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Glycerol esters of butyric and valeric acids counteract diet-related disorders

Prevention of metabolic disturbances induced by high-fat intake

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Glycerol esters of butyric and valeric acids counteract diet-related disorders

Prevention of metabolic disturbances induced by high-fat intake

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DEPARTMENT OF FOOD TECHNOLOGY, ENGINEERING AND NUTRITION | LUND UNIVERSITY



Glycerol esters of butyric and valeric acids counteract diet-related disorders

Prevention of metabolic disturbances induced by
high-fat intake

Thao Duy Nguyen



LUND
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DOCTORAL DISSERTATION

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Abstract		
<p>Short-chain fatty acids (SCFAs), gut metabolites formed from indigestible dietary components by the colonic microbiota, are associated with numerous health effects, and may be responsible for the beneficial effects, at least in part, associated with dietary fibre. High-fat diets are known to cause low-grade inflammation in the body, but SCFAs are suggested to counteract unfavourable effects induced by these types of diets. This project was conducted to investigate possible preventive effects of four glycerol esters of SCFAs – monobutyryl (MB), tributyrin (TB), monovalerin (MV) and trivalerin (TV) – in conventional and ApoE knockout (ApoE^{-/-}) rats fed high-fat diets.</p> <p>Glycerol esters of SCFAs were added to high-fat diet (HF) at different doses from 1 to 15 g/kg and given to rats for 3 to 5 weeks. A group fed a low-fat (LF) diet was included as a reference. Lipid and SCFA profiles, caecal microbiota, intestinal permeability, expression of genes involved in bile acid synthesis, tight junction proteins, SCFA receptors and some inflammatory markers were analysed to evaluate possible nutritional effects of the glycerol esters.</p> <p>In conventional rats, glycerol esters produced no apparent change in caecal SCFA concentrations. However, all the glycerol esters (supplemented to high-fat diets based on butter), at a dose of 5 g/kg, reduced total liver cholesterol, LDL-cholesterol, the ratio of LDL-to-HDL-cholesterol, and succinic acid. Butyrins had stronger effects on liver lipids than valerins, especially MB, which decreased liver total cholesterol by 51% and significantly downregulated <i>Cyp8b1</i> expression involved in bile acid synthesis. When supplemented to high-fat diets based on lard, this dose of MB also decreased total and LDL-cholesterol in blood, while liver HDL-cholesterol increased. The valerins, MV and TV, increased levels of serum valeric acid and brain acetic acid. For all the glycerol esters, caecal microbiota composition was shifted to lower relative abundances of bacteria associated with obesity and inflammation, although the monoforms showed a more distinct difference from the HF non-supplemented group. Increased doses of MB, 7.5 and 15 g/kg, resulted in consistent reduction in liver total cholesterol and triglycerides. Intestinal permeability was lower with the highest dose of MB compared with the non-supplemented HF group.</p> <p>In ApoE^{-/-} rats, supplementation of MB or MV at a dose of 10 g/kg upregulated the expression of occludin and ZO-1 in the brain and increased the mucosal thickness in the small intestine. MB increased the content of butyric acid in the brain, lowered plasma IL-10 concentration, and tended to improve intestinal barrier function. With MV, serum concentrations of HDL-cholesterol and valeric acid increased, while the amount of isovaleric acid was reduced in the brain.</p> <p>The results support a preventive role of butyrins and valerins from lipid disorders, impaired intestinal barrier and inflammation caused by high-fat diets and genetic predisposition. Their use as dietary supplements in human nutrition may therefore be promising for preventing or counteracting metabolic disorders and related diseases.</p>		
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MADE IN SWEDEN 

*To those who always support me.
And to those who are curious about how diet
can benefit health.*

