This is the published version of a paper published in *International Journal of Food Sciences and Nutrition*.

Citation for the original published paper (version of record):

Liu, J., Hefni, M E., Witthöft, C M., Bergström, M., Burleigh, S. et al. (2022)
On the effect of flavonoids and dietary fibre in lingonberries on atherosclerotic plaques, lipid profiles and gut microbiota composition in Apoe(-/-) mice
*International Journal of Food Sciences and Nutrition, 73*(8): 1080-1090
https://doi.org/10.1080/09637486.2022.2106358

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:
http://urn.kb.se/resolve?urn=urn:nbn:se:lnu:diva-116331
On the effect of flavonoids and dietary fibre in lingonberries on atherosclerotic plaques, lipid profiles and gut microbiota composition in Apoe\(^{-/-}\) mice

Jiyun Liu, Mohammed E. Hefni, Cornelia M. Witthöft, Maria Bergström, Stephen Burleigh, Margareta Nyman & Frida Hållenius

To cite this article: Jiyun Liu, Mohammed E. Hefni, Cornelia M. Witthöft, Maria Bergström, Stephen Burleigh, Margareta Nyman & Frida Hållenius (2022) On the effect of flavonoids and dietary fibre in lingonberries on atherosclerotic plaques, lipid profiles and gut microbiota composition in Apoe\(^{-/-}\) mice, International Journal of Food Sciences and Nutrition, 73:8, 1080-1090, DOI: 10.1080/09637486.2022.2106358

To link to this article: https://doi.org/10.1080/09637486.2022.2106358

© 2022 The Author(s). Published with license by Taylor & Francis Group, LLC.

Published online: 05 Aug 2022.

Article views: 1068
On the effect of flavonoids and dietary fibre in lingonberries on atherosclerotic plaques, lipid profiles and gut microbiota composition in Apoe<sup>−/−</sup> mice

Jiyun Liua, Mohammed E. Hefnia,b, Cornelia M. Witthöfta, Maria Bergströmab, Stephen Burleighb and Frida Hålleniusc

aDepartment of Chemistry and Biomedical Sciences, Faculty of Health and Life Sciences, Linnaeus University, Kalmar, Sweden; bFood Industries Department, Faculty of Agriculture, Mansoura University, Mansoura, Egypt; cDepartment of Food Technology, Engineering and Nutrition, Lund University, Lund, Sweden

ABSTRACT
It has not been clarified whether the anti-atherosclerotic effect of lingonberry can be ascribed to its content of flavonoids or dietary fibre or both. The aim of this study was to evaluate the metabolic effects of whole lingonberries compared with isolated flavonoid and fibre fractions on atherosclerotic plaques, plasma lipid profiles, gut microbiota and microbiota-dependent metabolites in an Apoe<sup>−/−</sup> mouse model. Mice fed whole lingonberries showed the lowest amount of atherosclerotic plaques, while mice fed the fibre fraction had the highest formation of caecal butyric acid. Flavonoids, rather than dietary fibre, were suggested to be the components that favour proliferation of Akkermansia, as judged by the lowest abundance of this bacterium in mice fed the fibre fraction. All groups fed lingonberry diets had both, lower Firmicutes/Bacteroidetes ratios and creatinine concentrations, compared with the control. To conclude, different components in lingonberries are associated with different physiological effects in Apoe<sup>−/−</sup> mice.

Introduction
Atherosclerosis is a chronic inflammatory disease characterised by accumulation of lipids and infiltration of macrophages into the arterial wall (Gui et al. 2012). Although the mechanisms of the disease are not fully understood, several risk factors are suggested to be involved in the development or progression of atherosclerosis, such as inflammation, hypercholesterolaemia, imbalance in high- and low-density lipoprotein (HDL and LDL) and lipid oxidation (Rafieian-Kopaei et al. 2014). Dysbiosis of gut microbiota, characterised by ‘imbalance’ due to changes in community members or in relative abundance of microbes (Messer and Chang 2018), has also been proven to contribute to the development of atherosclerosis (Jones et al. 2014). On the other hand, some bacterial species, for instance Akkermansia muciniphila, have been found to possess anti-atherosclerotic activity (Li et al. 2016).

A variety of gut microbiota-dependent metabolites, such as short-chain fatty acids (SCFAs) and trimethylamine N-oxide (TMAO), may mediate between gut microbiota and atherosclerosis (Ma and Li, 2018). SCFAs are products from fermentation of dietary fibre that show protective effects against cardiovascular disease (Richards et al. 2016). Butyric acid is the most potent SCFA, since it serves as the primary source for the epithelium and is important for the integrity of the intestinal epithelial barrier (Kelly et al. 2015). TMAO, which can be produced in the host liver from a microbiota-dependent metabolite (trimethylamine, TMA), has been associated with increased risk of atherosclerosis in humans (Senthong et al. 2016). The precursors of TMA, such as choline, betaine and L-carnitine, are commonly found in animal-based food, for example, meat and lard (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) 2016).

