatase produces glucose through glycogenolysis and gluconeogenesis, regulating blood glucose levels. |
| Cyp2b13 | ↓Li Bc Bi LF Ac | Cytochrome P450, family 2, subfamily b, polypeptide 13. |
| Dnajb1 | ↓Li Bc Bi LF Ac | Lanosterol synthase; Enzyme in cholesterol biosynthesis pathway. |
| Asb7 | ↓Li Bc Bi LF Ac | NAD(P) dependent steroid dehydrogenase-like; Enzyme in cholesterol biosynthesis pathway. |
| Snx11 | ↓Li Bc Bi LF Ac | Phosphomevalonate kinase; Enzyme in cholesterol biosynthesis pathway. |
| 3300001-P08Rik | ↓Li Bc Bi LF Ac | Unknown |

Fig. 2. **General trends in gene expression derived from microarray analysis of liver from mice receiving HF diet supplemented with different berries.** (A) Venn diagram displaying probes for genes differentially expressed compared to the HF control (FDR>.05). (B) Increased and decreased probes in different berry groups compared to HF control. (C) Name, regulation and function of the 10 genes found to be regulated by all diets.

Table 1
Comparison of GO (biological process) and KEGG pathways significantly changed by at least one diet using DAVID software.

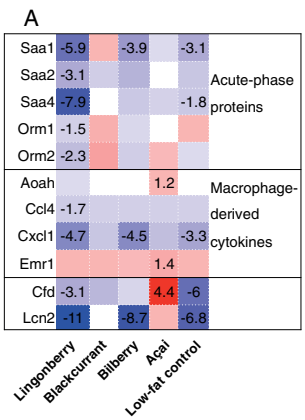
| Decreased pathways | Genes | % | *P* value | Increased pathways | Genes | % | *P* value |
|---|---|---|---|---|---|---|---|
| **Lingonberry** | | | | | | | |
| Acute inflammatory response | 9 | 2.2 | 2.5 E-04 | Metabolism of xenobiotics by cytochrome P450 | 17 | 3.7 | 7.0 E-11 |
| Acute-phase response | 6 | 1.5 | 3.3 E-04 | Drug metabolism | 17 | 3.7 | 5.5 E-10 |
| Lipid metabolic process | 29 | 7.1 | 5.3 E-04 | Glutathione metabolism | 10 | 2.2 | 2.3 E-05 |
| Oxidation reduction | 25 | 6.1 | 6.1 E-03 | Oxidation reduction | 32 | 7.0 | 5.2 E-04 |
| Lipid biosynthetic process | 13 | 3.2 | 1.5 E-02 | ABC transporters | 7 | 1.5 | 2.3 E-03 |
| | | | | Steroid hormone biosynthesis | 4 | 2.1 | 1.6 E-02 |
| | | | | Oxidation reduction | 13 | 6.8 | 3.8 E-02 |
| **Blackcurrant** | | | | | | | |
| Drug metabolism | 13 | 5.5 | 1.6 E-09 | Steroid hormone biosynthesis | 4 | 2.1 | 1.6 E-02 |
| Oxidation reduction | 25 | 10.5 | 9.8 E-06 | Oxidation reduction | 13 | 6.8 | 3.8 E-02 |
| Pyruvate metabolism | 6 | 2.5 | 4.5 E-04 | | | | |
| Glutathione metabolism | 6 | 2.5 | 1.4 E-03 | | | | |
| Lipid biosynthetic process | 10 | 4.2 | 1.4 E-02 | | | | |
| Fatty acid biosynthetic process | 5 | 2.1 | 2.3 E-02 | | | | |
| Glycolysis/gluconeogenesis | 5 | 2.1 | 2.3 E-02 | | | | |
| **Bilberry** | | | | | | | |
| Regulation of cell death | 13 | 6.7 | 1.1 E-02 | Cholesterol biosynthetic process | 14 | 7.3 | 2.3 E-20 |
| Response to wounding | 9 | 4.7 | 2.4 E-02 | Sterol metabolic process | 18 | 9.3 | 6.0 E-18 |
| Defense response | 10 | 5.2 | 3.8 E-02 | Lipid biosynthetic process | 22 | 11.4 | 5.7 E-12 |
| Acute-phase response | 3 | 1.6 | 3.7 E-02 | Steroid biosynthesis | 9 | 4.7 | 4.4 E-11 |
| | | | | Oxidation reduction | 26 | 13.5 | 8.7 E-08 |
| | | | | PPAR signaling pathway | 7 | 3.6 | 1.2 E-03 |
| | | | | Triglyceride metabolic process | 4 | 2.1 | 7.2 E-03 |
| | | | | Primary bile acid biosynthesis | 3 | 1.6 | 2.1 E-02 |
| | | | | Fatty acid metabolic process | 6 | 3.1 | 4.8 E-02 |
| **Açai** | | | | | | | |
| Enzyme-linked receptor protein signaling pathway | 5 | 5.4 | 4.6 E-02 | Lipid metabolic process | 35 | 19 | 1.10 E-15 |
| Cholesterol efflux | 2 | 2.2 | 4.8 E-02 | Lipid biosynthetic process | 24 | 13 | 2.6 E-15 |
| | | | | Sterol biosynthetic process | 11 | 6 | 1.1 E-13 |
| | | | | Cholesterol biosynthetic process | 10 | 5.4 | 3.5 E-13 |
| | | | | Cholesterol metabolic process | 12 | 6.5 | 5.0 E-11 |
| | | | | Oxidation reduction | 20 | 10.9 | 1.9 E-05 |
| | | | | Fatty acid biosynthetic process | 6 | 3.3 | 1.0 E-03 |
| | | | | Pyruvate metabolism | 5 | 2.7 | 1.8 E-03 |
| | | | | PPAR signaling pathway | 6 | 3.3 | 3.3 E-03 |
| | | | | Glutathione metabolism | 5 | 2.7 | 4.3 E-03 |
| | | | | Glycolysis/gluconeogenesis | 5 | 2.7 | 1.6 E-03 |
| | | | | Triglyceride metabolic process | 4 | 2.2 | 4.8 E-03 |
| | | | | Phospholipid biosynthetic process | 4 | 2.2 | 4.4 E-02 |
| **LF diet** | | | | | | | |
| Oxidation reduction | 60 | 11.7 | 2.9 E-18 | Lipid biosynthetic process | 24 | 5.7 | 4.1 E-07 |
| Drug metabolism | 20 | 3.9 | 1.2 E-13 | Lipid metabolic process | 40 | 9.5 | 7.5 E-07 |
| Fatty acid metabolism | 15 | 2.9 | 1.1 E-11 | Carboxylic acid metabolic process | 31 | 7.3 | 1.7 E-06 |
| Metabolism of xenobiotics by cytochrome P450 | 16 | 3.1 | 2.8 E-10 | Cholesterol biosynthetic process | 5 | 1.2 | 2.0 E-03 |
| Lipid metabolic process | 47 | 9.1 | 5.3 E-10 | PPAR signaling pathway | 9 | 2.1 | 2.4 E-03 |
| Fatty acid metabolic process | 22 | 4.3 | 4.0 E-09 | Cholesterol metabolic process | 7 | 1.7 | 6.7 E-03 |
| PPAR signaling pathway | 11 | 2.1 | 7.3 E-05 | Acetyl-CoA biosynthetic process | 3 | 0.7 | 8.1 E-03 |
| Acyl-CoA metabolic process | 6 | 1.2 | 9.4 E-05 | Triglyceride metabolic process | 5 | 1.2 | 1.1 E-02 |
| Glutathione metabolic process | 6 | 1.2 | 2.4 E-04 | Transmembrane receptor protein serine/threonine kinase signaling pathway | 7 | 1.7 | 1.1 E-02 |
| Biosynthesis of unsaturated fatty acids | 6 | 1.2 | 8.7 E-04 | Glycerolipid metabolic process | 9 | 2.1 | 01.3 E-02 |
| Synthesis and degradation of ketone bodies | 4 | 0.8 | 2.4 E-03 | Metabolism of xenobiotics by cytochrome P450 | 6 | 1.4 | 4.7 E-02 |

*Genes* indicates number of genes involved in the enriched term.
% indicates the percentage of the mapped genes/total number of genes.
*P* values were derived from Fisher's Exact Test.
The list is a mix of GOTERM_BP_ALL, GOTERM_BP_FAT and KEGG_PATHWAY generated using DAVID. Where the GO terms overlapped, they were combined and the highest *P* value is displayed.

reduction were affected by several of the berry diets (Table 1) and hence became focus for further analysis (complete dataset in Supplementary Table S2). In general, the lingonberry- and bilberry-fed mice were protected against a HF-induced proinflammatory gene expression, as seen by downregulation of *Saa1* and *Lcn2* (Fig. 3A–C). Several acute-phase proteins were expressed at even lower levels than in the LF diet group (Table 1 and Fig. 3). Lipid-related gene expression was affected by the berry diets (Fig. 4); for example, lingonberries and blackcurrants tended to downregulate genes encoding enzymes involved in lipid synthesis compared to the HF control. Mice receiving açai, bilberries and LF diet had a different profile with several lipid- and cholesterol-synthesizing genes being upregulated (Figs. 4 and 5).

### 3.3. Effect of berry supplementation on inflammation

Several inflammatory factors were among the top hits in the microarray data (Fig. 3A). The mRNA of *Saa* isoforms, *Lcn2* and *Cxcl1*




| Gene | Description |
|---|---|
| **Acute phase proteins** | |
| Saa1;2;4 | Acute-phase serum amyloid A (SAA) 1, 2 and 4; Acute-phase proteins and constitutive SAA protein. |
| Orm1;2 | Alpha-1-acid glycoprotein 1 and 2; Acute-phase proteins. |
| **Macrophage-derived cytokines and markers** | |
| Aoah | Acyloxyacyl hydrolase; Lipase inactivating bacterial lipopolysaccharide. |
| Ccl4 | C-C motif chemokine 4; Chemokine produced by macrophages in response to infection/inflammation. |
| Cxcl1 | Chemokine (C-X-C motif) ligand 1; Cytokine expressed by macrophages. |
| Emr1 | EGF-like module-containing mucin-like hormone receptor-like 1; Macrophage marker F4/80. |
| Cfd | Complement factor D (adipsin); Alternative complement pathway component. |
| Lcn2 | Neutrophil gelatinase-associated lipocalin (NGAL). |

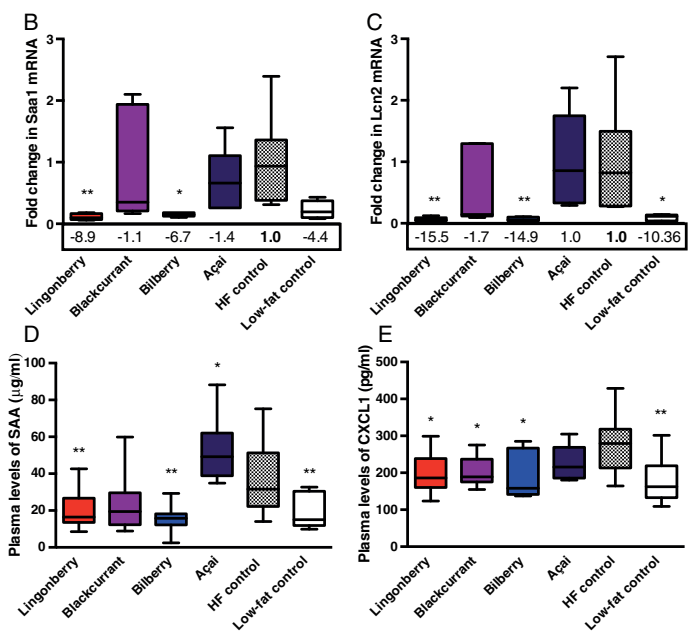
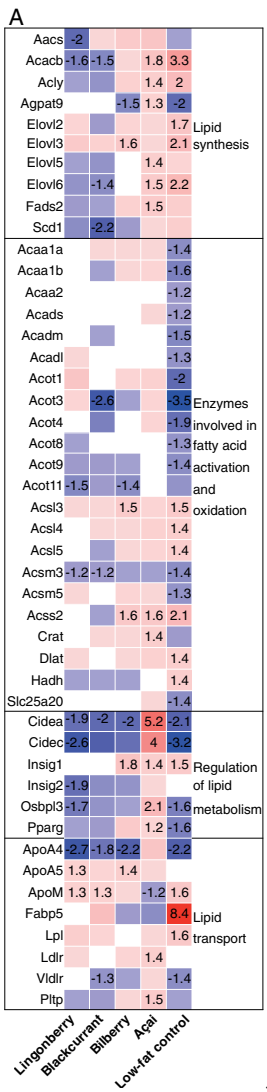



Fig. 3. **Effects on the expression of genes involved in inflammation.** (A) Genes changed by at least one diet involved in acute-phase response, defense response and response to inflammation and wounding (identified using DAVID). The fold change is displayed for genes that were significantly changed compared to the HF control. Shades of red and blue indicate the degree of upregulation or downregulation, respectively. White indicates no difference in fold change compared to the control. (B and C) qPCR results validating microarray results of *Saa1* and *Lcn2*, $n=5–6$, Kruskal–Wallis posttest. (D and E) Plasma concentration of total SAA and CXCL1, $n=10–12$, Dunnett's posttest. Values significantly different from the HF control are depicted $^{*}P<.05$ and $^{**}P<.01$.

were downregulated several-fold in livers from mice receiving lingonberries, bilberries and the LF diet in comparison to HF control mice. Specifically, in the lingonberry group, *Saa1*, *Saa2*, *Saa4*, *Cxcl1*, *Cfd* and *Lcn2* were downregulated 6, 3, 8, 5, 3 and 11 times, respectively, compared to the HF control. The decrease in expression of *Saa1* and *Lcn2* was validated by qPCR (Fig. 3B and C). The acute-phase SAA proteins are induced and secreted by the liver in response to inflammatory stimuli (reviewed in Ref. [25]). In line with the mRNA