Table of Contents

Abstract	9
Popular science summary	11
Tóm tắt nội dung luận văn.....	13
List of papers.....	15
Author's contributions	16
Abbreviations	17
Introduction	19
Background	21
High-fat diet and its devastating effects	21
Cholesterol-centred lipid disorders.....	21
Inflammation	23
Altered gut microbiota and gut barrier function.....	23
Apolipoprotein E – cholesterol transporter	25
Nutritional effects of short-chain fatty acids.....	27
Dietary fibre-originated metabolites.....	27
Formation	28
Modes of action	28
Strengthened barrier function	30
Glycerol esters of short-chain fatty acids	31
Why glycerol esters?	31
Monobutylin and tributyrin	33
Monovalerin and trivalerin	35
The rat model	35
Aim.....	37

Materials and Methods	39
Test products	39
Monobutyryn and tributyrin	39
Monovalerin and trivalerin	39
Diets	40
Study design	40
Statistical evaluation and calculations	41
Results and Discussion	45
Lipid profiles	45
Liver	46
Portal vein.....	51
Short-chain fatty acid profiles	53
Caecum.....	53
Portal vein.....	55
Brain	56
Intestinal permeability	59
Caecal microbiota composition	61
Succinic acid	63
Tight junction proteins and SCFA receptors.....	64
Cytokines.....	66
Where are the glycerol esters absorbed?	68
Conclusions	69
Future perspectives	71
Acknowledgements	73
References	77

Abstract

Short-chain fatty acids (SCFAs), gut metabolites formed from indigestible dietary components by the colonic microbiota, are associated with numerous health effects, and may be responsible for the beneficial effects, at least in part, associated with dietary fibre. High-fat diets are known to cause low-grade inflammation in the body, but SCFAs are suggested to counteract unfavourable effects induced by these types of diets. This project was conducted to investigate possible preventive effects of four glycerol esters of SCFAs – monobutyryl (MB), tributyrin (TB), monovalerin (MV) and trivalerin (TV) – in conventional and ApoE knockout (ApoE^{-/-}) rats fed high-fat diets.

Glycerol esters of SCFAs were added to high-fat diet (HF) at different doses from 1 to 15 g/kg and given to rats for 3 to 5 weeks. A group fed a low-fat (LF) diet was included as a reference. Lipid and SCFA profiles, caecal microbiota, intestinal permeability, expression of genes involved in bile acid synthesis, tight junction proteins, SCFA receptors and some inflammatory markers were analysed to evaluate possible nutritional effects of the glycerol esters.

In conventional rats, glycerol esters produced no apparent change in caecal SCFA concentrations. However, all the glycerol esters (supplemented to high-fat diets based on butter), at a dose of 5 g/kg, reduced total liver cholesterol, LDL-cholesterol, the ratio of LDL-to-HDL-cholesterol, and succinic acid. Butyrins had stronger effects on liver lipids than valerins, especially MB, which decreased liver total cholesterol by 51% and significantly downregulated *Cyp8b1* expression involved in bile acid synthesis. When supplemented to high-fat diets based on lard, this dose of MB also decreased total and LDL-cholesterol in blood, while liver HDL-cholesterol increased. The valerins, MV and TV, increased levels of serum valeric acid and brain acetic acid. For all the glycerol esters, caecal microbiota composition was shifted to lower relative abundances of bacteria associated with obesity and inflammation, although the mono-forms showed a more distinct difference from the HF non-supplemented group. Increased doses of MB, 7.5 and 15 g/kg, resulted in consistent reduction in liver total cholesterol and triglycerides. Intestinal permeability was lower with the highest dose of MB compared with the non-supplemented HF group.

In ApoE^{-/-} rats, supplementation of MB or MV at a dose of 10 g/kg upregulated the expression of occludin and ZO-1 in the brain and increased the mucosal thickness in the small intestine. MB increased the content of butyric acid in the brain, lowered plasma IL-10 concentration, and tended to improve intestinal barrier function. With MV, serum concentrations of HDL-cholesterol and valeric acid increased, while the amount of isovaleric acid was reduced in the brain. The results support a preventive role of butyrins and valerins from lipid disorders, impaired intestinal barrier and inflammation caused by high-fat diets and genetic

predisposition. Their use as dietary supplements in human nutrition may therefore be promising for preventing or counteracting metabolic disorders and related diseases.