Lingonberry (Vaccinium vitis-idaea) is commonly included in Nordic diets and has been shown to have protective effects against atherosclerosis and other metabolic diseases in mice by modulation of gut microbiota (Heyman-Linden et al. 2016; Matziouridou et al. 2016). Dietary fibre has also been ascribed to...
have above named effects, and in recent years even antioxidants, such as flavonoids, from non-cereal foods have received attention (Carlson et al. 2018; Chen et al. 2018; Pap et al. 2021). However, effects of these bioactive components (flavonoids, dietary fibre) on metabolic performance, gut microbiota and microbiota-dependent metabolites have not been studied systematically. Marungruang et al. (2020) studied effects of soluble and insoluble fibre from lingonberry on microbiota modulation and metabolic improvement in mice, but not the effects of flavonoid components.

The aim of the study was, therefore, to characterise and compare the effects of whole lingonberry (WLB) and two separate fractions, flavonoids and dietary fibre, on atherosclerotic plaque amount, plasma lipid profile, gut microbiota and microbiota-dependent metabolites (including caecal SCFAs and plasma concentrations of methyamines and creatinine) in an apolipoprotein E-deficient (Apoe−/−) mouse model. This animal model is well-established for atherosclerosis studies, since lack of apolipoprotein E in mice leads to great susceptibility to the disease (Lo Sasso et al. 2016).

Materials and methods

Preparation and characterisation of WLB and the flavonoid and dietary fibre fractions

The flavonoid fraction was isolated from milled lyophilised whole lingonberries using 99.5% ethanol (Liu et al. 2020) and the residue was taken to represent the dietary fibre fraction. The chemical composition of the WLB and the two fractions was further characterised using existing methods (see Supplementary Material). Details of the procedures and the nutritional composition of the samples (Table S1) are provided in Supplementary Material.

Animal experiment

The animal study was approved by the Malmö-Lund Ethics Committee on Animal Experiments (ethical approval number: 9874-20). A total of 50 female Apoe−/− mice aged 6–7 weeks were obtained from Taconic Biosciences Inc. (Silkeborg, Denmark). They were housed at 22°C under a 12 h light/dark cycle with free access to water. After acclimatisation to the environment for one week, the mice were randomly allocated into five groups (two cages per group, with five mice per cage). Four groups were given the following high fat (HF) diets (Table S2): (1) A HF control diet containing 60 g/kg cellulose, (2) a WLB diet containing 60 g/kg dietary fibre from lingonberry and 3.0 g/kg flavonoids, (3) a lingonberry flavonoid (LBFla) diet containing 60 g/kg cellulose and 2.0 g/kg flavonoids and (4) a lingonberry dietary fibre (LB Fiber) diet containing 60 g/kg dietary fibre from lingonberry. (5) A low fat (LF) control diet containing 60 g/kg cellulose was also included (Table S2). Body weight per animal and feed consumption per cage were recorded every week. During the experimental period, one mouse in the LF group, one in the WLB group and two in the LB Fiber group showed symptoms of sickness, which was considered to relate to poor physical constitution of individual mice, rather than the diets. Therefore, these four mice were excluded from the study. After 10.5 weeks of feeding, the mice were anaesthetised using Isoflurane (Abbott, Chicago, USA), blood samples (~1 mL) were collected from the heart, and the mice were sacrificed. Plasma was separated by centrifugation (2000 g for 15 min at 4°C). The caecum was weighed and immediately stored on dry ice. Gonadal fat pads, spleen and liver were weighed and discarded. Hearts were stored in OCT cryomount medium (Histoblab Products AB, Gothenburg, Sweden) in Tissue-Tek Cryomold® (Sakura Finetek, Torrance, USA) and frozen on dry ice. All samples were kept at −80°C prior to analyses.

Analysis of biological samples

Plasma lipid profiles

Total, HDL- and LDL-cholesterol and triglyceride levels in the mice plasma were measured using Infinity™ Cholesterol Reagents, Indiko™ and Konelab™ System Clinical Chemistry Reagents and Infinity™ Triglycerides Reagents (Thermo Fisher Scientific Inc., Waltham, USA), respectively, according to the protocols of the manufacturers.

Plasma creatinine, TMAO and its precursors

Plasma creatinine, TMAO and its precursors were measured using an LC-MS method with some modification (Hefni et al. 2021). Details are provided in Supplementary Material.

Atherosclerotic plaque area in aortic root

The samples for histological analyses were prepared at Histocenter (Gothenburg, Sweden). Details of the procedure are provided in Supplementary Material. Representative pictures of aortic sections were obtained using a camera-microscope system (Nikon Eclipse E400) with imaging software (NIS-Elements).
4.13; both Nikon Instruments Inc., Tokyo, Japan). Atherosclerotic plaque amount (ratio of plaque area to total vessel area, %) was quantified using BioPixQ 2.0 software (BIOPIX software, Gothenburg, Sweden).

**SCFAs in caecum content**
The content and profile of SCFAs in the caecum was analysed using a GC-FID method with some modifications (Zhao et al. 2006). For details, see Supplementary Material.

**Sequencing of bacterial 16S rRNA genes**
DNA extraction and PCR amplification and sequencing were conducted by Clinical Microbiomics, Copenhagen, Denmark. For details, see Supplementary Material.