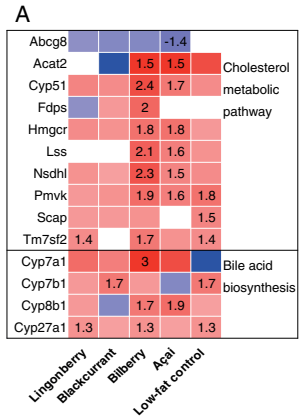



| Gene | Description |
|---|---|
| **Lipid synthesis** | |
| Aacs | Acetoacetyl-CoA synthetase; Activates acetoacetate to acetoacetyl-CoA. |
| Acacb | Acetyl CoA carboxylase beta (ACC2); Inhibits mitochondrial β-oxidation. |
| Acly | ATP citrate lyase; Synthesis of actyl-CoA, role in *de novo* lipid synthesis. |
| Agpat9 | 1-acylglycerol-3-phosphate O-acyltransferase 9; In glycerolipid synthesis. |
| Elovls | Elongation of very long chain fatty acids protein 2;3;5 and 6. |
| Fads2 | Fatty acid desaturase 2; Biosynthesis of highly unsaturated fatty acids. |
| Scd1 | Stearoyl-CoA desaturase 1; Synthesis of unsaturated fatty acids. |
| **Enzymes involved in fatty acid activation and oxidation** | |
| Acaas | Acetyl-CoA acyltransferase A;B and 2; Final step of β-oxidation. |
| Acads | Short; Medium and Long-chain specific acyl-CoA dehydrogenase; Catalyze the initial steps of mitochondrial beta-oxidation. |
| Acots | Acyl-CoA thioesterase 1;3;4;8;9 and 11; Catalyze hydrolysis of acyl-CoA. |
| Acsl3;4;5 | Long-chain-fatty-acid-CoA ligase 3;4 and 5; Convert long-chain fatty acids to acyl-CoA esters activating them for lipid synthesis or β-oxidation. |
| Acsm3;5 | Acyl-CoA synthetase medium-chain family member 3 and 5. |
| Acss2 | Acyl-CoA synthetase short-chain family member 2; Activates acetate. |
| Crat | Carnitine acyltransferase; regulates levels of acetyl-CoA. |
| Dlat | Dihydrolipoamide acetyltransferase; Converts pyruvate into acetyl-CoA. |
| Hadh | Hydroxyacyl-CoA dehydrogenase; Enzyme of mitochondrial β-oxidation. |
| Slc25a20 | Acylcarnitine translocase; Transports acyl-carnitines for β-oxidation. |
| **Regulation of lipid metabolism** | |
| Cidea;c | Cell death activator CIDE-A and C. Regulate lipid droplets. |
| Insig1;2 | Insulin-induced gene 1 and 2; Inhibits processing of SREBPs. |
| Osbpl3 | Oxysterol binding protein-like 3; Intracellular lipid receptor/sensor. |
| Pparg | Peroxisome proliferator-activated receptor gamma (PPARG). |
| **Lipid transport** | |
| ApoAs | Apolipoprotein A4; 5 and M; Association with VLDL and HDL. |
| Fabp5 | Fatty acid binding protein 5; Binds fatty acids, abundant in epidermis. Polymorphisms associated with type 2 diabetes. |
| Lpl | Lipoprotein lipase; Vascular lipase. |
| Ldlr | Low-density lipoprotein receptor; Binds LDL and transports it into cells. |
| Vldlr | Very low-density lipoprotein receptor; Binds VLDL and transports into cells. |
| Pltp | Phospholipid transfer protein; Transfer of lipids between lipoproteins. |

Fig. 4. **Effect on the expression of genes involved in lipid metabolism.** (A) Genes changed by at least one diet involved in pathways related to lipid metabolism, lipid biosynthetic processes, fatty acid and triglyceride metabolic processes, PPAR signaling pathways and glycerolipid metabolic processes (identified using DAVID). The fold change is displayed for genes that were significantly changed compared to the HF control. Shades of red and blue indicate the degree of upregulation or downregulation, respectively. White indicates zero difference in fold change compared to the control. (B and C) qPCR results of gene expression of *Acacb* and *Pparg2*, $n=6$. Values significantly different from the HF control (Dunnett's and Kruskal–Wallis posttest) are depicted *$P<.05$ and ***$P<.0001$.

expression data, the plasma levels of SAA were significantly decreased in plasma from the groups receiving lingonberry, bilberry and LF diet compared to HF control (Fig. 3D) and were elevated in the açai group.

The chemokine CXCL1 plays a role in inflammation by recruiting neutrophils and its expression was significantly downregulated in livers from the lingonberry, bilberry and LF diet compared to the HF




| Gene | Description |
|---|---|
| **Cholesterol metabolic process** | |
| Abcg8 | ATP-binding cassette sub-family G member 8 (sterolin-2); Cholesterol excretion from liver into bile. |
| Acat2 | Acetyl-CoA acetyltransferase 2; Cholesteryl ester synthesis. |
| Cyp51 | Cytochrome P450, family 51 (CYP51); Enzyme in de novo cholesterol biosynthesis pathway (CBP). |
| Fdps | Farnesyl diphosphate synthase; Enzyme in CBP. |
| Hmgcr | 3-hydroxy-3-methylglutaryl-CoA reductase; Rate limiting step of cholesterol synthesis. |
| Lss | Lanosterol synthase; Enzyme in CBP. |
| Nsdhl | NAD(P) dependent steroid dehydrogenase-like; CBP. |
| Pmvk | Phosphomevalonate kinase; Enzyme in CBP. |
| Scap | Sterol regulatory element-binding protein cleavage-activating protein; Regulates export of SCAP/SREB at low cholesterol. |
| Tm7sf2 | Transmembrane 7 superfamily member 2; In CBP. |
| **Bile acid biosynthesis** | |
| Cyp7a1 | CYP7A, polypeptide 1 (CYP7A1); Catalyzes rate limiting step of conversion of cholesterol to bile acids. |
| Cyp7b1 | CYP7B1; Involved in conversion of cholesterol to bile acids. |
| Cyp8b1 | CYP8B1; Regulates ratio of chenodeoxy- and cholic acid. |
| Cyp27a1 | CYP27A1 (sterol 27-hydroxylase) ; Involved in conversion of cholesterol to bile acids. |

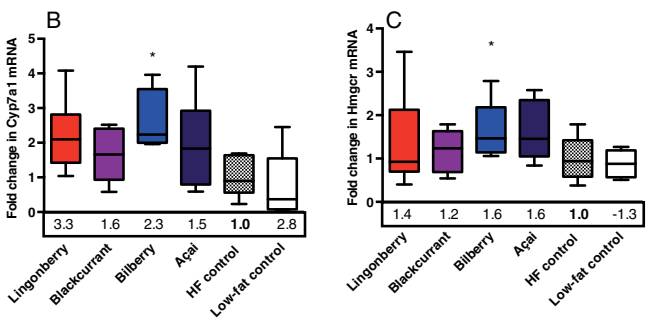



Fig. 5. **Effect on the expression of genes involved in cholesterol metabolism.** (A) Genes changed by at least one diet involved in pathways related to cholesterol biosynthesis and sterol and bile acid metabolism (identified using DAVID). The fold change is displayed for genes that were significantly changed compared to the HF control. Shades of red and blue indicate the degree of upregulation or downregulation, respectively. White indicates zero difference in fold change compared to the control. (B and C) qPCR results of gene expression of *Cyp7a1* and *Hmgcr*, $n=6$, Dunnett's posttest. Values significantly different from the HF control are depicted *$P<.05$.

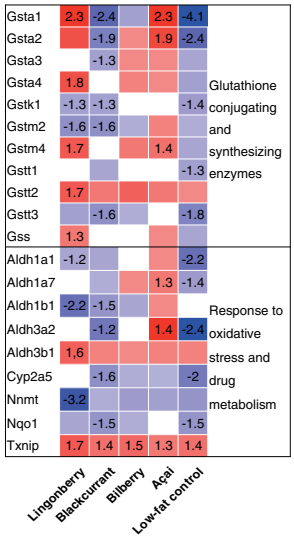
control (Fig. 3A). In addition, the plasma levels of CXCL1 protein were significantly decreased in all groups except the açai group (Fig. 3E) compared to the HF control. Genes for other macrophage-derived cytokines tended to be downregulated by lingonberries and upregulated by açai (Fig. 3A). Acyloxyacyl hydrolase, produced by Kupffer cells and involved in detoxification of lipopolysaccharide (LPS) [26], was upregulated 1.2-fold by açai supplementation.

### 3.4. Effects of berries on genes involved in lipid metabolism

Many genes involved in lipid synthesis, transport and regulation were differentially expressed compared to the HF control (Fig. 4). The general trends are that LF diet-fed mice show the most significantly changed gene expression profile, and the açai group displays upregulated expression of genes involved in lipid synthesis compared to the HF control group. *Cidea* and *Cidec*, involved in the regulation of lipid droplets, were both found to be downregulated in all berry groups except the açai group, where they were upregulated compared to HF control. In the LF group, several genes involved in acetyl and acyl-CoA metabolism were significantly regulated, such as the *Acot* and *Elovl* gene families. The mRNA levels for the gene transcribing the fatty acid-binding protein FABP5 were elevated 8.4 times in livers from the LF control group. FABPs are implicated in fatty acid uptake and metabolism, and polymorphisms in human *Fabp5* are associated with T2DM [27]. Except for açai, the mRNA levels for apolipoproteins ApoA5 and ApoM were induced by berry supplementation and LF diet, whereas *ApoA4* was downregulated (Fig. 4A). The *Acacb* gene, encoding ACCβ, was significantly downregulated in the lingonberry and blackcurrant groups and was upregulated in the açai and LF diet group compared to the HF control. The qPCR validation of *Acacb* and *Pparg2* shows the same trends in mRNA expression as in the array (Fig. 4B and C).

### 3.5. Effects of berries on the expression of cholesterol metabolism genes

In general, genes involved in cholesterol biosynthesis (such as *Cyp51*, *Fdps*, *Hmgcr*, *Lss*, *Nsdhl*, *Pmvk*, *Tm7sf2*) tended to be upregulated by all diets compared to HF control, but it was most pronounced in the bilberry, açai and LF diet groups (Fig. 5). Notably, the genes encoding




| Gene | Description |
|---|---|
| **Glutathione conjugating and synthetizing enzymes** | |
| Gsta1;a2;a4:k1;m4;t;t3 | Glutathione S-transferase alpha 1;alpha 2;alpha 4;kappa 1;mu 2;mu 4;theta 1;theta 2 and 3; Conjugation of glutathione to exogenous and endogenous hydrophobic electrophiles. |
| Gss | Glutathione synthetase; Catalyzes second step of glutathione synthesis. |
| **Response to oxidative stress and xenobiotic metabolism** | |
| Aldh1a1; 1a7;1b7;3a2;3b1 | Aldehyde dehydrogenase family 1 member A1, A7 and B1;family 3 member A2 and B1; Oxidation and detoxification of various substrates, including reactive lipid-derived aldehydes. |
| Cyp2a5 | CYP, family 2, subfamily A, polypeptide 5 (CYP2A5); Phase I xenobiotic metabolism |
| Nnmt | Nicotinamide N-methyltransferase; Catalyses N-methylation which is important for biotransformation of drugs and xenobiotic compounds. Proposed regulator of adiposity and energy expenditure. |
| Nqo1 | Nad(p)h:quinone oxidoreductase 1; Phase II xenobiotic metabolism |
| Txnip | Thioredoxin interacting protein; Possible oxidative stress mediator. |

Fig. 6. **Effect on the expression of genes involved in redox processes and glutathione metabolism.** Genes changed by at least one diet involved in pathways related to glutathione, xenobiotic and drug metabolism and redox activities (identified using DAVID). The fold change is displayed for genes that were significantly changed compared to the HF control. Shades of red and blue indicate the degree of upregulation or downregulation, respectively. White indicates zero difference in fold change compared to the control.

A

| *Top upstream regulators affected by lingonberries* | | *Effect of lingonberry supplementation* | *Z-score* | | | | |
|---|---|---|---|---|---|---|---|
| STAT3 | Transcription regulator | Inhibited | -2.1 | 0.03 | -2.5 | - | - |
| mTOR | Upstream kinase | Inhibited | -2.5 | -2.7 | -2.2 | 0.4 | - |
| SOX11 | Transcription regulator | Activated | 2.8 | - | 2.1 | - | - |

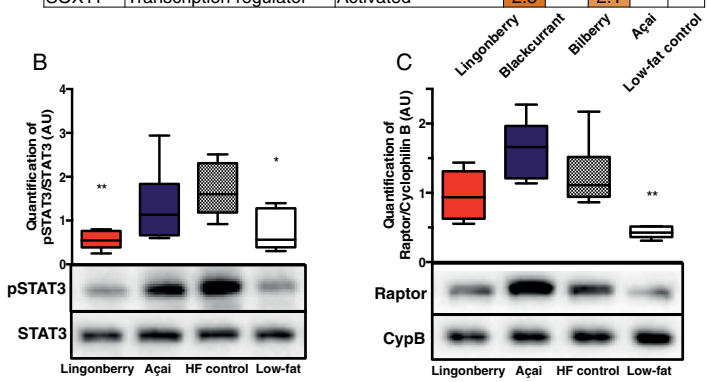



Fig. 7. **Upstream regulators predicted *in silico* to affect gene expression and validation by immunoblotting.** (A) The transcription regulators and kinase predicted to be most activated or inhibited by lingonberries (no upstream kinase was predicted to be activated). The prediction *z*-scores are depicted for all berry groups and represents inhibition (less than −2) or activation (more than 2) by the experimental diet compared to the HF control. Hepatic protein expression of the regulators was measured by immunoblotting in the lingonberry, açai and HF and LF control groups ($n$=6). Representative blots and quantification of the protein levels of (B) the phosphorylated signal transducer and activator of transcription pSTAT Tyr705 and total STAT3 and (C) the phosphorylated regulatory-associated protein of mTOR pRaptor are shown. pRaptor was normalized against CypB. Values significantly different from the HF control (ANOVA, Dunnett's posttest) are denoted *$P$<.05 and **$P$<.01 ($n$=6).

the rate-limiting steps of cholesterol (*Hmgcr*) and bile acid (*Cyp7a1*) synthesis were upregulated 1.8-fold and 3-fold, respectively, in the bilberry group. *Hmgcr* was also upregulated in the açai group (1.8-fold). The expression of *Cyp7a1* and *Hmgcr* was quantified by qPCR and found to be significantly upregulated by bilberries compared to HF control (Fig. 5B and C). The gene *Abcg8*, encoding a cholesterol efflux transporter, tended to be downregulated by all berry groups, although significantly only in the group receiving açai (−1.4-fold).

### 3.6. Effects of berries on the expression of genes related to oxidation/reduction processes, glutathione and xenobiotic metabolism

Overall, the gene expression profile of glutathione and redox enzymes in the blackcurrant group mimicked the downregulation observed in the LF diet group, whereas especially açai, but also lingonberry- and bilberry-fed mice, had a more upregulated expression profile of these enzymes compared to the HF control (Fig. 6), exemplified by *Gsta1* that was upregulated by lingonberries and açai (2.3- and 2.3-fold) and downregulated by blackcurrant and LF diet (−2.4- and −4.1-fold). *Cyp2a5* and *Nqo1*, involved in xenobiotic metabolism, were significantly downregulated in the blackcurrant and LF diet groups compared to the HF control group. Overall, several glutathione-conjugating and glutathione-synthesizing enzymes were downregulated by the LF diet. The pattern of gene expression of aldehyde dehydrogenases (ALDHs), involved in detoxification of lipid-derived aldehydes, tended to be downregulated in the lingonberry, blackcurrant and LF groups, whereas the opposite was observed in the açai group. Nicotinamide *N*-methyl transferase (*Nnmt*) was significantly downregulated in the lingonberry group (−3.2-fold) compared to the HF control.