Popular science summary

Diet is a central determinant of human health. Over the past 100 years, diet intake has dramatically shifted to consumption of foods that are fat-rich, energy-dense and low in dietary fibre. This shift coincides with the increased prevalence of obesity and its accompanying metabolic diseases. Nutritional research has revealed anti-obesity effects of dietary fibre, where the formation of metabolically active gut metabolites, short-chain fatty acid (SCFAs), is increasingly discussed as one factor of importance.

Dietary fibre is not digested by the digestive enzymes in the gastrointestinal tract and passes into the colon intact. In the colon, the microbiota can degrade dietary fibre to bioactive products, predominantly acetic, propionic and butyric acids, and smaller amounts of valeric acid. The SCFAs are absorbed through the portal vein into the blood where they may have different metabolic implications. Butyric acid is most emphasised in this respect. It has been discussed whether SCFAs could be added to the diet in some way, but butyric acid in particular has an unacceptable odour. This can be avoided by attaching the SCFAs to a glycerol backbone, forming glycerol esters of SCFAs. Another advantage is that the glycerol esters of SCFAs are probably hydrolysed and absorbed further down in the gastrointestinal tract than the single molecule. Where the glycerol esters are absorbed and their potential nutritional effects have not been examined to any greater extent.

To examine potential beneficial effects of glycerol esters of butyric acid and valeric acid – monobutyryl (MB), tributyrin (TB), monovalerin (MV) and trivalerin (MV) – they were therefore added (dose 5 g/kg) to high-fat diets and given to rats. High-fat diets were chosen since they are known to increase the inflammatory tone in the body. The glycerol esters did not increase the SCFAs in the colon, but after a 3-week dietary intervention, levels of cholesterol, LDL-cholesterol (commonly referred to as ‘bad’ cholesterol) and an inflammatory marker (succinic acid) were lower in the livers of rats fed the diets supplemented with the glycerol esters. The reduction in liver cholesterol was associated with decreased expression of genes involved in the synthesis of bile acids. Increasing the amounts of glycerol esters, especially MB, added to high-fat diets led to a concomitant decrease of cholesterol and triglycerides in the liver. Similar reductions of cholesterol circulating in the blood, and an increase in HDL-cholesterol (‘good’ cholesterol) in the liver were also seen when the MB diet was consumed for a longer time (4 weeks). The highest amount of MB (15 g/kg) strengthened intestinal barrier function. The presence of bacteria associated with the onset of obesity and inflammation was lower in the caecum of rats consuming diets containing the glycerol esters. Some changes in SCFA profiles at different locations or tissues in the body were seen, with higher valeric acid in the portal vein, and higher acetic acid but lower isovaleric acid (a minor SCFA) in the brain.

Potential effects of MB and MV at doses of 10 g/kg were explored in rats deficient in a specific gene that transports cholesterol (apolipoprotein E). Absence of this protein results in extremely high levels of blood lipids, mimicking dyslipidaemia seen in humans with heart diseases or lipid disorders. Supplementation of MV to the diet increased blood levels of HDL-cholesterol and valeric acid. MB tended to lower intestinal permeability and decrease blood levels of an anti-inflammatory marker (interleukin-10). Both MB and MV strengthened the gut-brain barrier, especially in the brain, by stimulating expression of tight junction proteins, structural components of the barrier. These effects were linked to changes of SCFAs, with MV in the diet increasing valeric acid in blood, while MB increased the content of butyric acid and suppressed that of isovaleric acid in the brain.

In conclusion, supplementing diet with glycerol esters of SCFAs is effective in lowering lipid disorders caused by high-fat intake or genetic deficiency. Improvements in lipid profile are accompanied by strengthened gut-brain barrier, a healthier microbiota composition and reduced inflammation, in association with changes in SCFA profiles. These outcomes suggest a promising role for glycerol esters in nutritional interventions preventing or counteracting metabolic disturbances and associated diseases.