**Analysis of sequence data**
Raw sequence data were analysed with the open-source bioinformatics pipeline Quantitative Insights Into Microbial Ecology (QIIME; Caporaso et al. 2010), together with our in-house developed pipeline. A total of 1,497,226 reads were obtained from 45 samples, with an average of 33,272 reads per sample. The sequences were normalised by rarefaction (depth of 18,000), grouped into operational taxonomic units (OTUs) at a minimum of 97% similarity using the closed reference method based on the Greengenes database (v.13.8), and filtered by removal of singletons and low abundance OTUs (minimum count fraction set at 0.001).

**Statistical analysis**
Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using both univariate and multivariate approaches. For univariate analysis, except for body weight and feed consumption data, the two control groups were compared using Student’s t-test or Welch’s t-test for parametric data with unequal SDs. Unless otherwise stated, significant differences between the HF and lingonberry intervention groups were analysed by one-way ANOVA followed by Tukey’s multiple comparisons test. In cases where the same SD was not assumed, Welch’s ANOVA test followed by Dunnett’s T3 multiple comparisons test was applied. As for body weight and feed consumption data, significant differences between all the groups were analysed by two-way ANOVA (diet and time as the variables) followed by Tukey’s multiple comparisons test. For the non-parametric data on bacterial abundance, the Kruskal-Wallis test followed by Dunn’s test for multiple comparisons was utilised. For multivariate analysis, non-parametric Spearman correlation was performed to measure the correlation between TMAO, atherosclerotic plaque and gut microbiota. Significance was set to \( p \leq 0.05 \), and \( p \leq 0.1 \) was considered to indicate a tendency (Prism 9.2.0, GraphPad, La Jolla, CA, USA). Principal component analysis (PCA) was performed to investigate the overall effects of the diets on metabolic parameters, using R programming language. A QIIME-based permanova (using the pseudo-F statistical test and 999 permutations) was used to test for overall differences between the microbiomes in the five treatments.

**Results**

**Characterisation of lingonberry and its fractions**
The total content of dietary fibre in the whole lingonberries and the isolated fibre fraction was 18 and 61 g/100 g dry weight (dwt), respectively. The fibre consisted mainly of uronic acids (29%), glucose (24%), arabinose (19%) and xylose (16%) (Figure S1). The total content of flavonoids, including anthocyanidins, proanthocyanins, flavan-3-ols and flavonols, in the WLB diet was 672 mg/100 g dwt, and the content in the LBFla diet was 590 mg/100 g dwt (Table S1).

**Feed consumption, organ and fat pad weights**
In the two control groups (HF and LF diets), no significant differences in body or organ weight were observed (Figure S2). Mice fed the three lingonberry diets had 50%–90% higher feed consumption per week \( (p < 0.0001) \) compared with the HF control. The WLB group attained higher liver \( (p < 0.05) \) and caecum \( (p < 0.01) \) weight than the HF group. Mice fed the LBFla fraction had higher caecum weights \( (p < 0.05) \) but lighter fat pads compared with mice fed the HF diet \( (p < 0.05) \), while mice fed the LBFiber fraction had higher liver weights \( (p < 0.05) \) and body weights \( (p < 0.05) \) than mice fed the HF diet (Figure S2).

**Plasma lipid profiles and atherosclerotic plaques**
The LF diet resulted in 69% lower concentration of plasma cholesterol (Figure 1(A)) in the mice and 70% lower atherosclerotic plaque amount \( (p < 0.05) \) compared with the HF diet (Figure 1(D)). The mice fed the WLB and LBFiber diets had 40%–50%
higher levels of triglyceride in plasma ($p < 0.01$) compared with mice fed the HF diet (Figure 1(C)). The atherosclerotic plaque amount in the WLB group was 50% lower ($p < 0.05$) than in the HF group (Figure 1(D)).

Table 1. Caecal pools (µmol) and proportions (%) of individual SCFAs in the caecum of mice fed the LF, HF, WLB, LBFla and LBFiber diets for 10.5 weeks.1

|                | LF2 | HF2,3 | WLB3 | LBFla4 | LBFiber4 |
|----------------|-----|-------|------|--------|----------|
| **Amount (µmol)** |     |       |      |        |          |
| Acetic acid     | 5.7 ± 1.7 | 6.5 ± 1.3a | 16.1 ± 6.4b | 12.3 ± 5.1c | 12.6 ± 3.5c |
| Propionic acid  | 1.1 ± 0.2 | 1.1 ± 0.3a | 3.4 ± 1.8b | 2.5 ± 0.7c | 2.3 ± 0.9c |
| Butyric acid    | 0.8 ± 0.2a | 0.6 ± 0.1a | 1.2 ± 0.8ab | 1.3 ± 1.0bc | 1.8 ± 0.2bc |
| Minor acids4    | 0.5 ± 0.2 | 0.4 ± 0.1a | 0.5 ± 0.2ab | 0.7 ± 0.1bc | 0.6 ± 0.2bc |
| Total           | 8.0 ± 1.6 | 8.6 ± 1.6a | 21.2 ± 9.0b | 16.8 ± 6.7b | 17.2 ± 5.3b |
| **Proportion (%)** |     |       |      |        |          |
| Acetic acid     | 70 ± 4a | 76 ± 3a | 77 ± 4a | 73 ± 3a | 73 ± 3a |
| Propionic acid  | 13 ± 2  | 12 ± 2a | 16 ± 2b | 15 ± 3b | 13 ± 2ab |
| Butyric acid    | 10 ± 2a | 8 ± 2a  | 5 ± 2b  | 7 ± 2ab | 10 ± 2b |
| Minor acids     | 6 ± 3  | 4 ± 1a  | 2 ± 1b  | 5 ± 1a  | 3 ± 1a  |

1Values shown are mean ± SD ($n = 8–10$).
2Means in the LF and HF groups were compared using Student’s t-test.
3$p < 0.05$, $^{*}p < 0.01$.
4Means in the HF, WLB, LBFla and LBFiber groups were compared using one-way ANOVA followed by Tukey’s multiple comparison test. Means within rows with different lowercase letters are significantly different ($p < 0.05$).
5Minor acids include isobutyric-, valeric-, isovaleric-, caproic- and heptanoic acid.