### 3.7. Identification of upstream regulators driving gene expression changes induced by berry supplementation

Next, we sought to identify transcription factors responsible for the gene expression changes observed following supplementation with lingonberries, the berry with the most pronounced health-promoting effect according to our previous study [10] and with the strongest effects on gene expression (Fig. 2A). Based on the differentially expressed genes, an *in silico* tool for prediction of upstream regulators revealed that the most inhibited transcription regulator was STAT3, the most inhibited kinase was mammalian target of rapamycin (mTOR) and the most activated transcription regulator was sex-determining region Y box (SOX11) (no kinase was predicted to be activated by lingonberries) (Fig. 7A; for total lists, see Supplementary Tables S3 and S4). These results were validated at the protein level by immunoblotting, and the phosphorylation of STAT3 protein was decreased in the lingonberry and the LF control group compared to the HF control (Fig. 7B). LF diet significantly decreased the protein expression of Raptor compared to HF control (Fig. 7C). Protein expression of AMPK and ACC, downstream targets of mTOR, was also measured as effects on AMPK signaling have been described to mediate metabolic effects of resveratrol and other polyphenols [28]. However, no significant differences in total AMPK (subunit $\alpha_1/\alpha_s$, $\alpha_1$, $\beta_1/\beta_2$, $\beta_2$ or $\gamma_1$), phosphorylation of AMPK or phosphorylation of the target protein ACC were observed (Supplementary Fig. S1).

The *in silico* upstream analysis predicted that NF-κB and SREBP1c were regulated in several berry groups (Supplementary Table S4 and Fig. 8). As these transcription factors are master regulators of inflammation and fatty acid synthesis, respectively, we measured their concentration in nuclear extracts of all groups. Interestingly, the NF-κB complex was predicted to be significantly inhibited by lingonberries (*z*-score of −2.3) and tended to be inhibited also by blackcurrants and bilberries, when compared to the HF control. Immunoblot analysis showed that NF-κB protein was significantly

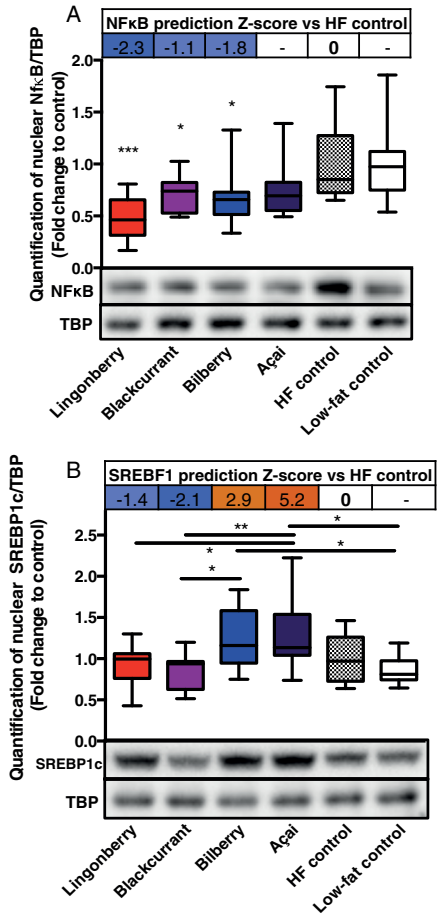
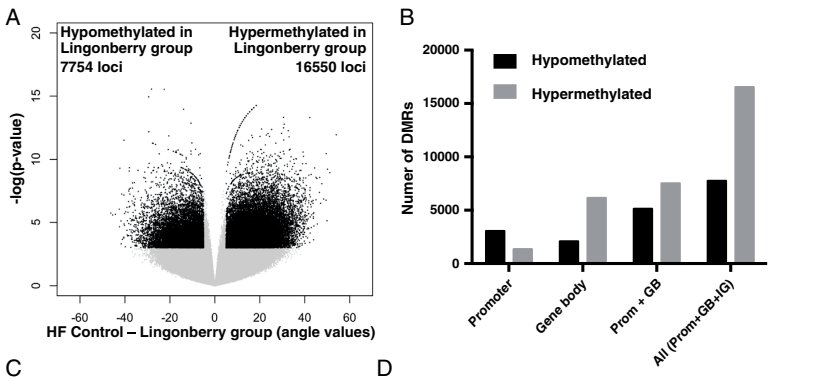



Fig. 8. **Immunoblot analysis of transcription factors NF-κB and SREBP1c in nuclear extracts.** (A) NF-κB was predicted by IPA to be significantly inhibited by lingonberries (*z*-score of less than −2), with a tendency to be inhibited also by blackcurrants and bilberries, when compared to the HF control. Nuclear extracts were subjected to immunoblotting and quantification showed that NF-κB (p65) protein was significantly reduced in liver nuclear fractions from mice receiving lingonberries, blackcurrants and bilberries. Values significantly different from the HF control (ANOVA, Dunnett's posttest) are denoted *$P$<.05 and ***$P$<.0001 ($n$=11–12). (B) *Srebf1* encodes SREBP1c and was predicted to be highly regulated by different berries, e.g., significantly activated by açai and bilberry (*z*-score of more than 2) and inhibited by blackcurrant (*z*-score of less than −2). Compared to the HF control, there were no significant differences in nuclear SREBP1c protein concentration (ANOVA, Dunnett's posttest). However, there were significant differences among the berry groups, as well as the LF control group (ANOVA, Tukey's posttest), *$P$<.05 and **$P$<.01, ($n$=11–12). The protein expression was normalized against the nuclear marker TBP.

reduced in nuclear fractions of livers from mice receiving lingonberries, blackcurrants and bilberries compared to the HF control (Fig. 8A). SREBF1 was predicted to be significantly activated by açai and bilberry (*z*-scores of 5.4 and 2.9) and inhibited by blackcurrant




D

| Location | DNA methylation (%) | | | | mRNA | |
|---|---|---|---|---|---|---|
| *Closest gene* | *Lingon-berry* | *HF diet* | *Diffe-rence* | *P-value* | *FC* | *P-value* |
| *Ncor2* (GB) | 81.7 | 68.2 | +13.5 | 2.2 $E^{-3}$ | -2.8 | 0.22 |
| *Il16* (Prom) | 78.7 | 70.2 | +8.5 | 4.3 $E^{-3}$ | +1.2 | 0.67 |

E

| Differentially methylated pathways | DMR-associated genes | % | P-value |
|---|---|---|---|
| transcription | 395 | 13.4 | 9,10E-14 |
| embryonic morphogenesis | 114 | 3.9 | 1,10E-13 |
| regulation of transcription | 473 | 16.0 | 8,00E-13 |
| regulation of transcription from RNA polymerase II promoter | 158 | 5.3 | 4,20E-10 |
| negative regulation of nitrogen compound metabolic process | 111 | 3.8 | 2,60E-09 |
| negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 110 | 3.7 | 2,90E-09 |
| negative regulation of transcription | 104 | 3.5 | 4,90E-09 |
| embryonic organ development | 75 | 2.5 | 6,90E-09 |
| embryonic organ morphogenesis | 56 | 1.9 | 1,00E-08 |
| pattern specification process | 84 | 2.8 | 1,10E-08 |

Fig. 9. **General trends in DNA methylation derived from HELP-tagging analysis of liver from mice receiving HF diet supplemented with lingonberries.** (A) Volcano plot comparing DNA methylation in liver of mice receiving HF diet supplemented with lingonberries versus control mice receiving only HF diet [plot of loci-specific methylation differences (*x*-axis) vs. −log *P* value (*y*-axis)]. The black dots represent the loci with a significant methylation difference greater than 5, $P<.05$. (B) Visualization of significantly DMRs in promoter (Prom), gene body (GB) or intergenic regions (IG). Black bars represent DMRs with hypomethylation, and gray bars represent DMRs with hypermethylation in lingonberry-supplemented mice. (C) The analyzed CpG sites were mapped according to gene region, here defined as being in the promoter [−2 kb/+2 kb of the transcription start site (TSS)] or inside the gene body. All other loci were classified as intergenic. (D) Supplementation with 20% lingonberries to the HF control diet induced hypermethylation at sites associated with *Ncor2* and *Il16* (mean values and difference, $n=6$, MassArray). The mean fold changes (FC) in mRNA expression in the lingonberry group vs. HF control are reported; however, the changes were not significant ($P>.05$). (E) The top most significant pathways enriched in genes with differentially hypomethylated or hypermethylated loci (>5, $P<.05$) in the promoter region are visualized. The number of DMR-associated genes in each pathway, percentage of mapped genes/total number of genes and *P* values are displayed.

(*z*-score of −2.1). Immunoblot analysis showed a tendency ($P=.13$) to increase nuclear SREBP1c protein in the açai group (Fig. 8B). Comparison of nuclear SREBP1c between all groups revealed that SREBP1c was increased in the açai and bilberry groups compared to the blackcurrant and LF groups. There was also a significant increase of nuclear SREBP1c in the açai group compared to the lingonberry group.

### *3.8. Epigenome-wide DNA methylation patterns in mice receiving HF diet with or without lingonberry supplementation*

Since lingonberry supplementation had large effects on liver gene expression and phenotype, we decided to further investigate the mechanisms behind the transcription pattern in this group by assessing genome-wide DNA methylation at specific CpG sites. The DNA methylation profile in liver from mice receiving lingonberries was distinct from the profile in control mice receiving HF diet, as seen in Fig. 9A. A total of 24,304 loci were differentially methylated (methylation score difference: >5, $P<.05$), 16,550 loci were hypermethylated and 7754 loci were hypomethylated in lingonberries compared to the HF control group. Out of these, 4413 were in promoter regions, 8253 were in the gene body and 11,638 loci were intergenic (Fig. 9B). Methylation in all samples at 14 CpG sites was assessed using both HELP tagging and pyrosequencing (negative correlation value between techniques: −0.74, $P<.0001$) (Supplementary Fig. S2A). Validation by MassArray of two loci of interest

showed that a CpG site (loci chr5:125521063) in the gene body of nuclear receptor corepressor 2 (*Ncor2*) and a CpG site (loci chr7:90885449) in the promoter region of interleukin 16 (*Il16*) were significantly hypermethylated in the lingonberry group compared to HF control (+13.5 and +8.5, respectively, $P<.01$) (Fig. 9D). The hypermethylation of *Ncor2* was validated in a biological replicate cohort (+11.3, $P<.05$); however, the smaller difference in methylation of *Il16* (+1.8) was not significant (Supplementary Fig. S2B). Gene expression of *Ncor2* tended to be downregulated in the lingonberry group (Fig. 9D); however, the effect was not significant ($P=.2$). The 100 top most significant DMRs with a difference in methylation score of >5 are listed in Supplementary Table S5.

Several DMRs were in the promoter or gene body region of genes that were differentially expressed or characterized as important for the phenotype based on the microarray gene expression analysis, for example the inflammatory-related genes *Cfd*, *Cxcl11* and *Ccl5* and lipid/cholesterol genes *Apoa5*, *Elovl6* and *Cyp7a1* (Supplementary Table S6). Overall, the pathways associated with genes containing differentially methylated loci in the promoter region were related to categories such as transcriptional regulation, morphogenesis and regulation of metabolic processes (Fig. 9E).

## 4. Discussion

The liver plays a central role in energy homeostasis and maintenance of insulin sensitivity, and disturbance of its function by nutritional overload such as HF feeding may result in steatosis, inflammation and insulin resistance. We have previously described the prevention of steatosis and obesity by lingonberries, bilberries and blackcurrants [10]. In the present study, we extend these findings and show that intake of berries modulates the liver response to a HF diet. The protective effects of lingonberries and bilberries result in a strong inhibition of acute-phase reaction and inflammation. Blackcurrant supplementation inhibits hepatic lipid synthesis, thereby contributing to the lean phenotype. In contrast, the addition of açai berry to a HF diet results in a pronounced upregulation of pathways of lipid and cholesterol metabolism, explaining the exacerbation of fatty liver development by this berry.

### 4.1. Trends in gene expression correlate with mouse phenotype

As expected, the LF diet group had the highest number of differentially expressed genes compared to the HF diet control group. However, it is interesting to note that the lingonberry diet, which had the same fat content as the HF control diet, mirrored the LF diet by affecting nearly as many genes. On the same note, the obese mice in the açai group, with large steatotic livers [10], had the lowest number of changed genes compared to the HF control mice, indicating that açai is less efficient than other berries in providing protection against HF diet-induced metabolic changes. Further supporting this notion is the finding that, out of the 10 genes identified as being affected by all diets, *Cidea* and *Anxa2* were upregulated only in the group receiving açai berries. *Cidea* induction is closely related to fat accumulation in hepatic steatosis [29] and *Cidea*-null (−/−) mice are resistant to diet-induced obesity and insulin resistance [30]. *Anxa2* may be upregulated by oxidative stress [31] and has been proposed to be a marker of adipose tissue dysfunction and insulin resistance that can be targeted by dietary interventions [32]. In contrast to açai, supplementation with lingonberry, blackcurrant and bilberry resulted in mice with similar body weight and liver status to mice fed a LF diet [10]. However, as shown in the present study, hepatic gene expression and regulated pathways differed between the groups, implying that the berries exert their beneficial metabolic effects by different mechanisms.

### 4.2. Lingonberry and bilberry intake have antiinflammatory effects

Inflammation is a key factor in the progression of fatty liver into more severe stages, and it appears to be required for development of hepatic insulin resistance [33–35]. Furthermore, obesity is closely associated with a state of chronic low-grade inflammation [2]. From this perspective, it is interesting to observe that pathways related to acute-phase and inflammatory responses were inhibited and several inflammatory genes were several-fold downregulated in the lingonberry and bilberry groups. SAA proteins are proposed mediators of inflammation with increased levels being associated with obesity and insulin resistance [36]. SAA1 is produced in the liver and the levels rise during inflammation and upon LPS challenge [37]. The fact that three isoforms of *Saa* were considerably downregulated by lingonberries and that also the plasma levels of SAA were decreased suggest that the mice receiving HF diet supplemented with lingonberries were protected against HF diet-induced inflammation. Neutrophil chemoattractant CXCL1 levels are elevated in obese *db*/*db* mice and T2DM patients [38,39] and were affected by berry supplementation in our study. Similar to *Saa*, the mRNA expression as well as the plasma levels of CXCL1 was decreased by lingonberries, bilberries and LF diet.