Tóm tắt nội dung luận văn

Chế độ ăn uống là yếu tố quyết định sức khỏe con người. Trong vòng 100 năm qua, việc ăn uống đã thay đổi đáng kể theo hướng tiêu thụ thực phẩm nhiều chất béo và năng lượng, nhưng lại thiếu chất xơ. Sự thay đổi này trùng hợp với sự gia tăng tỉ lệ béo phì và các bệnh liên quan đến trao đổi chất. Các nghiên cứu dinh dưỡng cho rằng chất xơ thực phẩm có khả năng chống béo phì, trong đó các axit béo mạch ngắn (ABMN), hình thành từ sự lên men của chất xơ trong đường ruột, được cho là nhân tố quan trọng cho các tác dụng có lợi của chất xơ.

Chất xơ thực phẩm không thể bị phân hủy bởi các enzyme tiêu hóa trong dạ dày và ruột non nên chúng di chuyển xuống ruột già ở tình trạng nguyên vẹn. Tại đây, các vi khuẩn đường ruột phân hủy chất xơ thành các ABMN, bao gồm ba loại axit chính: acetic axit, propionic axit, và butyric axit; valeric axit cũng được tạo ra, nhưng ở lượng ít hơn. Qua tĩnh mạch, các ABMN được hấp thụ vào trong máu và có thể ảnh hưởng đến các quá trình trao đổi chất khác nhau. Butyric axit được quan tâm nhiều nhất ở khía cạnh này. Nhiều ý kiến đặt ra liệu có cách nào để thêm các ABMN vào chế độ ăn mặc dù chúng có mùi hăng rất khó chịu, đặc biệt là butyric axit. Nhược điểm này có thể được loại bỏ bằng cách kết nối các axit béo với glycerol, tạo ra glycerol este của ABMN. Lợi điểm chính của các ABMN glycerol este là chúng có thể được hấp thụ ở những phân thấp hơn của hệ đường ruột. Nhưng chính xác ở đâu các glycerol este được hấp thụ và liệu chúng có ảnh hưởng gì về mặt dinh dưỡng vẫn chưa được nghiên cứu ở diện rộng.

Để tìm hiểu về các tác dụng dinh dưỡng của ABMN glycerol este, bốn loại este của butyric và valeric axit - monobutyryn, tributyrin, monovalerin, và trivalerin - được thêm vào các khẩu phần ăn nhiều chất béo, với liều lượng 5 g/kg, và được thử nghiệm trên chuột. Khẩu phần ăn nhiều chất béo được chọn vì chúng thúc đẩy sự viêm nhiễm trong cơ thể. Sau 3 tuần trên chế độ ăn thử nghiệm, tổng số cholesterol, cholesterol 'xấu' và một chất chỉ thị sự viêm nhiễm (succinic axit) giảm đáng kể trong gan của các nhóm chuột với chế độ ăn có bổ sung ABMN glycerol este. Cholesterol trong gan giảm cũng liên quan đến sự giảm biểu hiện của các gen tổng hợp axit mật. Gia tăng lượng ABMN glycerol este, đặc biệt là monobutyryn, lên 7.5 và 15 g/kg cũng làm giảm cholesterol và axit béo trung tính trong gan. Hàm lượng cholesterol trong máu cũng giảm đáng kể, trong khi lượng cholesterol 'tốt' lại gia tăng khi chế độ ăn có bổ sung monobutyryn được kéo dài tới bốn tuần. Hơn thế nữa, lượng thử nghiệm cao nhất của monobutyryn (15 g/kg) giúp tăng cường chức năng phòng vệ của hệ đường ruột. Sự hiện diện của các vi khuẩn liên quan đến sự phát triển của chứng béo phì và viêm nhiễm giảm rõ rệt trong đường ruột của các nhóm chuột có chế độ ăn được bổ sung ABMN glycerol este. Vài thay đổi trong thành phần của các ABMN được ghi nhận tại các vị trí hay mô khác nhau của cơ thể, với valeric axit trong máu tăng, hay acetic axit tăng nhưng isovaleric axit (một ABMN phụ) lại giảm trong não.