SCFAs in caecum

Mice fed the LF diet had a higher caecal pool and proportion of butyric acid ($p < 0.05$) compared with mice fed the HF diet (Table 1). Inclusion of WLB or lingonberry fractions in the diets led to higher caecal...
pools of acetic and propionic acids in the mice, as well as total SCFAs, compared with the HF diet. Moreover, compared with the HF diet, the WLB diet resulted in a higher proportion of propionic acid with concurrently lower proportions of butyric and minor acids ($p < 0.05$); the LBFla diet resulted in a higher pool and proportion of propionic acid ($p < 0.05$); the LBFiber group resulted in a higher pool of butyric acid ($p < 0.05$) and an increasing tendency regarding the proportion ($p = 0.08$) (Table 1).

**Methylamines and creatinine in plasma**

Regarding the two control groups, mice in the LF group had higher plasma concentrations of TMAO and LL-carnitine and lower plasma creatinine concentrations ($p < 0.05$) compared with the HF control. Moreover, compared with the HF diet, the WLB diet resulted in lower plasma concentrations of LL-carnitine ($p < 0.05$), the LBFla diet resulted in higher concentrations of TMAO, betaine and acetyl-carnitine ($p < 0.05$), and the LBFiber diet resulted in higher TMAO concentrations ($p < 0.05$; Figure 2).

Plasma TMA concentrations ($<3\mu M$) were detected in all groups and no significant differences were found (data not shown).

**Gut microbiota**

Significant differences were observed in the composition of gut microbiota between the five diets, based on a QIIME permanova ($p < 0.01$), and these differences were also visually apparent at both phylum (Figure 3(D)) and genus (Figure 3(E)) level. The
lowest alpha-diversity was found in the WLB group and the highest in the LBFiber group (Figure 3(A)). Beta-diversity analysis using unweighted and weighted UniFrac principal coordinates analysis (PCoA) plot based on OTU level showed that the three lingonberry groups were clearly separated from the two controls (Figure 3(C)).

At phylum level, Firmicutes was the predominant species (46%–60%) in all groups except the WLB group, where Verrucomicrobia was present in the highest proportion (34%; Figure 3(D)). The LF group had lower abundance of Deferrribacteres and Proteobacteria ($p < 0.05$) compared with the HF group. Compared with mice fed the HF diet, mice fed the WLB diet had higher abundance of Actinobacteria and Bacteroidetes ($p < 0.05$) and lower abundance of Deferrribacteres ($p < 0.01$), mice fed the LBFla diet had higher abundance of Actinobacteria ($p < 0.05$), and mice fed the LBFiber diet had higher abundance of Bacteroidetes ($p < 0.05$). Mice in the WLB group also had higher abundance of Verrucomicrobia ($p < 0.05$) and lower abundance of Deferrribacteres compared

**Figure 3.** (A) Chao1 rarefaction curves, (B) unweighted and (C) weighted UniFrac PCoA plots, relative abundance at (D) phylum level and (E) genus level of gut microbial taxa and (F) Firmicutes/Bacteroidetes ratio in the caecum of mice fed the LF, HF, WLB, LBFla and LBFiber diets for 10.5 weeks. Firmicutes/Bacteroidetes ratio was compared between the LF and HF groups using Mann-Whitney test, and between the HF, WLB, LBFla and LBFiber groups using Kruskal-Wallis test followed by Dunn’s test for multiple comparisons. Different lowercase letters within panels indicate significant difference ($p \leq 0.05$). ns, not significant.
with the LBFiber group \((p < 0.01)\). All lingonberry diets resulted in lower Firmicutes/Bacteroidetes \((F/B)\) ratio than the HF diet \((\text{Figure 3(F)}\).

At genus level, the LF group had higher abundance of *Dehalobacterium* and lower abundance of *Lactobacillus, Mucispirillum* and *Bilophila* \((p < 0.05)\) than the HF group. Compared with mice fed the HF diet, mice fed the WLB diet had lower abundance of *Oscillospira, Lactobacillus, Mucispirillum* and *Bilophila* \((p < 0.05)\) and higher abundance of *Bifidobacterium* \((p < 0.05)\), mice fed the LBFla diet had lower abundance of *Lactobacillus* \((p < 0.05)\) and higher abundance of *Bifidobacterium* \((p < 0.05)\), and mice fed the LBFiber diet had higher abundance of unclassified *S24_7* and *Clostridiales* \((p < 0.05; \text{Figure S3})\).