Another highly regulated gene was *Lcn2*, encoding lipocalin 2, which was 10- to 15-fold downregulated in groups receiving lingonberries, bilberries and LF control. The lipocalin family plays a role in antimicrobial and antiinflammatory response and Lcn2 has been shown to be expressed mainly in liver upon acute-phase response [40]. Lcn2 has been linked to obesity and insulin resistance and was recently proposed to be a key modulator of hepatic lipid homeostasis by controlling intracellular lipid droplet formation [41]. Furthermore, Lcn2 is an indicator of liver damage and *Lcn2* expression in liver correlates with steatosis in obese human subjects [42]. The finding that *Lcn2* was highly downregulated in mice receiving lingonberries and bilberries further strengthens that these berries attenuate HF-induced hepatic steatosis and inflammation, resulting in a liver phenotype similar to the LF-fed mice.

An interesting observation was that *Cfd*, encoding complement factor D, was downregulated by lingonberries and LF diet, whereas it was upregulated 4.4 times by açai intake compared to mice receiving the HF control diet. Complement factor D, also known as adipsin, plays a role in humoral suppression of infectious agents and it has been shown that *Cfd* mRNA is dramatically induced by HF diet in the liver of C57BL/6 mice concomitant with the development of steatosis [34,43,44]. Upregulation of *Cfd* by açai supplementation to HF diet further strengthens the notion that açai promotes, rather than prevents, hepatic steatosis.

Based on the pathway analysis and regulation of specific genes, we conclude that HF-induced hepatic inflammation is counteracted by supplementation with lingonberries and also bilberries. In our previous study [10], we showed that mice receiving a LF diet had smaller livers with lower triacylglycerol content than the bilberry-supplemented group. However, the pathway analysis of the livers from the LF group did not indicate inactivation of inflammatory pathways, even if several genes related to inflammation were downregulated in liver of these mice compared to the HF diet control group. This implies that supplementation with lingonberries and bilberries to HF diet have antiinflammatory effects beyond reduction of fat accumulation.

### 4.3. Lingonberries, blackcurrants, bilberries and açai exert their effects on lipid metabolism by different mechanisms

Several factors may contribute to the development of liver steatosis, such as increased dietary lipid intake, impaired VLDL secretion, increased hepatic lipid synthesis (*de novo* lipogenesis), impaired hepatic fatty acid oxidation and increased free fatty acid flux

from adipose tissue [7,8,45]. In this study, we observed that HF diet downregulates expression of genes in lipid and cholesterol biosynthesis, fatty acid synthesis and lipogenesis compared to mice receiving the normal chow LF diet. Similar results have been reported by others [34] and are expected since intake of LF diet results in an increased demand for endogenous lipid synthesis, and the observed upregulation of *Fabp5* could be an attempt to optimize fatty acid uptake. Furthermore, we found that HF diet-induced fatty liver occurred concurrently with increased expression of several genes previously shown to be associated with steatosis (e.g., *Cfd*, *Acaa1b*, *Cidea*, *Cidec*, *Anxa2* [5,34]).

Supplementing the HF diet with different berries significantly altered hepatic gene expression, even though the dietary fat content was kept constant. Compared to the HF control group, the decreased steatosis of the lingonberry and blackcurrant groups [10] was accompanied by reduced expression of genes involved in lipid biosynthesis pathways, such as *Elovl6* and *Scd1*, whereas the reduction in *Acacb* in these groups could imply reduced inhibition of β-oxidation. *Scd1* knock-out mice are protected against diet-induced obesity and hepatic steatosis [46] and *Elovl6* knock-out mice are resistant to diet-induced insulin resistance [47]. Based on these results and phenotype, we suggest that lingonberries and blackcurrants protect against HF-induced steatosis at least in part by downregulating lipid synthesis to a level where harmful lipid accumulation is prevented.

The upregulation of lipid biosynthetic pathways observed in the bilberry group may represent a compensatory mechanism to the increased fecal excretion of triacylglycerol and cholesterol previously observed in this group [10]. In addition, the mice in this group had a significantly higher food intake compared to the HF control. Based on this, we conclude that the prevention of hepatic steatosis as well as adiposity and body weight gain by bilberries is at least in part due to reduced intestinal lipid absorption. The prominent upregulation of a large number of genes involved in cholesterol biosynthesis and bile acid synthesis, including *Hmgcr* and *Cyp7a1*, provides further support for this conclusion. The upregulation of *Cyp7a1* (3-fold) implies an increased need for bile acid synthesis to compensate for reduced lipid absorption and/or loss of bile acids, likely due to increased bulk in the intestine hindering bile acid reuptake. Polyphenol-rich extracts from berries have been shown to inhibit pancreatic lipase *in vitro* [48], and it is possible that this mechanism plays a role also *in vivo*. Interestingly, a dietary bilberry extract reduced liver and serum triglycerides and cholesterol as well as liver weight in T2DM mice, without affecting body weight gain or food intake [49]. In a human study, daily consumption of bilberry puré and dried bilberries upregulated markers of cholesterol synthesis, and a marker of cholesterol absorption tended to be downregulated [50]. Furthermore, a recent study by Mykkänen *et al.* [51] validates our previous finding that supplementation with 20% of freeze-dried bilberries prevents weight gain in a HF diet mouse model [10]. Mykkänen *et al.* show that 10% of freeze-dried whole bilberries supplemented to HF diet prevent body weight gain in C57BL/6J mice [51]. Based on our results and these findings, we propose that intake of whole bilberries in larger doses reduces cholesterol and lipid absorption, potentially contributing to prevention of weigh gain.

Livers from mice receiving açai displayed a strong upregulation of several pathways and genes related to triacylglycerol as well as cholesterol synthesis compared to the HF diet control group. In addition, the gene encoding the transporter of cholesterol from the liver into bile (*Abcg8*) was downregulated. Several steatosis-associated (*Crat*, *Cidec* and *Cfd*) and lipid-synthesizing (*Pparg* and *Elovl6*) genes were upregulated in the açai group compared to HF diet-fed mice. The upregulation of *Acacb* could imply less β-oxidation of fatty acids, further aggravating the steatosis. In contrast to LF and bilberry, we propose that the increased fat and cholesterol synthesis in response to açai is maladaptive since it led to a large increase in liver size and fat content compared to the mice receiving HF diet alone [10].

Apolipoproteins are important in systemic lipid metabolism, and emerging evidence suggests a role for apolipoproteins in intracellular lipid homeostasis and crosstalk between adipose tissue and liver [52]. ApoA5 is predominantly expressed in the liver and was upregulated by lingonberry and bilberry diets. Studies have shown that plasma levels of ApoA5 are reduced in obese subjects and inversely correlated with measures of insulin resistance [53]. In hepatocytes and adipocytes, ApoA5 localizes on the surface of cytoplasmic lipid droplets, and incubation with ApoA5 has been shown to reduce cellular triglyceride content in human adipocytes [54,55]. *ApoM* overexpression has been shown to improve insulin sensitivity in rats [56] and *ApoM* was found to be upregulated by all berries, except açai, which downregulated *ApoM*. In a study by VerHague *et al.*, HF-fed C57BL/6 mice developed steatosis, which was associated with increased hepatic *ApoA4* mRNA expression [57]. In our study, HF diet-induced *ApoA4* expression was decreased by all berries except açai.

### 4.4. Lingonberries and açai induce expression of genes related to oxidation/reduction processes, glutathione and xenobiotic metabolism

The liver, as the major organ for biotransformation of drugs and foreign compounds, is continuously exposed to reactive oxygen species (ROS). This may cause peroxidation of polyunsaturated fatty acids in lipid membranes, producing unstable lipid hydroperoxides that decompose into cytotoxic aldehydes [58]. Berries are among the plants with the highest content of antioxidants and have been shown to protect against oxidative stress-related pathologies *in vivo* [59]. Lingonberries possess high *in vitro* antioxidant activity and can increase glutathione reductase activity in livers of rats on HF diet [60,61]. HF diet is proposed to cause oxidative stress in liver, and we observed an upregulation of antioxidant enzymes and pathways of oxidation/reduction and drug, xenobiotic and glutathione metabolism by HF diet compared to LF diet. This presumably represents an adaptive response to protect against ROS, which is line with previous findings in the same mouse model [34].

Reactive lipid aldehydes have shown to be increased in livers of NAFLD patients [62]. ALDHs may be induced to metabolize lipid aldehydes to less toxic compounds [58]. In our study, several *Aldh*s had lower expression in mice receiving LF diets as well as HF diet supplemented with lingonberries and blackcurrants, suggesting less lipotoxicity, whereas *Aldh* mRNA was increased by açai supplementation. Another enzyme family important for protective biotransformations is the family of enzymes catalyzing N-methylations, including nicotinamide *N*-methyltransferase (Nnmt). A recent study states a role for Nnmt in insulin resistance and obesity as a mediator of weight gain [63]. Moreover, *Nnmt* expression is increased in livers of obese and diabetic mice, and Nnmt knockdown protects against diet-induced obesity. We observe that the gene expression of *Nnmt* was downregulated 3-fold in the lingonberry group.

Dietary polyphenols have been studied for their strong antioxidative capacity and more recently for their potential to activate the endogenous antioxidant defense enzymes, which protect against lipid peroxidation under metabolic oxidative stress [64]. These, and other effects, could be mediated by polyphenols interfering with redox-sensitive thiol groups influencing transcription factors and kinases [65]. An interesting target of polyphenols is activation of the transcription factor Nrf2, which can bind to antioxidant-responsive elements and induce, for example, glutathione *S*-transferases, which mediate protection against reactive compounds by conjugation to glutathione [66]. Interestingly, the genes of several enzymes in this family were upregulated by lingonberry and açai intake, compared to ingestion of HF diet alone. In addition, Nrf2 (gene name NFE2L2) was

predicted to be activated by both lingonberries and açai (Supplementary Table S4).

All berries increased pathways involved in oxidation/reduction. However, pathways related to metabolism of glutathione, drug and xenobiotics were most prominently increased by lingonberries and were decreased by blackcurrants. We speculate that the different gene expression profiles induced by the different berries reflect not only effects on hepatic redox homeostasis but also adaptations to the metabolism of the polyphenols themselves as a direct consequence of the different phenolic profiles of the berries [67].

### 4.5. Berries mediate effects on inflammation and steatosis by affecting STAT3, mTOR and NF-κB pathways in vivo

In an effort to elucidate the upstream regulatory factors, we used an *in silico* prediction program designed to cluster regulated genes under known regulatory proteins. The analysis revealed that STAT3 was predicted to be inhibited by lingonberry and bilberry supplementation. STAT3 is activated in response to various cytokines and growth factors, and it plays a role in many cellular processes, including inflammation and cell growth. STAT3 may be activated by phosphorylation, dimerization and translocation to the nucleus where it induces transcription of for example acute-phase proteins [68]. We could verify at the protein level that the lingonberry-fed mice had a reduced level of STAT3 phosphorylation compared to the HF control, which is well in line with the reduced expression of several STAT3 target genes (*Saas*, *Cxcls*, *Ccl4* and *Socs3*; Supplementary Table S3) in this group. Hence, we propose that lingonberries inhibit the inflammatory response in the liver of HF-fed mice in part by suppressing phosphorylation and activation of STAT3. Reduced STAT3 phosphorylation has previously been reported *in vitro* in response to black raspberry extract and several polyphenols [69,70]; however, the underlying mechanisms are not understood. Furthermore, hepatic STAT3 overexpression results in increased circulating lipids and increased expression of lipogenic genes [71], and in humans, variants of STAT3 have been shown to be associated with NAFLD [72]. STAT3 phosphorylation is stimulated by LPS [73]. It is possible that compounds in lingonberries and bilberries modulate the gut microbiota in a beneficial manner, resulting in improved barrier function and less leakage of LPS into the blood stream. Indeed, upstream analysis predicted an inhibition of LPS-mediated gene expression in livers from mice receiving lingonberries and bilberries (Supplementary Table S4).

The proinflammatory transcription factor complex NF-κB was also predicted *in silico* to be inhibited by lingonberries. In agreement, we found that the nuclear protein content of NF-κB p65 was reduced by lingonberries, blackcurrants and bilberries compared to the HF control, implying that NF-κB translocation and thus activation of proinflammatory gene transcription are prevented by these berries. Studies on cancer inflammation suggest that there are interactions between NF-κB and STAT3 inflammatory signaling pathways creating a loop of prolonged NF-κB activation important for chronic inflammation [73,74]. Furthermore, NF-κB activity in liver has been shown to increase concomitant with the appearance of hepatic insulin resistance in a study investigating the time and tissue dependence of HF diet-induced insulin resistance [33].

Another interesting upstream regulator is the mTOR kinase, which was predicted to be inhibited by lingonberries, blackcurrants and bilberries. Protein expression analysis of Raptor, which forms the mTOR complex 1 (mTORC1) together with mTOR and other proteins, revealed that HF diet significantly upregulated Raptor compared to LF diet. This upregulation tended to be inhibited by lingonberries and exacerbated by açai supplementation. mTORC1 integrates environmental cues, such as nutrients, in the regulation of energy balance and metabolism. Overfeeding and high levels of nutrients and cytokines activate lipogenesis via mTORC1, and inhibition of hepatic mTORC1 impairs SREBP function and protects mice against HF-induced hepatic steatosis [75–77]. In addition, mice lacking Raptor in liver macrophages were protected against inflammation and insulin resistance [78]. Considering their regulatory role in nutrient, lipid and inflammatory processes, Raptor and mTOR are additional important regulators found to be targeted by berry supplementation. Previous studies have found that compounds such as flavonols can target mTOR, but the precise mechanism has not been defined [77]. SREBP1c is a major regulator of fatty acids and triacylglycerol synthesis and was among the top predicted upstream regulators. The prediction was based on the compelling number of SREBP1c target genes that were upregulated by açai and downregulated by in particular blackcurrants. The pattern of nuclear translocation of SREBP1c corresponded well to the observed phenotype and predictions, although none of the changes were significant compared to the HF control. However, it should be noted that immunoblot analysis of nuclear extracts does not take into account posttranscriptional modifications and interacting factors that mediate SREBP1c transcription activation. In view of the large effect of açai on SREBP1c target genes, it is tempting to speculate that açai berries contain one or several bioactive components that activate SREBP1c signaling, thereby promoting development of hepatic steatosis.

We conducted a thorough AMPK analysis since activation of AMPK has been implicated in the antidiabetic effects of a wide variety of polyphenols and plant extracts, including lingonberry and bilberry extracts [28,49,79,80]. Eid *et al.* have shown that lingonberry extracts may increase the phosphorylation of the AMPK target ACC in myotubes and that mice with diet-induced obesity supplemented with lingonberry extract have higher levels of phosphorylated AMPK in the liver. In addition, pharmacological activation of AMPK has been shown to suppress IL-6-induced STAT3 phosphorylation in C57BL/6N mouse liver [81], and AMPK is proposed to negatively regulate Raptor/mTOR [82]. In the present study, we did not observe lingonberry-induced phosphorylation and activation of AMPK or its target ACC. In addition, there was no increased expression of AMPK subunits at the gene or protein level. We conclude that hepatic AMPK activation is likely not involved in the health-promoting effects of lingonberries observed in our study [10], although we cannot rule out that the AMPK pathway is affected in other tissues and/or other time points than what was studied or that the discrepancy with previous literature is explained by the use of whole lingonberries, as supposed to extracts.