Hiệu quả tiềm năng của hai loại glycerol este monobutylin và monovalerin, ở mức 10 g/kg, đã được khảo sát trong chuột thiếu một loại protein vận chuyển cholesterol trong cơ thể (apolipoprotein E). Sự thiếu hụt protein này dẫn đến mỡ máu cao đột biến, tương tự như sự rối loạn mỡ máu trong bệnh nhân mắc bệnh tim mạch hay rối loạn trao đổi chất béo. Bổ sung monovalerin vào chế độ ăn nhiều chất béo giúp gia tăng cholesterol ‘tốt’ và valeric axit trong máu. Về phần mình, monobutylin có xu hướng làm giảm tính thấm của màng ruột và một chất chống viêm (IL-10). Điều đáng chú ý là cả monobutylin và monovalerin giúp tăng cường hệ thống màng bảo vệ trong hệ đường ruột-não, đặc biệt trong não, bằng việc kích thích biểu hiện của các gen liên kết hệ thống màng bảo vệ. Các ảnh hưởng này liên quan đến sự thay đổi thành phần của các ABMN, với valeric axit tăng trong máu khi monovalerin hiện diện trong chế độ ăn, trong khi monobutylin gia tăng số lượng butyric axit nhưng làm giảm isovaleric axit trong não.

Nói tóm lại, bổ sung ABMN glycerol este vào chế độ ăn làm giảm rối loạn chuyển hóa chất béo gây ra bởi chế độ ăn nhiều chất béo hay do khiếm khuyết gen. Những thay đổi có lợi trong thành phần chất béo đồng hành cùng với hệ thống màng bảo vệ đường ruột và não bộ cũng như một hệ vi khuẩn đường ruột khỏe mạnh, và những thay đổi trong thành phần của các ABMN. Các kết quả nghiên cứu này chỉ ra tiềm năng đầy hứa hẹn của các ABMN glycerol este trong các can thiệp dinh dưỡng nhằm ngăn chặn hay chống lại bệnh béo phì, các rối loạn chuyển hóa và các bệnh liên quan.

List of papers

Paper I

Effects of monobutyryl and tributyrin on liver lipid profile, caecal microbiota composition and short-chain fatty acids in high-fat diet fed rats.

Thao Duy Nguyen, Olena Prykhodko, Frida Fåk Hållenius and Margareta Nyman
Journal of Nutritional Science (2017), <https://doi.org/10.1017/jns.2017.54>

Paper II

Monovalerin and trivalerin increase brain acetic acid, decrease liver succinic acid, and alter gut microbiota in rats fed high-fat diets.

Thao Duy Nguyen, Olena Prykhodko, Frida Fåk Hållenius and Margareta Nyman
European Journal of Nutrition (2018), <https://doi.org/10.1007/s00394-018-1688-z>

Paper III

Monobutyryl reduces liver cholesterol and improves intestinal barrier function in rats fed high-fat diets.

Thao Duy Nguyen, Olena Prykhodko, Frida Fåk Hållenius and Margareta Nyman
Nutrients (2019), <https://doi.org/10.3390/nu11020308>

Paper IV (*submitted manuscript*)

Monobutyryl and monovalerin reinforce gut-brain barrier and improve brain short-chain fatty acid profile in ApoE-knockout rats.

Thao Duy Nguyen, Frida Fåk Hållenius, Xue Lin, Margareta Nyman and Olena Prykhodko (2019)

Paper not included in the thesis

Lingonberries reduce atherosclerosis in ApoE^{-/-} mice in association with altered gut microbiota composition and improved lipid profile.

Chrysoula Matziouridou, Nittaya Marungruang, Thao Duy Nguyen, Margareta Nyman and Frida Fåk

Molecular Nutrition and Food Research (2016), DOI: 10.1002/mnfr.201500738

Author's contributions

Paper I

The author was involved in the design of the study, performed the animal experiments, conducted all the analyses except the caecal microbiota sequencing, analysed the data and interpreted the results, and was responsible for writing the manuscript.