**Overall effects of diets on organ and body weight and biomarkers**

Organ and body weight, caecal SCFAs, plasma lipids and methylamines in mice fed the different diets were compared using PCA. Each group clustered separately in the PCA plot \((\text{Figure S4})\). Mice in the HF group were separated likely in part due to high plasma creatinine concentration, while the LF group was characterised by high plasma 1-\(\text{p-carnitine}\) concentration. The WLB group was separated from the other lingonberry groups by high caecum weights and high caecal pools of acetic acid, propionic acid and total SCFAs. The LBFla group was characterised by a significant plasma betaine concentration, while the LBFiber group clustered based on weight gain and liver weight. The effects of the WLB and LBFla diets were more similar to each other, suggesting that the WLB and LBFla groups were closely associated.

**Correlations between methylamines, atherosclerosis plaque and caecal microbiota**

There was no significant association between plasma TMAO concentration and atherosclerotic plaque amount. As regards to the associations between the concentrations of TMAO and its precursors in plasma and caecal microbiota, a total of 29 bacterial taxa at genus level were identified \((\text{Table S3})\). The genera *Mucispirillum, Streptococcus, Peptococcaceae* and *Bilophila* were positively associated with atherosclerotic plaques \((p < 0.05)\).

**Discussion**

The aim of this study was to characterise and compare the effects of WLB, LBFla and LBFiber fractions on atherosclerotic plaque, plasma lipid profiles, gut microbiota and microbiota-dependent metabolites in Apoe\(^{-/-}\) mice. The results showed that WLB, but not the LBFla or LBFiber fraction, had the potential to decrease atherosclerotic plaque amount, but none of the three lingonberry diets positively affected lipid profile.

**Effects of WLB and its fractions on gut microbiota**

The alpha-diversity of gut microbiota was lower with the WLB group compared with the HF group \((\text{Figure 3(A)})\), which is in line with a previous study \((\text{Matziouridou et al. 2016})\). Low diversity of gut microbiota is often linked to microbiota dysbiosis \((\text{Kriss et al. 2018})\). The low alpha-diversity in the WLB group needs to be carefully interpreted because *Akkermansia*, which is regarded as beneficial against metabolic diseases and atherosclerosis in some studies \((\text{Everard et al. 2013; Li et al. 2016; Zhao et al. 2017})\), was the predominant genus in gut microbiota in mice fed the WLB diet \((\text{Figure 3(E)})\). Stimulation of the growth of *Akkermansia* by WLB has been reported by others \((\text{Marungruang et al. 2020})\). Because of higher relative abundance of *Akkermansia* in the LBFla group \((\text{Figure 3(E)})\), it may be questioned, whether the flavonoid content, rather than the dietary fibre, in lingonberry favours the proliferation of *Akkermansia*. Similarly, Rodríguez-Daza et al. \((2020)\) found that polyphenol-rich cranberry and blueberry promoted the growth of *Akkermansia* in an obese mouse model, while the fibrous fraction had no effect.

All the lingonberry diets reduced the \(F/B\) ratio, high values of which have been linked with obesity and disorders of energy balance \((\text{Koliada et al. 2017; Ley et al. 2005})\). The low \(F/B\) ratio observed in the berry groups may indicate potential beneficial effects of WLB and its fractions on gut microbiota at phylum level. At genus level, the WLB and LBFla diets showed similarities in decreasing the relative abundance of *Lactobacillus* and increasing the abundance of *Bifidobacterium* \((\text{Figure S3})\). Similarly, in a human trial the relative abundance of *Bifidobacterium* has been observed to increase after six weeks of consumption of a blueberry drink \((\text{Vendrame et al. 2011})\). The increase in *Bifidobacterium* might be attributable to the high amount of flavonoids in berries, as this species can metabolise flavonoids \((\text{Le Roes-Hill et al. 2011})\). The WLB group also had lower abundance of...
Oscillospira and Mucispirillum, both of which are associated with inflammatory conditions (Chen et al. 2020; Herp et al. 2021). In contrast, the LBFiber group had higher abundance of unclassified S24_7 and Clostridiales (Figure S3), both of which are involved in metabolism of complex carbohydrates (Lagkouvardos et al. 2019).

Overall, the WLB and LBFla diets had similar effects on gut microbiota composition as seen for the LBFiber diet.

Effects of WLB and lingonberry fractions on microbiota-related metabolites

The profile of caecal SCFA depends on several factors, such as the monomeric composition and physiochemical properties of dietary fibre and the gut microbiota composition (Fåk et al. 2015). We observed a significantly higher pool and proportion of butyric acid in the LBFiber group than the HF group, which did not occur in the WLB or LBFla groups (Table 1). A previous study on rats found that a HF diet decreased butyric acid formation, whereas this effect was reversed by intake of pure dietary fibre, such as guar gum and pectin (Jakobsdottir et al. 2013). Pectin, the main component of LBFiber, is known to give a high amount of acetic acid, but a combination of pectin with other indigestible carbohydrates may change the pattern of SCFAs (Henningsson et al. 2002). One may also wonder why the LBFla diet, which contained cellulose, a fibre very resistant to fermentation in colon, led to high amounts of caecal SCFAs. It could be due to the high content of fructose (210–296 mg/g dwt; Vilkickyte et al. 2019) in this lingonberry fraction, which may be malabsorbed in the small intestine and therefore reach colon for fermentation. An intake of about 50 g fructose may be enough for malabsorption in humans (Rao et al. 2007). This corresponds to about 20 mg in a mouse with 20 g body weight. The fructose was extracted together with the flavonoids and remained in the LBFla fraction. Part of fructose in the LBFla fraction reached the colon and may explain the high formation of SCFAs. Further studies are needed to clarify the role of fructose on SCFA formation in lingonberries.