### 4.6. Lingonberry supplementation alters genome-wide DNA methylation profile

Epigenetic modification is one way for environmental factors, such as diet, to interact with metabolism and fine-tune gene expression. A growing body of literature suggests that aberrant DNA methylation may play a role in diseases such as NAFLD and T2DM [83–85], although the understanding of these mechanisms remains limited. The effect of HF diet on hepatic methylation patterns has been studied previously in mouse models, but mainly in *in utero* studies or in a context of deficiency of or supplementation with methyl donors [86]. We hypothesized that lingonberries may modulate HF-induced gene expression by acting on DNA methylation. Interestingly, we observed that lingonberry supplementation to an obesogenic HF diet induced a promoter-specific hypomethylation and a global shift toward DNA hypermethylation. This may be of importance as HF diet has been shown to induce DNA hypomethylation in mouse liver [87]. Furthermore, in our study, several DMRs were associated with genes in pathways that were highly affected by lingonberry supplementation, such as inflammation and lipid metabolism pathways. *Ncor2*, a key transcription corepressor, was found to be hypermethylated in livers from mice receiving lingonberries compared to mice receiving HF diet only. Notably, a recent human study found that *Ncor2* was

| Berry | Effect on HF-induced steatosis and obesity | Proposed regulators and mechanisms |
|---|---|---|
| Lingonberry | Prevention | **Anti-inflammation**<br>-reduced STAT3, mTOR and NF-κB signalling. |
| Blackcurrant | Prevention | **Decreased lipid synthesis**<br>-likely involving inhibition of SREBP1c transcriptional activity.<br>**Anti-inflammation**<br>-reduced NF-κB translocation to nucleus. |
| Bilberry | Prevention | **Anti-inflammation**<br>-reduced NF-κB translocation to nucleus.<br>**Reduced intestinal lipid absorption**<br>-compensatory upregulation of lipid synthesis likely mediated by activation of SREBP1c transcriptional activity |
| Açai | Aggrevates hepatic steatosis and obesity | **Increased lipid synthesis**<br>-increased mTOR signaling<br>-likely involving activation of SREBP1c transcriptional activity. |

Fig. 10. Summary of findings and suggested main regulators and mechanisms explaining the effects on liver of berry supplementation to a HF diet.

hypermethylated in adipose tissue in response to exercise [88]. NCoR (also known as SMRT) is implicated in the regulation of lipid, inflammation and oxidative stress pathways. For example, NCoR/SMRT has been shown to mediate effects on Nrf2-induced *Gst* expression [89] and cytokine expression in macrophages by interaction with PPARγ2/NF-κB [90].

In general, the overlap of differentially methylated and expressed genes was low in our data and methylation of specific genes did not explain their expression changes at this time point. The analysis of differentially methylated pathways showed that genes with DMRs in the promoter region were involved in broad functional categories such as transcription and morphogenesis rather than the categories that were most transcriptionally affected. DNA methylation is typically associated with gene repression; however, crosstalk between transcription factors and methylation of CpG sites in regulatory elements may affect chromatin state and gene expression in a manner that is difficult to predict with current knowledge and tools. To the best of our knowledge, this is the first study utilizing a genome-wide technique assessing methylation of individual CpG sites in liver in response to HF diet and HF diet supplemented with a natural compound effective in preventing adiposity and NAFLD. The HELP-tagging technique provides data for approximately 1.8 million CpG sites throughout the genome covering many but not all sites. Putative CpG sites of importance, not located in HpaII/MspI recognition sites, are overlooked by this technique. Furthermore, a diet modification such as lingonberry supplementation of HF diet may not induce dramatic changes in DNA methylation such as those observed following early-life perturbation of nutrient provision during fetal and/or neonatal development [22,86]. It is also possible that a larger number of animals are required to identify with certainty small, but meaningful, changes in DNA methylation associated with lingonberry supplementation of HF diet. Also, it is possible that methylation crosstalk with other epigenetic modifications such as histone marks in the liver and/or other tissues is important in our model. Given the large number of epigenetic modifiers and marks, we still know very little about the ways in which environmental cues may alter chromatin landscapes and regulatory factors to induce global chromatin modifications and control the expression of individual genes [91].

### 4.7. Concluding remarks

We report that consumption of lingonberries, and also blackcurrants and bilberries, modulates the liver response to HF diet of an *in vivo* model by altering the expression of genes related to mainly inflammation and lipid metabolism. We propose that reduced activities of NF-κB, STAT3 and mTOR are important mechanisms by which lingonberries, in particular, but also bilberries and blackcurrants, prevent HF-induced steatosis, low-grade inflammation and insulin resistance (summarized in Fig. 10).

Given the scarce knowledge of the complex interactions of nutrients and host metabolism *in vivo*, we believe that this study provides valuable mechanistic insight into how chronic intake of certain foods may help prevent disease. Knowledge regarding the mechanisms behind the capacity of lingonberries, blackcurrants and bilberries to prevent steatosis can be used to better target and design future studies, which should include studies in humans, as increased intake of these berries may be a useful strategy to prevent disease development caused by an unbalanced Western-style diet.

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## Authors' contribution

LHL conducted the study; extracted RNA/DNA; performed DAVID analysis, qPCR, immunoblotting and plasma measurements; and wrote the paper. LHL together with YS performed the HELP tag assay and data analysis, with invaluable advice on design and data interpretation from MJC. YS conducted MassArray and contributed to writing the paper. LHL and HAJ performed upstream analysis, and HAJ conducted database searches and took active part in all steps of interpreting results, developing and writing the manuscript. PS performed bioinformatics analysis of the microarray data and contributed to the manuscript. LHL, KB and CH designed the study. KB and CH supported in interpreting data and writing of the paper.

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# Paper III

# Lingonberries alter the gut microbiota and prevent low-grade inflammation in high-fat diet fed mice.

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## ABSTRACT

The gut microbiota plays an important role in the development of obesity and obesity-associated impairments such as low-grade inflammation. Lingonberries have been shown to prevent diet-induced obesity and low-grade inflammation, however it is not known weather the effect of lingonberry supplementation is related to modifications of the gut microbiota. The aim of the present study was to describe whether consumption of different batches of lingonberries alters the composition of the gut microbiota, which could be relevant for the protective effect against HF-induced metabolic alterations. Three groups of C57BL/6J mice were fed high-fat (HF) diet with or without a supplement of 20% lingonberries from two different batches (Lingon1 and Lingon2) during 11 weeks. The composition and functionality of the cecal microbiota were assessed by 16S rRNA sequencing and PICRUSt. In addition, parameters related to obesity, insulin sensitivity, hepatic steatosis, inflammation and gut barrier function were examined. HF-induced obesity was only prevented by the Lingon1 diet, whereas both batches of lingonberries reduced plasma levels of markers of inflammation and endotoxemia (SAA and LBP) as well as modified the composition and functionality of the gut microbiota, compared to the HF control group. The relative abundance of *Akkermansia* and *Faecaliebacterium*, genera associated with healthy gut mucosa and anti-inflammation, were found to increase in response to lingonberry intake. In conclusion, our results show that supplementation with lingonberries to a HF diet prevents low-grade inflammation and is associated with significant changes of the microbiota composition. Notably, the anti-inflammatory properties of lingonberries seem to be independent of effects on body weight gain.

## INTRODUCTION

The increasing prevalence of obesity is a worldwide health problem closely linked to diet and lifestyle factors. Obesity and its metabolic complications, such as non-alcoholic fatty liver disease (NAFLD), insulin resistance and dyslipidemia, contribute to a higher risk of developing type 2 diabetes. Accumulating evidence suggests that low-grade chronic inflammation is a common denominator for these metabolic diseases[1], and recent research demonstrates the important role of the gastrointestinal tract in contributing to this subclinical inflammation[2]. The gut and the composition of the gut microbiota are crucial for nutrient handling and energy harvest and influence whole body metabolism, immune response and insulin sensitivity[3-7]. The metabolic endotoxemia and associated inflammation observed in obesity are proposed consequences of a dysfunctional gut barrier resulting in leakage of lipopolysaccharide (LPS) and pro-inflammatory cytokines into the circulation[5 7]. The importance of the interaction between the diet and the gut is demonstrated by studies in mice showing that high-fat (HF) diet-induced inflammatory changes in the intestine develop before the onset of obesity and other metabolic complications [6].

Lingonberries (*Vaccinium vitis-idaea* L.) are commonly consumed in Scandinavia and have attracted increasing interest due to their nutritional properties[8] and putative role as a food with beneficial health effects[9-13]. Recently, it was found that lingonberry supplementation prevents weight gain and associated negative effects of HF diet consumption in mice [9], however several questions regarding the cause of the preventive effect remain to be further investigated. High antioxidant and antimicrobial activities of lingonberries have been described[14-17] and might be of particular relevance for interactions with the gastrointestinal milieu. The present study was conducted to investigate if supplementation with different batches of lingonberries modifies the gut microbiota of HF fed C57BL/6J mice, as this may be an important factor to assess in order to promote understanding of the metabolic effects of lingonberry intake.

## RESEARCH DESIGN AND METHODS

*Preparation and analysis of diets.* HF diet (control) and HF diets supplemented with 20% (w/w) of freeze-dried lingonberries were prepared by Research Diets (New Brunswick, NJ, USA). The HF diet and HF diet supplemented with lingonberries from Batch1 (referred to as Lingon1) have been characterized and described previously [9], and the HF diet has been shown to induce obesity, insulin resistance and low-grade inflammation compared to a low-fat diet [9 10]. In this study, an independent second batch of lingonberries (Batch2) was obtained from the same provider that supplied Batch1 (MOLDA AG, Dahlenburgh, Germany). Batch2 was used to manufacture a second lingonberry diet, referred to as Lingon2. All diets contained 45% of kcal from fat, 20% kcal from protein and 35% kcal from carbohydrate and were designed to have matching nutrient content (Table 1). After manufacturing, all diets were analyzed for fiber content (Table 1), and Lingon1 and Lingon2 were subjected to detailed analyses of fatty acids, cholesterol, benzoic and sorbic acid (Eurofins, Lidköping, Sweden).

*Animals and study design.* The study was approved by the Animal Ethics Committee in Lund, Sweden (Permit Number: M185-11) in accordance with the Council of Europe Convention (ETS 123). Male C57BL/6JBomTac mice, 6 weeks old with an average body weight of 23.8 ± 1.0 g, were obtained from Taconic (Skensved, Denmark). The animals were housed in a controlled environment (12h light-dark cycle, 7 am – 7 pm). After 9 days of acclimatization the mice were separated into 3 groups based on mean body weight per cage and housed in groups of 5 mice per cage. Mice were fed Lingon1 diet, Lingon2 diet or HF diet (n = 10 mice/diet group) for 11 weeks *ad libitum*. At week 7, 8 and 10 weeks post diet introduction, the mice were placed in clean cages on grids and feces was collected over 24 hours. At the end of the study, 4 h fasted animals were anesthetized with an intraperitoneal injection of midazolam (Midazolam (5 mg/mL), Panpharma S.A., Luitré, France) and a mixture of fluanisone 10 mg/mL and fentanyl citrate 0.315 mg/mL (Hypnorm, VetaPharma, Leeds, UK). Body composition was determined by dual-energy X-ray absorptiometry (DEXA) using a Lunar PIXImus (GE Lunar, Madison, WI, USA). Blood samples were taken by intraorbital puncture and animals were sacrificed by cervical dislocation and selected tissues were dissected, weighed and saved for further analysis.

*Body weight, food intake and fecal energy.* Body weight and food intake were monitored weekly. The energy intake was expressed per mouse to adjust for the loss of 1 mouse in Lingon2 during week 9 of the study. Estimated mean intake per mouse was calculated as follows: [weekly food consumption (kcals) per group (mean of 2 cages/group)] / [number of mice per diet group]. The energy content of

dried feces collected at week 7, 8 and 10 was determined using a bomb calorimeter (C6000, IKAWerke GmbH, Germany) and expressed as energy content of excreted feces during 24 hours per cage. Ingested digestible energy intake was established by calculating the mean daily energy intake at week 7, 8 and 10 related to the energy excreted in feces sampled at the same time points. Feed efficiency was obtained by calculating body weight gain per calories consumed and not excreted into feces.

*Plasma analysis and assessment of insulin resistance.* Plasma levels of triacylglycerol, total cholesterol, high density lipoprotein (HDL)-cholesterol, alanine aminotransferase (ALT), glucose, insulin and homeostasis model assessment-estimated insulin resistance (HOMA-IR) were determined as previously described[9]. Serum amyloid A (SAA) and LPS-binding protein (LBP) were measured in plasma using commercial ELISA-kits (Tridelta Development Ltd, Wicklow, Ireland and Nordic Biosite, Täby, Sweden).

*Real-time PCR of intestine tissue.* Jejunum (n = 6-7) was snap-frozen and ground to a powder in a mortar under liquid nitrogen. The powder was subjected to total RNA extraction and reverse transcription followed by quantitative PCR (qPCR) analysis as previously described [18]. Expression of *Tlr4* (toll-like receptor 4, forward: GCCTTTCAGGGAATT; reverse: AGATCAACCGATGGA), *Occludin* (forward: ATGTCCGGCCGATGC; reverse: TTTGGCTGCTCTTGG) (Dna Technologies A/S, Aarhus, Denmark), *Emr1* (EGF-like module-containing mucin-like hormone receptor-like, Mm00802529_m1), *Gcg/proglucagon* (Mm01269055_m1), *Reg3g* (regenerating islet-derived protein 3 gamma, Mm00441127_m1) and *Muc2* (mucin 2, Mm01276696_m1) (Applied Biosystem, Foster City, CA, USA) were quantified and related to the expression of the reference gene *Actb* (beta-actin, Mm00607939_s1).