Paper II

The author was involved in the design of the study, performed the animal experiments, conducted all the analyses except the caecal microbiota sequencing, analysed the data and interpreted the results, and was responsible for writing the manuscript.

Paper III

The author was involved in the design of the study, performed the animal experiments, conducted the analyses, analysed the data and interpreted the results, and was responsible for writing the manuscript.

Paper IV

The author was involved in the design of the study, performed the animal experiments, conducted part of the analyses, performed the evaluation of the data and interpretation of results, and was responsible for writing the manuscript.

Abbreviations

AD:	Alzheimer's disease
AMPK:	5' adenosine monophosphate-activated kinase
ApoE:	apolipoprotein E
APP:	amyloid precursor protein
Arg:	arginine
A β :	amyloid-beta
BAs:	bile acids
BBB:	blood-brain barrier
BSH:	bile salt hydroxylases
Cys:	cysteine
EGFR:	epidermal growth factor receptor
GABA:	gamma-aminobutyric acid
GPRs:	G protein-coupled receptors
HDACs:	histone deacetylases
HDL-c:	high-density lipoprotein cholesterol
HMG-CoA	reductase: 3-hydroxy-3-methylglutaryl-coenzyme A reductase
IL:	interleukin
IVD:	isovaleric acidaemia
kcal:	kilocalorie
LC-MS: 1	liquid chromatography-mass spectrometry
LDL-c:	low-density lipoprotein cholesterol
LPS:	lipopolysaccharide
MB:	monobutyrim
MCT:	monocarboxylate transporter
MMPs:	matrix-metalloproteinases
mRNA:	messenger ribonucleic acid
MV:	monovalerin
NAFLD:	non-alcoholic fatty liver disease
NF- κ B:	nuclear factor-kappaB
PET:	positron emission tomography
PPARs:	peroxisome proliferator-activated receptors
SCFAs:	short-chain fatty acids
SMCT:	sodium-coupled monocarboxylate transporter
sn:	stereospecific numbering
TB:	tributyrim
TG:	triglycerides
TLR:	toll-like receptors
TNF- α :	tumour necrosis factor-alpha
TV:	trivalerin
ZO-1:	zonula occludens-1

Introduction

Food is not only an energy source but also a metabolic indicator of health. Eating the right food benefits health, but an unhealthy diet exposes the body to a wide range of metabolic disorders. In the long term, such disorders may lead to a series of associated diseases affecting many organs, such as obesity, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD), inflammatory bowel disease, and even Alzheimer's disease (AD). More people suffer and die from these diseases than from both crime and war, so there is a need to understand and prevent or combat the onset of these diseases. Thanks to the findings from nutritional research, it is known that high-fat diets are one of the initial environmental factors causing metabolic disorders. In contrast, fibre-rich foods are associated with reducing these unfavourable health effects.

Dietary fibres are indigestible by human digestive enzymes, but they are degraded by the colon microbiota to form bioactive products. Among these, short-chain fatty acids (SCFAs) have attracted great attention and are suggested to be responsible, in part, for the observed health benefits of dietary fibre. Main members of this group are acetic, propionic and butyric acids, while valeric, isobutyric, and isovaleric acids are formed to a lesser extent. SCFAs, especially butyric acid, are well-known as anti-inflammatory agents and have beneficial effects against metabolic disorders. However, strong odour hinders their incorporation into food or oral administration. This can be circumvented by combining them with glycerol, generating glycerol esters of SCFAs. Some glycerol esters of SCFAs are used as feed additives, but their nutritional and metabolic effects, especially in dietary interventions targeting metabolic problems, remain unexplored.

To fill this gap in current understanding, this project was conducted to examine potential preventive effects of glycerol esters of butyric and valeric acids when mixed with high-fat diets and given to rats. High-fat diets were used to provoke inflammatory status, and rat models were chosen because they mimic fermentability in humans and are commonly used in cardiovascular and neurological research. Changes in SCFA profiles were tracked by measuring their concentrations in different locations within the body. The caecal microbiota was determined, since these are responsible for the main formation of SCFAs. Lipid levels, inflammation and intestinal permeability were investigated. Whether gut-brain barriers were affected by the glycerol esters was examined by gene expression of tight junction proteins and SCFA receptors.