To our knowledge, our data is the first report on TMAO concentrations in mice after lingonberry intake (Table S3). Unexpectedly, the WLB diet and the LF diet resulted in lower atherosclerotic plaque amount (Figure 1(C)), but not in lower plasma TMAO concentrations (Figure 2(A)). This may be due to the use of the Apoe<sup>−/−</sup> mouse model instead of other healthy mouse models. In several previous studies using the Apoe<sup>−/−</sup> model, the mice did not have higher atherosclerotic plaque amount despite higher plasma TMAO concentrations (Collins et al. 2016; Koay et al. 2021; Lindskog Jonsson et al. 2018). Besides, female mice were used in the present study and female mice are reported to have high expression levels of TMA-converting enzymes, leading to higher basic plasma TMAO concentrations (Falls et al. 1995). It is also worth noting that all three lingonberry diets resulted in lower plasma creatinine concentrations compared with the HF diet (Figure 2(C)). Elevated plasma creatinine is a strong predictor of renal failure (Aronson and Burger, 2010). Our findings hint at potential protective effects of lingonberry against kidney diseases.

Effects of WLB and lingonberry fractions on plasma lipids and atherosclerotic plaque amount

High total or LDL cholesterol level has been identified as an important risk factor in atherosclerosis (Kohsaka et al. 2010; Ravnskov, 2002). Previous studies have shown that intake of WLB can decrease total plasma cholesterol (Matziouridou et al. 2016) and enhance HDL cholesterol concentrations (Marungruang et al. 2020). However, the lingonberry diets did not affect plasma cholesterol concentrations in our study (Figure 1(A)).

Our results on plasma triglyceride levels and liver weights (Figure S2) were inconsistent with previous findings of a triglyceride-decreasing effect of lingonberry (Madduma Hewage et al. 2021; Matziouridou et al. 2016). We do not have a good explanation for this discrepancy, but it was perhaps due to the long-term high amount of feed intake exaggerating the health status of Apoe<sup>−/−</sup> mice with a known genetic defect regarding lipid metabolism (Kypreos et al. 2009).

An anti-atherosclerotic effect of WLB (Figure 1(C)) was also observed by Matziouridou et al. (2016), using the same mouse model. This effect might be related to the gut microbiota. Studies have indicated a role of gut microbiota in atherosclerosis development, for example, Gregory et al. (2015) found that transplantation of gut microbiota from atherosclerosis-prone mice led to transmission of atherosclerosis susceptibility. The bacterial species Akkermansia muciniphila has been demonstrated to possess anti-atherosclerotic activity (Li et al. 2016). Akkermansia was the most abundant genus in the WLB group (34%; Figure 3(E)) and might have contributed to the lower atherosclerotic plaque amount (Figure 1(C)) seen for that group.
In summary, WLB, rather than the single lingonberry fractions, caused lower atherosclerotic plaque amount, although none of the diets positively affected the lipid profile in Apoe\textsuperscript{−/−} mice. All the lingonberry diets resulted in lower F/B ratios compared with the HF diet, but all lingonberry diets showed different modulating effects on gut microbiota and microbiota-dependent metabolites. WLB, but not the fibre fraction, stimulated growth of Akkermansia. The LBFiber diet led to higher formation of caecal butyric acid. Whereas both lingonberry fraction diets caused higher plasma TMAO concentrations, resulted all the lingonberry diets in lower plasma creatinine.

**Author contributions**

J.L.: Conceptualisation, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft. M.E.H.: Conceptualisation, Methodology, Writing - Review & Editing, Supervision. C.M.W.: Conceptualisation, Methodology, Resources, Writing - Review & Editing, Supervision. M.B.: Methodology and Writing - Review & Editing, Supervision. S.B.: Data Curation, Software, Writing - Review & Editing. M.N.: Conceptualisation, Methodology, Resources, Writing - Review & Editing, Supervision. F.F.H.: Conceptualisation, Methodology, Resources, Writing - Review & Editing, Supervision, Funding Acquisition.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

The study was funded by the Direktör Albert Pählsson Foundation [grant number 15/12 2015 FF]. Research funding (for analysis of TMAO and related metabolites) from Crafoordskå Foundation [grant number: 20180874] and a Ph.D. scholarship from China Scholarship Council (CSC) for J.L. are gratefully acknowledged.

**ORCID**

Jiyun Liu http://orcid.org/0000-0001-8097-3483
Mohammed E. Hefni http://orcid.org/0000-0003-0550-5828
Cornelia M. Witthöft http://orcid.org/0000-0003-0387-4312
Maria Bergström http://orcid.org/0000-0001-7753-3823
Stephen Burleigh http://orcid.org/0000-0003-4261-6180
Margareta Nyman http://orcid.org/0000-0003-3660-2364
Frida Hälenius http://orcid.org/0000-0001-8203-9635

**References**

Aronson D, Burger AJ. 2010. The relationship between transient and persistent worsening renal function and mortality in patients with acute decompensated heart failure. J Card Failure. 16(7):541–547.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, et al. 2010. QIME allows analysis of high-throughput community sequencing data. Nat Methods. 7(5):335–336.