*Immunocyto- and histochemistry in liver and adipose tissue.* The medial lobe of the liver and epididymal visceral fat (n = 3 per group) were dissected, fixated in 4 % paraformaldehyde, embedded in paraffin (SVA, Uppsala, Sweden) and sectioned (5 µm) for immunocyto- and histochemistry to illustrate effects on hepatic steatosis and inflammatory cell infiltration in adipose tissue. To study liver steatosis and Kupffer cells in the liver, paraffin sections were deparaffinised, hydrated and rinsed in hydroxymethylaminomethane (TRIS) buffer (pH 7.6). To eliminate endogenous peroxidase activity and unspecific background staining, sections were exposed to 0.3% hydrogen peroxide containing TRIS buffer for 20 min and Protein K (code: S3004, Dako, Glostrup, Denmark) for 6 min before being treated with 2% bovine serum albumin for 20 min. Sections were incubated with an antibody against the surface-specific glycoprotein F4/80 for macrophages

(dilution 1:50, ST-MCA497R, Nordic Biosite, Täby, Sweden) over night at 4°C, before being treated with biotinylated anti-rat IgG (code 4001; dilution 1:50; Vector BA) for 30 min. The VECTASTAIN ABC kit (code PK-6100; Vector Laboratories Inc., CA, USA) (DAB; code ab 644238; Abcam, Cambridge, MA, USA) was used in combination with hydrogen peroxide in accordance to manufacturer's instructions o visualize the biotinylated F4/80. The sections were counter stained with Mayer's hematoxylin (Histolab, Göteborg, Sweden) before being dehydrated, mounted and scanned using a computerized image analyzing system Imagescope (Aperio Scan Scope, Vista, CA, USA). The F4/80 immunoreactive macrophages stained brown. Microvesicular steatosis was evaluated according to Brunt et al.[19], with a few minor modifications. Hepatocyte nuclei, macrophages and macrovesicles were analyzed with ImageJ 1.49 (Canadian content interactive media, Ontario, Canada). The analysis procedure was automated using a Macro program specifically developed for the project. Five images each from zone 1 and 3 were collected per animal. Zone 1, located around the portal triads, and Zone 3, located around central veins, were analyzed separately due to their functional differences. Hepatocyte nuclei, macrophages and macrovesicles were identified and quantified, giving the objects per $mm^2$ in each image. To study inflammatory infiltration in visceral fat, paraffin sections were stained with Hematoxylin and Eosin (H&E), scanned and analyzed in Imagescope (Aperio Scanscope) and the number of leukocytes per $mm^2$ were quantified using Image J[20].

*Sequencing and analysis of bacterial 16S rRNA genes.* Whole cecum was dissected, weighed and snap-frozen in liquid nitrogen (n = 9-10). DNA extraction, PCR amplification and sequencing were performed at GATC Biotech AG, Konstanz, Germany. Briefly, the cecal tissue and content were thawed on ice and DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), with a bead beating step included. The V1-3 region of 16S rRNA genes were amplified by PCR with forward and reverse primers containing Illumina adapter sequences and unique dual indexes used to tag each PCR product[21]: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3'). Paired-end sequencing with a read length of 2x250 bp was carried out on a Miseq instrument using a Miseq reagent kit v2 (Illumina, San Diego, CA, USA). Sequences were analyzed with the free software package Quantitative Insights into Microbial Ecology (QIIME) using default parameters, except where specified[22]. Sequences were removed if they were shorter than 200 nucleotides or longer than 1000 nucleotides and contained ambiguous bases, primer mismatches, homopolymer runs in excess of six bases or uncorrectable barcodes. Similar sequences were binned into operational taxonomic units (OTUs) using UCLUST[23], with a minimum pairwise identity of 97%. The most abundant sequence in each OTU was chosen to represent its OTU.

Representative sequences from each OTU were aligned using PyNAST (a python-based implementation of NAST in QIIME[24]) and taxonomy was assigned using the Greengenes[25] database (v. 13_8) and the RDP classifier[26] using a minimum percent identity of 90%.

*Statistical analysis of bacterial 16S rRNA genes.* Graphpad Prism 6 software (GraphPad Software, San Diego, CA, USA) was used to identify significant differences in bacterial relative abundances between groups using one-way ANOVA and Tukey's test to adjust for multiple comparisons at each taxonomic level. Further, alpha- and beta-diversity were analyzed in QIIME, using a non-parametric t-test and Bonferroni correction for multiple comparisons and the ANOSIM and Adonis non-parametric statistical tests, respectively. To investigate whether bacterial taxa could be identified as biomarkers related to lingonberry intake, Linear Discriminant Analysis (LDA) of effect size (LEfSe) was applied on the OTU table according to Segata et al.[27] The online software tool PICRUSt was applied on the OTU table to infer the functional capacity from 16S rRNA gene sequencing data, and resulting significant pathways were collapsed into three levels of pathways and visualized using the LEfSe cladogram with a LDA score > 3 [28].

*Statistical analysis.* Unless stated otherwise, data are displayed as mean ± SD and analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. In cases where Gaussian distribution could not be assumed, groups were compared using Kruskal-Wallis post test. The ROUT test[29] was performed to statistically identify outliers with 99% confidence level. Statistical analyses were performed using GraphPad Prism 6.0 and differences with a p-value $< 0.05$ were considered significant.

## RESULTS

*Lingonberry supplementation affects body weight, metabolic and inflammatory plasma parameters.* After 11 weeks, the mice receiving HF diet supplemented with Lingon1 weighed 39 ± 3.9 g, which was significantly lower ($p = 0.0003$) compared to the control group receiving HF diet without berries (46 ± 2.2 g) (Figure 1A). Mice in the Lingon2 group weighed less than mice in the control group at week 5, 6 and 7, but the final weight of 44 ± 2.8 g was not significantly different from the control group ($p = 0.43$). In addition, there was a significant difference in body weight between Lingon1 and Lingon2 ($p = 0.01$). The percentage of body fat was significantly lower in the Lingon1 group compared to Lingon2 and control (Figure 1B). There were no significant differences in the lean body mass or weight of epididymal fat pads amongst the groups (data not shown). Cecum weight was higher ($p < 0.0001$) in the Lingon1 (0.65 ± 0.09 g) and Lingon2 (0.62 ± 0.13 g) groups compared to the control (0.23 ± 0.03 g). In the Lingon1 group, the fasting plasma levels of glucose and cholesterol were significantly lower compared to the control group (Figure 1C and D), whereas there was only a tendency ($p = 0.056$) towards reduced glucose levels in the Lingon2 group. The HDL-cholesterol levels in the Lingon1 group (1.8 ± 0.23 mM) were significantly lower ($p = 0.018$) compared to control (2.1 ± 0.28 mM), whereas the change in HDL-cholesterol in the Lingon2 group was not statistically significant (1.9 ± 0.19 mM), $p > 0.05$). There were no significant differences in plasma insulin levels (Lingon1: 1059 ± 848.7 pM, Lingon2: 1547 ± 1622 pM, Control: 1933 ± 1730 pM) ($n = 9$-$10$). The calculated HOMA-IR index was not significantly different between the groups (data not shown), however it tended to be lower in Lingon1 compared to the control. SAA and LBP in plasma reflect inflammation and LPS-levels, respectively, and were significantly reduced in plasma from mice receiving Lingon1 and Lingon2 compared to the control group (Figure 1E and F).

*Diet composition and food intake.* The analysis of the composition of the Lingon1 and Lingon2 diets did not reveal any difference in the content of compounds such as total fiber, insoluble fiber, specific fatty acids, cholesterol, benzoic and sorbic acid (data not shown). According to the margin of error of the utilized methods, the only indicated difference was in soluble fiber content (Table 1). Data illustrating energy intake, fecal energy content, ingested digestible energy and feed efficiency of mice receiving the different diets are presented in Table 2.

*Histology analysis of lingonberry-mediated effects on liver and adipose tissue inflammation.* The average mass of the livers in group Lingon1 was significantly reduced compared to the control (Figure 2A). The group receiving Lingon2 diet

displayed a tendency ($p = 0.12$) to reduced liver mass compared to the control group. The plasma levels of ALT, a marker of liver dysfunction, were significantly reduced by both lingonberry diets compared to control mice not receiving lingonberries (Figure 2B). The histochemical analysis of liver is illustrated by one representative slide per group in Figure 2E, F and G. The evaluation according to Brunt et al.[19] graded the microvesicular steatosis as absent or mild in Lingon1, mild or marked in Lingon2 and marked in all samples in the control group. The data generated using ImageJ to analyze area of hepatocytes, macrophages and macrovesicular steatosis are visualized in Figure 2C and 2D as the area occupied by macrophages (brown stained) and macrovesicles related to the area occupied by hepatocytes. The relative area occupied by macrophages appeared to be higher in livers from the control group, especially in the zone 3 area around the central vein, compared to mice receiving lingonberries. The area of macrovesicular steatosis was relatively similar amongst the groups in zone 1, surrounding the portal triads. In zone 3, the macrovesicular steatosis was 20-fold higher in the control group compared to Lingon1 and Lingon2. In epididymal adipose tissue, histochemistry revealed a higher number of leukocytes, most of them arranged in crown-like structures, in the control group (median: $177 \pm 22$ counts/mm$^2$) compared to mice receiving Lingon1 ($121 \pm 26$ counts/mm$^2$) and Lingon2 ($138 \pm 47$ counts/mm$^2$) (representative slides displayed in Fig 2H, I and J).

*Lingonberry supplementation affects expression of genes involved in intestinal barrier function and inflammation.* The results from the jejunal gene expression analysis are displayed in Figure 3. Compared to the control group, Lingon2 group displayed decreased expression of LPS-sensing *Tlr4* and macrophage marker *Emr1,* and increased expression of the tight-junction protein-encoding *occludin* gene. There was a tendency to increased expression of *Gcg* (proglucagon) ($p = 0.07$) in the Lingon1 group compared to control, whereas there were no significant differences between the groups in expression of *Reg3g* and *Muc2* (data not shown).

*Effects of lingonberry intake on the composition and functionality of cecal gut microbiota.* After quality filtering, a total number of 9,125,002 sequences were generated, with an average of 314,655 sequences per sample in the dataset. Two samples in the control group failed to produce sequences and were excluded from further analyses. Remaining samples had between 229,369 and 377,540 sequences per sample. The overall composition of the bacterial community was influenced by diet (Figure 4D), with significant alterations between lingonberry diets and the control group in the phyla Bacteroidetes, Firmicutes, Verrucomicrobia and Proteobacteria (Figure 4A). The abundance of Proteobacteria was only significantly different between the control and the Lingon1 group ($p < 0.01$), and

Verrucomicrobia differed between lingonberry groups and control ($p < 0.0001$), as well as between the Lingon1 and Lingon2 group ($p < 0.01$). Foremost, analysis at the phylum level showed that the relative abundance of Bacteroidetes was significantly increased and the relative abundance of Firmicutes was decreased by lingonberry supplementation ($p < 0.0001$) (Figure 4A). The Firmicutes/Bacteroidetes ratio was significantly reduced by both Lingon1 and Lingon2 diet compared to the control ($p < 0.0001$) (Figure 4B). At genus level, 14 bacterial taxa differed significantly between the control and lingonberry groups, and 5 taxa differed between Lingon1 and Lingon2 ($p < 0.05$, significantly different genera with a relative abundance > 4% in at least one group are displayed in Figure 4C. The HF diet-induced increase in Firmicutes was largely accounted for by increase of reads assigned to an unclassified genus within the *Lachnospiraceae* family and to a smaller extent from the genera *Ruminococcus* and *Oscillospira* (Figure 4C).

The increase of Bacteroidetes in the lingonberry groups was to a large extent caused by increased relative abundance of bacteria belonging to an unclassified genus in the *S24-7* family (Figure 4C). The genus *Parabacteriodes* was also significantly increased in the Lingon1 (relative abundance 15%) and Lingon2 (13%) groups compared to the control group (3%) (Figure 4C). The genus *Odoribacter* was not present in the control group, but had a relative abundance of approximately 9% in the lingonberry groups (Figure 4C). Furthermore, the genus *Akkermansia*, belonging to the Verrucomicrobia phylum, was significantly increased in both lingonberry groups compared to the control ($p < 0.0001$, relative abundance 7%) and *Akkermansia* was also significantly higher in the Lingon2 group (20%) compared to the Lingon1 group (16%) ($p < 0.0001$)(Figure 4C). The alpha-diversity, analyzed with the observed species test, was higher in the control group ($554 \pm 32$) compared to Lingon1 ($203 \pm 22$) and Lingon2 ($209 \pm 19$) ($p = 0.003$)(Figure 4E). Unweighted UniFrac analysis (Figure 4D) revealed that supplementation with lingonberries promoted modification of gut microbiota and PC1 explained 48% of the observed variation ($p < 0.001$, ANOSIM). In addition, the second principal component explained 7% of the variation in microbiota community, which accounts for the difference in microbiota driven by supplementation with lingonberries from different batches (Lingon1 vs Lingon2). LEfSe analysis confirmed the described changes in the cecal microbiota of mice in the Lingon1 and Lingon2 groups and further identified additional bacterial genera related to the control, Lingon1 or Lingon2 groups of mice (Figure 5A).

The comparison of functional pathways enriched in the control, Lingon1 and Lingon2 groups (Figure 5B) revealed an enrichment of genes belonging to pathways related to metabolism in the Lingon-groups, and an enrichment of genes involved in transport and motility in the control group.

## DISCUSSION

There is growing evidence that increased consumption of fruit and vegetables reduce the risk of chronic disease such as cardiovascular disease and stroke, and may also prevent body weight gain and development type 2 diabetes[30]. The protection of fruits and vegetables against obesity-related disorders has partly been attributed to polyphenols, and intake of flavonoid-rich foods, such as berries [31], has been associated with a lower risk of developing type 2 diabetes [32 33]. In agreement with a previous study[9], we show that supplementation with lingonberries (Lingon1) prevents HF diet-induced weight gain, increased liver weight, body fat accumulation and elevated plasma levels of glucose and cholesterol. Surprisingly, we found that supplementation with a different batch of lingonberries (Lingon2) did not have the same capacity to attenuate weight gain. Nevertheless, both batches of lingonberries altered the gut microbiota composition and were effective in preventing HF-induced low-grade inflammation and endotoxemia, demonstrating that the effect of lingonberries on these parameters are independent of effects on body weight.

The gut microbiota may partly influence host metabolism by leakage of proinflammatory factors into the circulation, such as LPS, which may trigger inflammatory response in the liver and other tissues[34 35]. In our study, intake of both batches of lingonberries led to reduced plasma levels of LBP, which indicates reduced endotoxemia compared to mice receiving HF diet[36]. SAA proteins are acute phase proteins secreted by the liver in response to inflammatory stimuli, including LPS[37], and mice receiving lingonberry diets displayed a marked reduction in the plasma levels of SAA compared to control. Furthermore, HF feeding as well as increased LPS and SAA-levels are associated with lipid accumulation into the liver, which may contribute to development of liver inflammation and hepatic insulin resistance. Even though only mice in the Lingon1 group had reduced liver size compared to the control, both lingonberry batches prevented a rise in plasma ALT and tended to reduce hepatic macrovesicular steatosis and the presence of macrophages. These results suggest that liver function was improved by lingonberry supplementation to a HF diet, and are in line with previous studies showing that lingonberry-supplementation reduce HF-induced hepatic lipid accumulation[9]. Interestingly, the findings presented here demonstrate that both batches of lingonberries protected against HF-induced liver steatosis and inflammation, which may be important for preventing development of systemic low-grade inflammation. Notably, as only one batch of lingonberries prevented diet-induced obesity, we show that the anti-inflammatory effect of lingonberries is more than a result of reduced body weight. Furthermore, as germ-free C57BL/6J mice are protected against HF-induced liver triglyceride accumulation and have reduced plasma levels of SAA [38] one might speculate that interactions with the gut is an involved mechanism in the metabolic effects of lingonberries.