Background

High-fat diet and its devastating effects

Obesity is now recognised as a global epidemic health problem, affecting >1.9 billion adults according to the World Health Organization [1,2]. Although genetic factors may be responsible for a small proportion, approximately 2 - 3% of the obese population of European ancestry, the prevalence of obesity is mostly caused by excess energy intake, due to a combination of environmental factors such as insufficient physical activity and an unhealthy or unbalanced diet [3].

Consumption of a high-fat diet (41 - 60% total energy in kcal from fat) may lead to obesity and metabolic disorders in several organs, such as fat accumulation in the liver. This condition is now recognised as NAFLD, the most common form of chronic liver disease worldwide [4]. Patients with NAFLD have an increased risk of type 2 diabetes and hepatocellular carcinoma [5,6]. Like obesity, no effective treatments are currently available for NAFLD, so reducing the effects of environmental factors such as an unbalanced diet and a sedentary lifestyle remains a first-line recommendation.

At present, diet-induced obesity is not only a metabolic pandemic but also an inflammatory disease, associated with conditions such as diabetes, cardiovascular diseases and inflammatory bowel disease [7-9]. Recent data demonstrate that obese subjects are at an increased risk of developing neurodegenerative diseases in the long term [10]. For example, prospective studies suggest that mid-life obesity increases late-life risk of dementia, including AD [11], and several studies have revealed possible links between obesity and cognitive impairment, with high-fat diet-induced inflammation being one of the commonly identified contributors [3].

Cholesterol-centred lipid disorders

An inflammatory high-fat diet leads to progressive metabolic changes in many organs. For example, accumulation of triglycerides in the liver, also known as steatosis, can induce alterations in lipid metabolism, resulting in obesity-related disturbances such as elevated low-density lipoprotein-cholesterol (LDL-c), hypertriglyceridaemia, and decreased levels of high-density lipoprotein-cholesterol (HDL-c). These change the phenotype of resident macrophages in the liver, called

Kupffer cells, to a proinflammatory profile, further exacerbating an inflammatory environment [3,12]. The triglyceride deposit-stimulated inflammation also occurs in adipose tissue.

Since high cholesterol levels in blood are strongly associated with the development of cardiovascular disease, maintaining cholesterol homeostasis is of vital importance. The gut-liver axis plays a key role in cholesterol homeostasis. Cholesterol accumulated in tissues can be reduced by reverse cholesterol transport, a process in which HDL transports cholesterol derived from peripheral tissues back to the liver for excretion. However, inflammation can compromise this process, reducing cholesterol efflux from macrophages to serum and lipid-carrying lipoproteins such as HDL and apoA-I [13].

An alternative pathway to reducing cholesterol levels is the use of cholesterol for generation of bile acids (BAs). This synthesis takes place in the liver using the enzymes *Cyp7a1*, mainly responsible for the total BA pool, and *Cyp8b1*, determining the composition of BAs [14]. BAs are then secreted into the small intestine, where they act as detergents facilitating digestion and absorption of fats. Gut microbiota can influence intestinal cholesterol absorption as well as BA composition.

Elevated cholesterol in blood is detrimental in peripheral systems, especially for the heart and vascular system, but it is currently not clear whether this also applies in the central nervous system. However, it cannot be ignored that cholesterol is a vital molecule acting as a component of cell membranes and as a precursor of steroid hormones, BAs and vitamin D. In the brain, cholesterol is an essential architectural component of myelin surrounding many nerve cells and is present in membranes of brain cells such as neurons and glial cells and bound to lipoproteins. Brain cells can synthesize cholesterol, and cholesterol transport is maintained via Apolipoprotein E (ApoE)-binding receptors [15,16].