Carlson JL, Erickson JM, Lloyd BB, Slavin JL. 2018. Health effects and sources of prebiotic dietary fiber. Curr Dev Nutr. 2(3):nyz005.

Chen C, You LJ, Huang Q, Fu X, Zhang B, Liu RH, Li C. 2018. Modulation of gut microbiota by mulberry fruit polysaccharide treatment of obese diabetic db/db mice. Food Funct. 9(7):3732–3742.

Chen YR, Zheng HM, Zhang GX, Chen FL, Chen LD, Yang ZC. 2020. High Oscillospora abundance indicates constipation and low BMI in the Guangdong Gut Microbiome Project. Sci Rep. 10(1):9364.

Collins HL, Drazul-Schrader D, Sulpizio AG, Koster PD, Williamson Y, Adelman SJ, Owen K, Sanli T, Bellamine A. 2016. L-Carnitine intake and high trimethylamine N-oxide plasma levels correlate with low aortic lesions in ApoE(-/-) transgenic mice expressing CETP. Atherosclerosis. 244:29–37.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). 2016. Dietary Reference Values for choline. EFSA J. 14(8):4484.

Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, Guiot Y, Derrien M, Muccioli GG, Delzenne NM, et al. 2013. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci U S A. 110(22):9066–9071.

Falls JG, Blake BL, Cao Y, Levi PE, Hodgson E. 1995. Gender differences in hepatic expression of flavin-containing monoxygenase isofoms (FMO1, FMO3, and FMO5) in mice. J Biochem Toxicol. 10(3):171–177.

Fåk F, Jakobsdottir G, Kulcinskaia E, Marunguang N, Matziouridou C, Nilsson U, Stålbrand H, Nyman M. 2015. The physico-chemical properties of dietary fibre determine metabolic responses, short-chain Fatty Acid profiles and gut microbiota composition in rats fed low- and high-fat diets. PLoS One. 10(5):e0127252.

Gregory JC, Buffa JA, Org E, Wang Z, Levison BS, Zhu W, Wagner MA, Bennett BJ, Li L, DiDonato JA, et al. 2015. Transmission of atherosclerosis susceptibility with gut microbial transplantation. J Biol Chem. 290(9):5647–5660.

Gui T, Shimokado A, Sun Y, Akasaka T, Muragaki Y. 2012. Diverse roles of macrophages in atherosclerosis: from inflammatory biology to biomarker discovery. Mediators Inflamm. 2012:693083.

Hefni ME, Bergström M, Lennqvist T, Fagerström C, Wittlöf CM. 2021. Simultaneous quantification of trimethylamine N-oxide, trimethylamine, choline, betaine, creatinine, and propionyl-, acetyl-, and l-carnitine in clinical and food samples using HILIC-LC-MS. Anal Bioanal Chem. 413(21):5349–5360.

Hennevissson AM, Björck IME, Nyman EMGL. 2002. Combinations of indigestible carbohydrates affect short-
chain fatty acid formation in the hindgut of rats. J Nutr. 132(10):3098–3104.

Herp S, Durai Raj AC, Salvado Silva M, Woelfel S, Stecher B. 2021. The human symbiont *Mucispirillum schaedleri*: causality in health and disease. Med Microbiol Immunol. 210(4):173–179.

Heyman-Linden L, Kotowska D, Sand E, Bjursell M, Plaza M, Turner C, Holm C, Fäk F, Berger K. 2016. Lingonberries alter the gut microbiota and prevent low-grade inflammation in high-fat diet fed mice. Food Nutr Res. 60:29993.

Jakobsdottir G, Xu J, Molin G, Ahrné S, Nyman M. 2013. High-fat diet reduces the formation of butyrate, but increases succinate, inflammation, liver fat and cholesterol in rats, while dietary fibre counteracts these effects. PLoS One. 8(11):e80476.

Jones ML, Martoni CJ, Ganopolsky JG, Labbe A, Prakash S. 2014. The human microbiome and bile acid metabolism: dysbiosis, dysmetabolism, disease and intervention. Expert Opin Biol Ther. 14(4):467–482.

Kelly CJ, Zheng L, Campbell EL, Saeedi B, Scholz CC, Bayless AJ, Wilson KE, Glover LE, Kominsky DJ, Magnuson A, et al. 2015. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. Cell Host Microbe. 17(3):662–671.

Koay YC, Chen YC, Wali JA, Luk AWS, Li M, Doma H, Kohsaka S, Jin Z, Rundek T, Homma S, Sacco RL, Di Tullio. 2011. Actinobacterial dysbiosis, dysmetabolism, disease and intervention. PLoS One. 8(11):e80476.

Koliada A, Syzenko G, Moseiko V, Budovska L, Puchkov K, Kriss M, Hazleton KZ, Nusbacher NM, Martin CG, Lagkouvardos I, Lesker TR, Hitch TCA, Galvez EJC, Smit N, le Roes-Hill M, Khan N, Burton SG. 2016. Lingonberries alter the gut microbiota and prevent low-grade inflammation in high-fat diet fed mice. Food Nutr Res. 60:29993.