Our study shows that supplementation of HF diet with lingonberries leads to profound changes in the cecal microbiota structure, including a decrease in the ratio of Firmicutes to Bacteroidetes. A high Firmicutes/Bacteroidetes ratio is characteristic for obesity-driven dysbiosis and associated with HF diet consumption, and the reduction of Firmicutes/Bacteroidetes ratio observed in response to lingonberries is similar to what have been reported previously in lean mice and upon dietary modifications including intake of low-fat diets and polyphenol supplementation [39-41]. In the present study, HF diet induced an increase of unclassified members of the *Lachnospiraceae* family as well as the genera *Ruminococcus* and *Oscillospira*. Increased abundance of the same groups of bacteria have been found to associate with promotion of diabetes pathogenesis in the NOD mouse model of type 1 diabetes [42]. In the same model, an increase in unclassified bacteria belonging to the *S24-7* family correlated to changes in gut immune parameters and was associated with protection against development of diabetes. Furthermore, bacteria assigned to the family *Lachnospiraceae* and genus *Bacteroides* have been shown to decrease in response to dietary treatments preventing HF-induced metabolic syndrome[40 43], and species belonging to *Lachnospiraceae* have been linked to development of obesity and type 2 diabetes in *ob/ob* mice [44]. Consequently, the fact that lingonberries promoted an increase in *S24-7,* as well as a decrease in *Lachnospiraceae*, *Ruminococcus, Oscillospira* and *Bacteroides*, indicates that lingonberries may confer beneficial effects on the gut microbiota composition in relation to diabetes development.

Analysis of bacteria at the genus level revealed that the relative abundance of the genus *Akkermansia* was increased in response to lingonberry consumption. The genus *Akkermansia* mainly consists of the mucin-associated species *Akkermansia muciniphila,* which has been associated with healthy gut mucosa [45 46] and has attracted considerable interest as a potentially beneficial gut bacteria. For example, administration of *A. muciniphila* reverses HF diet-induced endotoxemia, adipose tissue inflammation and insulin resistance in C57BL/6 mice [47], potentially by improving the mucus layer function and thereby preventing toxin translocation. Moreover, the presence of *A. muciniphila* inversely correlates with body weight in rodents and humans [18 48 49]. In the present study, we found that supplementation of HF diet with the Lingon2 batch increased the levels of *Akkermansia* more (+4%) than the Lingon1 batch, and LEfSe analysis ranked the increase of *Akkermansia* as the strongest biomarker of Lingon2 supplementation. This finding is notable as only the Lingon1 batch reduced body weight gain. Generally, our results are similar to studies in the same mouse model where supplementation with polyphenols [40] or cranberry extracts [43] prevented negative metabolic effects of HF diet and increased abundance of *A. muciniphila*. However, in the present study the increase of *Akkermansia* in response to lingonberries was independent of body weight. Furthermore, the genus *Faecaliebacterium* was identified as a marker for

Lingon2 supplementation and the butyrate-producing species *F. Prausnitzii* has anti-inflammatory properties in both mice and humans [50 51]. However, these observations require further studies to determine the importance of specific bacteria for the effects of lingonberries.

We also addressed the impact of lingonberries on the functionality and metabolic activities of the gut microbiota. We found that the microbiome in the HF control group had an increased abundance of genes belonging to categories such as transporters, ABC transporters, membrane transporters, cell motility, bacterial motility proteins, bacterial chemotaxis, flagellar assembly, two component system, transcription and signal transduction compared to the lingonberry groups. Notably, the same gene categories were found by Hildebrandt et al. to be increased in the fecal microbiome of mice fed HF diet compared to mice fed standard chow[52]. The observed enrichment of genes for transporters is paralleled by results from Turnbaugh et al. [41] where a Western diet induced an enrichment of genes for transporters and ABC transporters in the cecal microbiota of humanized gnotobiotic mice compared to mice receiving a low-fat diet. Furthermore, in our study, the microbiome in mice receiving lingonberries was enriched with genes related to metabolism of energy, lipids, amino acids, nucleotides. Similar findings were reported by Hildebrandt et al. in mice fed chow diet. The authors hypothesized that a HF-induced increase of nutrient transporters and a decrease of metabolic genes may be an adaptation to lower amounts of nutrients reaching the colonic microbiota, thus favoring bacteria with increased numbers of nutrient transporters[52]. Although more research is needed to interpret the implications of these shifts in functionality, it appears clear that supplementing HF diet with lingonberries has a large impact on both gut microbiota composition and functionality.

Gut permeability is controlled by tight-junction proteins such as occludin, which is a proposed key marker of tight-junction integrity [34]. In the present study, *occludin* was significantly upregulated in jejunum in response to the Lingon2 diet compared to the HF control. Moreover, the genes encoding toll-like receptor 4 (*Tlr4*) and the macrophage marker F4/80 (*Emr1*) were downregulated in mice receiving Lingon2 diet compared to the control. TLR4 activation in response to fatty acids or LPS is implicated as one mechanism by which HF diet and dysbiosis induce impaired barrier function, low-grade inflammation and endotoxemia with detrimental effects on whole-body metabolism [53]. Kim et al. described that HF diet increased expression of intestinal *Tlr4* and inflammatory mediators, whereas tight-junction proteins were decreased, compared to lean C56BL/6J mice fed a low-fat diet [53]. However, as no effects on intestinal markers were observed in response to Lingon1, it seems that this mechanism is not a major contributor to the capacity of lingonberries to prevent HF-induced low-grade inflammation, at least not in the jejunum.

The ability of lingonberries in batch Lingon1 to prevent weight gain has been described before [9] and was replicated in this study, whereas the new lingonberries in batch Lingon2 did not significantly prevent HF-induced weight gain and adiposity. In contrast to the previous study, we here observe a tendency to reduced energy intake in mice supplemented with lingonberries, especially Lingon1. There is also a tendency to increased energy content in feces from mice receiving lingonberries, potentially caused by fiber or due to less digestion and absorption of nutrients. Studies in humans suggest that lingonberries may delay the digestion of sucrose [54], and there is a need for additional studies describing how lingonberries affect absorption and digestibility, since these processes may be influenced by gut microbiota and phytochemical intake [55].

The reason for the difference in metabolic outcomes in response the Lingon1 and Lingon2 diets is intriguing, and should be the subject for further investigations. The quantity of nutrients and secondary metabolites in plants is highly variable and depends on several factors including cultivar, growth conditions, time of harvest and environmental exposures[56 57]. It has been theorized that new plant tissues produce defense compounds that affect growth and metabolism of for example microorganisms (e.g. acids and flavonoids), whereas more ripe tissues produce digestibility reducers that act on general digestive processes [58 59] however extensive further research is required to identify which factors and compounds define specific metabolic effects of lingonberries. The presented results highlight that variability between different batches is an important factor to take into consideration in nutritional research.

## CONCLUSIONS

To the best of our knowledge, this is the first work describing that lingonberry intake modifies the gut microbiota composition and prevents endotoxemia and low-grade inflammation. The present study replicates the previous finding that lingonberries exert anti-obesity effects when supplemented to a HF diet, however, the magnitude of the effect varies depending on the batch of berries. Regardless of the berry batch and effect on body weight, lingonberries promote modifications of the gut microbiota and protects against low-grade inflammation, including reduced inflammation in liver and adipose tissue. Specifically, lingonberries decrease the Firmicutes/Bacteroidetes ratio and the relative abundance of *Akkermansia* independently of changes in body weight. We propose that modification of the gut microbiota is important for the anti-inflammatory effect of lingonberries, and that lingonberries should be further investigated for its potential role in dietary strategies to prevent metabolic disease.

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**Author contributions**

LHL designed the study, performed the animal study, carried out measurements of plasma parameters, conducted statistical analysis and wrote the manuscript. FF aided in conceiving the hypothesis, performed analysis and statistical analysis of the 16S amplicon sequencing, came with valuable input and ideas throughout the study and aided in writing the manuscript. ES performed histology analysis, data interpretation and aided in writing the manuscript. DK performed RNA extraction and gene expression analysis of intestine, and gave valuable advice on the manuscript. MB carried out the bomb calorimetric measurements and participated in interpretation of fecal and food intake data. CH gave advice, support and provided valuable comments on the manuscript. KB took part of study design, interpretation of data and writing of the manuscript.

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**Table 1. Composition of the experimental diets**[a]

| | Control | Lingon1 | Lingon2 |
|---|---|---|---|
| *Calculated energy (kcal)* | | | |
| Protein | 812.0 | 812.0 | 812.0 |
| Carbohydrate | 1422.4 | 1422.4 | 1422.4 |
| Starch | 731.2 | 731.2 | 723.2 |
| Sucrose | 347.2 | 347.2 | 347.2 |
| Fructose | 172.0 | 172.0 | 172.0 |
| Glucose | 172.0 | 172.0 | 180.0 |
| Fat | 1822.5 | 1822.5 | 1822.5 |
| Total kcals | 4057 | 4057 | 4057 |
| *Calculated energy per gram diet (kcal/g)* | | | |
| Kcal/g | 4.5 | 4.3 | 4.4 |
| *Calculated energy (kcal%)* | | | |
| Protein | 20 | 20 | 20 |
| Carbohydrate | 35 | 35 | 35 |
| Fat | 45 | 45 | 45 |
| *Analyzed fiber (g/100 g diet)*[b] | | | |
| Insoluble fiber | 10.1 | 7.9 | 8.7 |
| Soluble fiber | <1 | 1.6 | 1.2 |
| Total fiber | 10.5 | 9.5 | 9.9 |

[a]The diets are formulated to have matched macronutrient composition by energy (Research Diets, NB, USA).

[b]Fiber analyzed by Eurofins, Lidköping, Sweden.

**Table 2. Energy intake and fecal energy content**

| | Lingon1 | Lingon2 | Control |
|---|---|---|---|
| Energy intake† (kcal/mouse) | 84.1 ± 3.8 | 89.6 ± 4.5 | 94.0 ± 5.0 |
| Fecal energy content‡ (kcal/24h/cage) | 11.7 ± 0.9 | 10.6 ± 1.1 | 8.2 ± 0.5 |
| Ingested digestible energy§ (kcal/day) | 80.2 ± 1.7 | 82.7 ± 0.7 | 87.2 ± 1.1 |
| Feed efficiency of digestible energy# (g/kcal*$10^6$/day) | 0.18 ± 0.15 | 0.25 ± 0.19 | 0.22 ± 0.08 |

† Mean weekly energy intake expressed per mouse.

‡ Mean fecal energy content excreted per cage per 24h sampled at week 7, 8 and 10.

§ Mean of ingested energy – fecal energy.

# Mean weekly body weight gain per consumed energy per cage.

Values are represented as group mean ± SD.

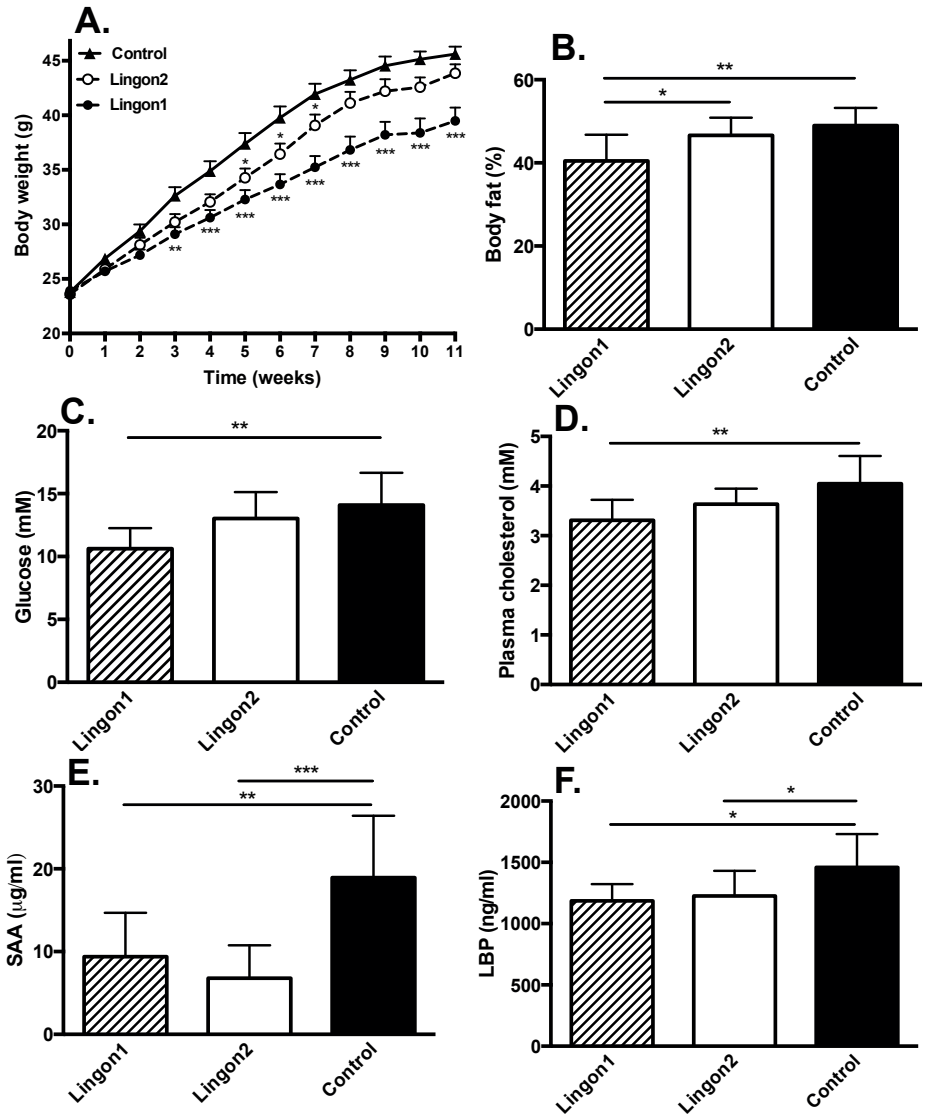


**Figure 1. Metabolic and inflammatory characteristics of mice fed high-fat diet (control) supplemented with different batches of lingonberries.** A: Lingon1 (black circles) and Lingon2 (white circles) supplementation had effects on body weight gain compared to the control (black triangles). The stars next to Lingon1 and Lingon2 data points denotes significant differences compared to the control group for each week (two-way ANOVA mean ± SEM; Dunnett's post-hoc test). The percentage of body fat (B), plasma levels of glucose (C) and cholesterol (D) were reduced by supplementation with Lingon1. E: The plasma concentration of the inflammatory marker serum amyloid A (SAA) and the endotoxemia marker LPS-binding protein (LBP) were reduced by both lingonberry diets compared to the control. Values represent mean ± SD, n = 9-10. One-way ANOVA; Tukey's post-hoc test * $p < 0.05$, **$p < 0.01$ or ***$p < 0.001$.