Liu J, Hefni ME, Witthoft CM. 2020. Characterization of flavoron compounds in common Swedish berry species. Foods. 9(3):358.

Lo Sasso G, Schlage WK, Boue S, Veličkovic E, Petitsch MC, Hoeng J. 2016. The ApoE(-/-) mouse model: a suitable model to study cardiovascular and respiratory diseases in the context of cigarette smoke exposure and harm reduction. J Tranal Med. 14(1):146.

Ma J, Li H. 2018. The role of gut microbiota in atherosclerosis and hypertension. Front Pharmacol. 9:1082.

Madduma Hewage S, Prashar S, O K, Siow YL. 2021. Lingonberry improves non-alcoholic fatty liver disease by reducing hepatic lipid accumulation, oxidative stress and inflammatory response. Antioxidants. 10(4):565.

Marungruang N, Kovalenko T, Osachenko I, Voss U, Huang F, Burleigh S, Ushakova G, Skibo G, Nyman M, Prykhodko O, et al. 2020. Lingonberries and their two separated fractions differently alter the gut microbiota, improve metabolic functions, reduce gut inflammatory properties, and improve brain function in ApoE(-/-) mice fed high-fat diet. Nutr Neurosci. 23(8):600–612.

Matziouridou C, Marungruang N, Nguyen TD, Nyman M, Fäk F. 2016. Lingonberries reduce atherosclerosis in ApoE(-/-) mice in association with altered gut microbiota composition and improved lipid profile. Mol Nutr Food Res. 60(5):1150–1160.

Messer JS, Chang EB. 2018. Microbial physiology of the digestive tract and its role in inflammatory bowel diseases. In: Said HM, editor. Physiology of the Gastrointestinal Tract. Cambridge: Academic Press; p. 795–810.

Pap N, Fidelis M, Azevedo L, do Carmo MAV, Wang D, Molan A, Pereira EPR, Xavier-Santos D, Sant’Ana AS, Yang B, et al. 2021. Berry polyphenols and human health: Evidence of antioxidant, anti-inflammatory, microbiota modulation, and cell-protecting effects. Curr Opin Food Sci. 42:167–186.

Rafieian-Kopaei M, Setorki M, Doudi M, Baradaran A, Nasri H. 2014. Atherosclerosis: process, indicators, risk factors and new hopes. Int J Prev Med. 5(8):927–946.

Rao SSC, Attaluri A, Anderson L, Stumbo P. 2007. Ability of the normal human small intestine to absorb fructose: evaluation by breath testing. Clin Gastroenterol Hepatol. 5(8):963.

Rao SSC, Attaluri A, Anderson L, Stumbo P. 2007. Ability of the normal human small intestine to absorb fructose: evaluation by breath testing. Clin Gastroenterol Hepatol. 5(8):963.

Ravnskov U. 2002. Is atherosclerosis caused by high cholesterol? QJM. 95(6):397–403.

Richards LB, Li M, Esch B, Garssen J, Folkerts G. 2016. The effects of short-chain fatty acids on the cardiovascular system. PharmaNutrition. 4(2):68–111.

Rodriguez-Daza MC, Roqium M, Dudonné S, Pilon G, Levy E, Marette A, Roy D, Desjardins Y. 2020. Berry polyphenols and fibers modulate distinct microbial metabolic functions and gut microbiota enterotype-like clustering in obese mice. Front Microbiol. 11:2032.

Senthong V, Wang Z, Li XS, Fan Y, Wu Y, Tang WH, Hazen SL. 2016. Intestinal microbiota-generated by preventing metabolic endotoxemia-induced inflammation in Apoe(-/-) mice. Circulation. 133(24):2434–2446.

Lindskog Jonsson A, Caesar R, Akrami R, Reinhardt C, Fäk Hällenius F, Borén J, Bäckhed F. 2018. Impact of gut microbiota and diet on the development of atherosclerosis in Apoe(-/-) mice. Arterioscler Thromb Vasc Biol. 38(10):2318–2326.
metabolite trimethylamine-N-oxide and 5-year mortality risk in stable coronary artery disease: the contributory role of intestinal microbiota in a COURAGE-like patient cohort. J Am Heart Assoc. 5(6):e002816.

Vendrame S, Guglielmetti S, Riso P, Arioli S, Klimis-Zacas D, Porrini M. 2011. Six-week consumption of a wild blueberry powder drink increases *Bifidobacteria* in the human gut. J Agric Food Chem. 59(24):12815–12820.

Vilkickyte G, Raudonis R, Motiekaityte V, Vainoriene R, Burdulis D, Viskelis J, Raudone L. 2019. Composition of sugars in wild and cultivated lingonberries (*Vaccinium vitis-idaea* L.). Molecules. 24(23):4225.

Zhao G, Nyman M, Åke Jönsson J. 2006. Rapid determination of short-chain fatty acids in colonic contents and faeces of humans and rats by acidified water-extraction and direct-injection gas chromatography. Biomed Chromatogr. 20(8):674–682.

Zhao S, Liu W, Wang J, Shi J, Sun Y, Wang W, Ning G, Liu R, Hong J. 2017. *Akkermansia muciniphila* improves metabolic profiles by reducing inflammation in chow diet-fed mice. J Mol Endocrinol. 58(1):1–14.