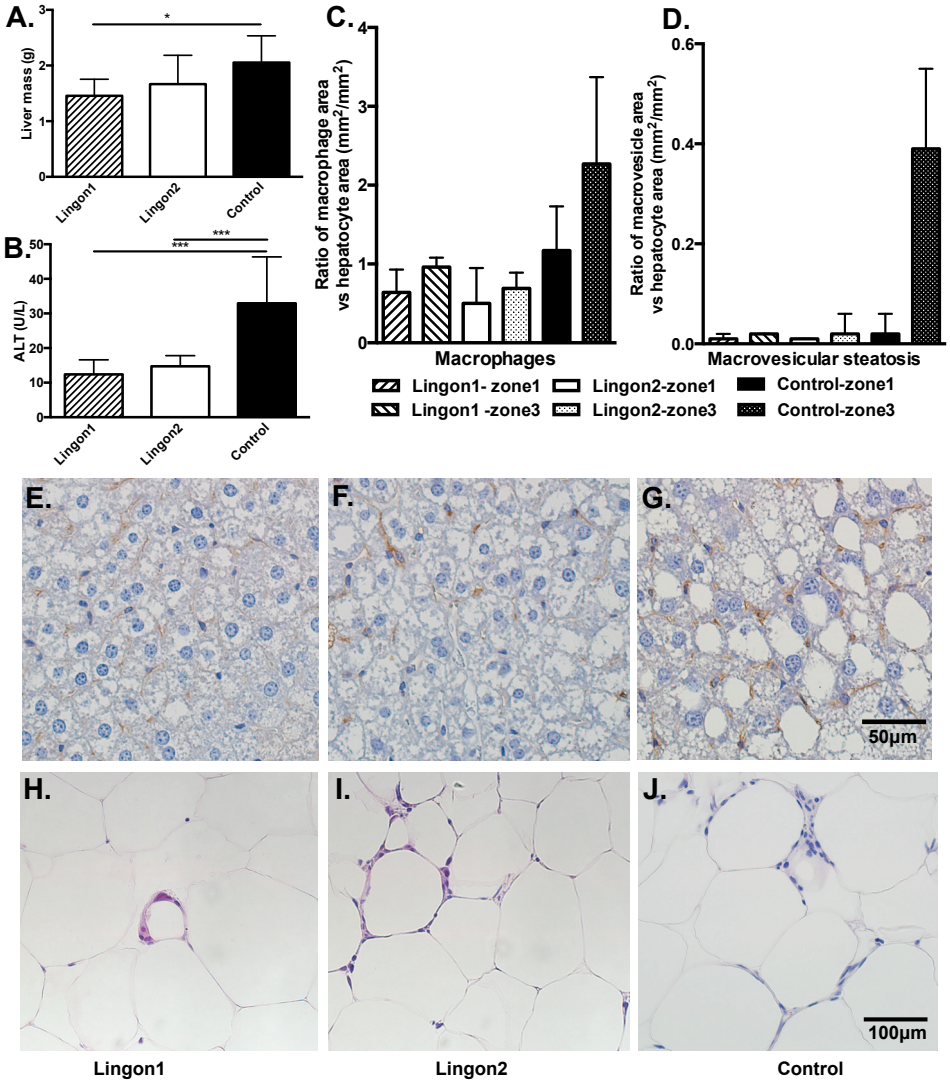


**Figure 2. The effect of lingonberry supplementation on liver steatosis and inflammation.** A: Liver mass was significantly lower in mice supplemented with lingonberries from batch1 (Lingon1) compared to mice receiving HF without berries (Control). B: The plasma levels of ALT, a marker for liver dysfunction, were reduced in groups receiving both Lingon1 and Lingon2 compared to control (mean ± SD, n = 8-10) * $p < 0.05$ or ***$p < 0.001$. The livers and epididymal fat pads from three mice per group were subjected to histological analysis. Quantification of slides revealed differences in the relative prevalence of macrophages (C) and macrovesicular steatosis (D) between groups, as well as between different liver zones within the groups; data plotted as median and interquartile range (n = 3, 2 sections analyzed per group, 5 areas per zone). Representative slides of livers stained with an antibody specific for macrophages (in brown) and counterstained with hematoxylin are shown in (E) Lingon1, (F) Lingon2 and (G) Control. Figure (H-J) display hematoxylin and eosin stained epididymal adipose tissue with leukocyte nuclei forming crown-like structures around adipocytes.

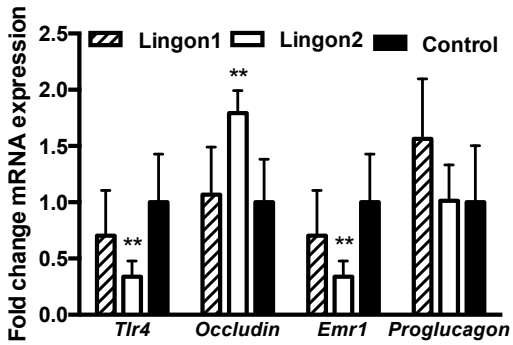


**Figure 3. Intestinal gene expression of markers related to inflammation and gut barrier integrity.** The fold change of jejunal mRNA levels of indicated genes are displayed relative to the expression in the control group. Lingon2 significantly increased expression of the gene for Tlr4 (LPS-stimulated receptor), the tight junction protein occludin and the macrophage marker Emr1 (F4/80). Values significantly different from control are depicted ** $p < 0.01$ (one-way ANOVA), mean ± SD, n = 6-7.

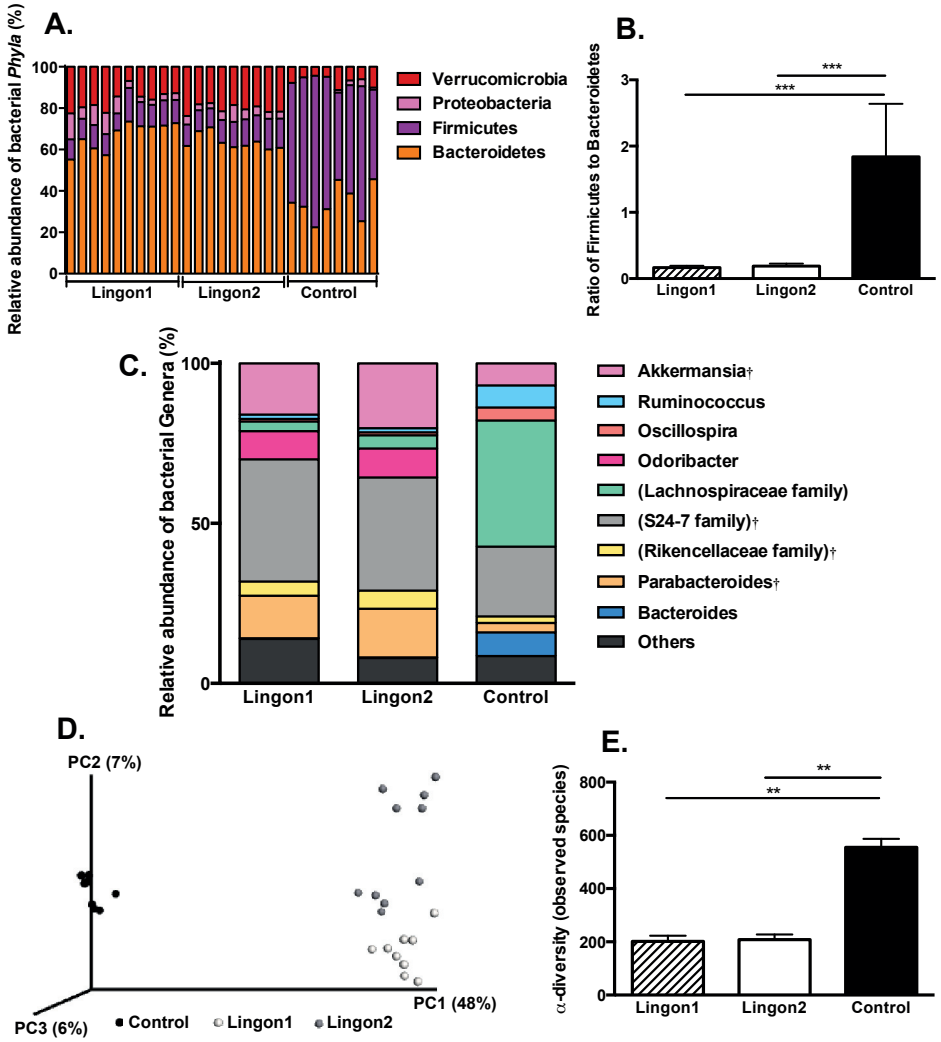


**Figure 4. Lingonberry intake promotes modification of gut microbiota composition in high-fat fed mice.** Cecal gut microbiota was analyzed using 16S rRNA sequencing in mice fed high-fat diets for 11 weeks. Two experimental groups were fed high-fat diet supplemented with two different batches of lingonberries, (Lingon1 and Lingon2) and were compared to a group receiving high-fat diet without berries (Control). A: Bars represent the relative abundance (%) of bacterial phyla and the Firmicutes/Bacteroidetes-ratio is displayed in (B). C: The relative abundance of bacterial genera (%) that were significantly modified by lingonberry supplementation ($p < 0.0001$) and had a relative abundance above 4% in one or more of the groups. † denotes genera significantly different between the Lingon1 and Lingon2 groups ($p < 0.05$) and each color represents a separate genera. In cases where the genus was unclassified, the family name is written in parenthesis. D: Unweighted PCA plot showing the degree of bacterial taxonomic similarity between samples at the genus level; the larger the distance between samples, the more different they are with respect to the axes (PC1, PC2 and PC3). Lingon1: white circles; Lingon2: grey circles; Control: black circles. F: The alpha-diversity was decreased in groups receiving lingonberries compared to the control group (non-parametric t-test and Bonferroni correction). n = 8-10, mean ± SD, ** $p < 0.01$, *** $p < 0.0001$.

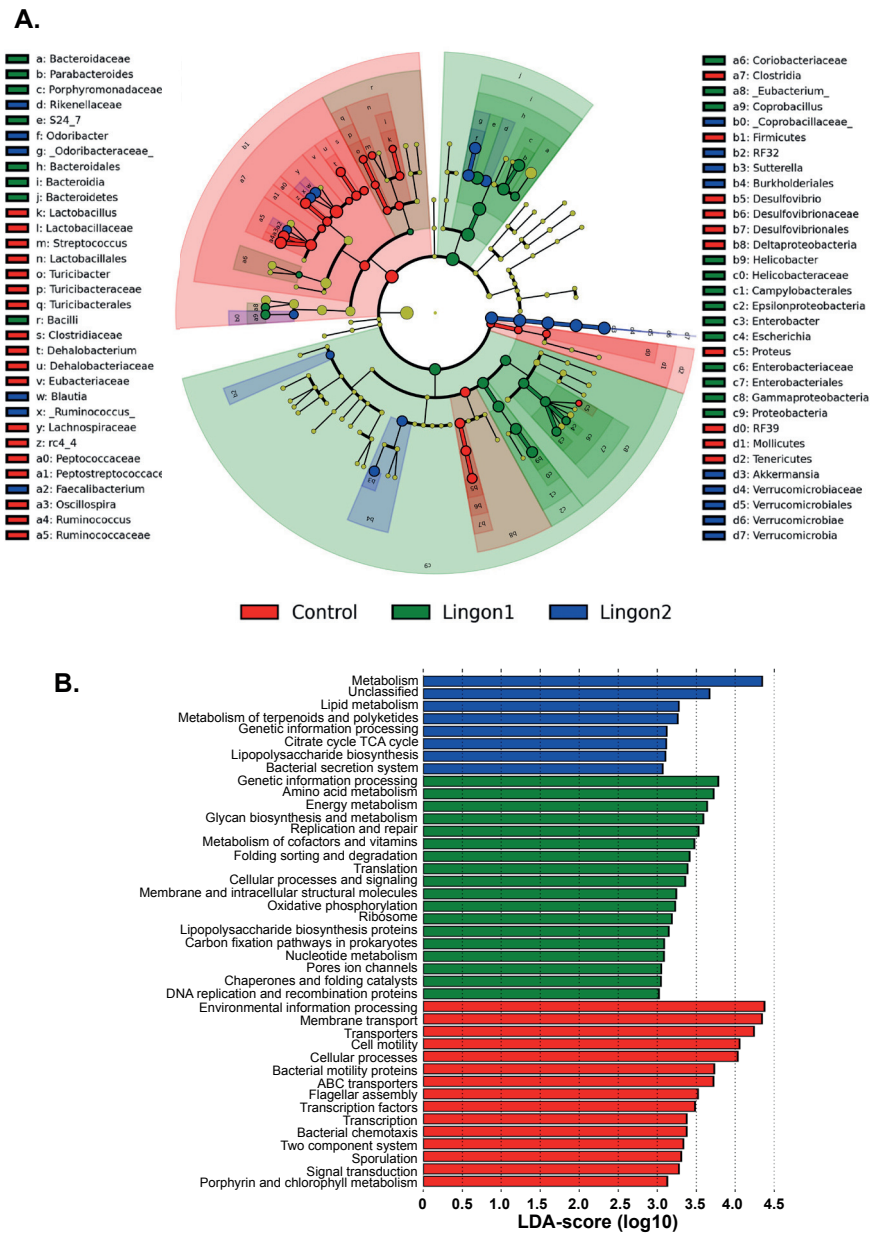


**Figure 5. Taxonomic and functional diversity of the cecal microbiota of mice receiving high-fat diet with or without supplementation with lingonberries.** A: Significant changes in relative abundance (proportional to circle size) are marked in red (elevated in control), green (elevated in Lingon1) and blue (elevated in Lingon2). Data are derived using LEfSe and differences with a LDA-score greater than 2 are considered significant, whereas non-significant changes are marked with yellow. Going towards the center of the figure, letter symbols represent phylum, class, order, family and genus, which are identified in the legend. B: Metabolic pathways (KEGG) altered by diet were identified using PICRUSt and LEfSe (LDA > 3.0). Pathways enriched in the microbiota of mice in the control group are displayed in red, Lingon1 in green and Lingon2 in blue.