The importance of cholesterol for neuronal development and maintenance is indicated by failure of neurotransmission and declined synaptic plasticity due to lack of cholesterol delivery to neurons [17]. The role of cholesterol in AD development has been demonstrated. Increased levels of cholesterol in neurons, following long-term consumption of cholesterol-rich diets, promote beta-site cleavage of amyloid precursor protein (APP), and may therefore increase amyloid-beta ($A\beta$) [18]. In contrast, lack of membrane cholesterol inhibits activity of gamma-secretase, the enzyme facilitating formation of $A\beta$ from APP [19]. APP and another enzyme, beta-secretase, are co-present in the cholesterol-rich microdomains within cellular membranes, suggesting a potential connection between high cholesterol and AD [20]. Although circulating and brain cholesterol are separated by the blood brain barrier (BBB), there is evidence that plasma cholesterol is associated with the incidence of AD [21]. A meta-analysis of prospective studies in humans showed a strong correlation between high midlife serum total cholesterol with the risk of any type of dementia, including AD, and cognitive impairment [22]. The allele $\epsilon 4$ of

ApoE, or ApoE4 isoform, has been established as the strongest risk factor for late onset of sporadic AD [21]. For instance, dyslipidaemia is more pronounced in stroke patients carrying ApoE4, indicated by higher serum total cholesterol and triglycerides values [23].

Inflammation

Obesity and its accompanying comorbidities, including type 2 diabetes and cardiovascular diseases, is accepted to be linked to chronic low-grade inflammation [24]. The high-fat diet itself may cause a rapid inflammatory response. For example, human studies with healthy subjects have indicated that consumption of a high-fat meal (41% kilocalories from fat) rapidly increased levels of markers related to inflammation such as plasma endotoxins (lipopolysaccharide/LPS), mRNA expression of tumour necrosis factor-alpha (TNF- α), interleukin-1beta (IL-1 β) and toll-like receptor-4 (TLR-4) within five hours after consumption of the meal [25]. One component of the high-fat diet, saturated fatty acids, can bind to TLRs expressed on immune cells, activating transcriptional factors such as nuclear factor-kappaB (NF- κ B) and consequently induce elevated production of proinflammatory cytokines (IL-1 β and TNF- α) [26]. This series of inflammatory responses can be ignited in several tissues, including liver, adipose tissue, pancreas and muscle [12].

Besides systemic inflammation, high-fat diet also stimulates inflammation in the central nervous system. Long before any signs of obesity, a very short-term (1-3 days) exposure to a high-fat diet increased neuroinflammation, gliosis (changes of glial cells in response to damage of the brain) and neuronal injury-related markers in the hypothalamus (a brain structure essential for the regulation of food intake and energy expenditure) of both rats and mice [27,28]. Increased hypothalamic gliosis was also found in obese patients [27].

It has recently been shown that obesity-derived neuroinflammation also affects other structures of the brain, including the hippocampus and the cortex, which are involved in mood regulation, memory formation and cognitive function [3]. Animal studies also indicated that inflammation-induced memory deficits are specifically hippocampus-dependent [29]. In a study of high-fat-mediated translocation of bacterial components, decreased memory performance, depressed mood and anxiety were concurrently displayed with increased levels of circulating cytokines after injection of a low dose of Salmonella endotoxin in healthy subjects [30].

Altered gut microbiota and gut barrier function

A high-fat diet can stimulate inflammation, not only by activating immune cells but also by disturbing the gut microbial composition, commonly known as gut dysbiosis. The gut microbiota is highly dependent on the diet, and its composition

is rapidly changed in just one day when switching from a low-fat diet rich in plant polysaccharides to a high-fat, high-sugar Western diet [31]. Consumption of a diet low in fibre and rich in fat not only reduces microbial diversity but also feeds the colon microbiota with undesirable substrates, such as dietary fat, through the BAs seen in humans and mice [32-34]. This results in toxic metabolites in the colon, such as secondary BAs known to have negative effects on colonic epithelium and function [35]. For example, intestinal hyperpermeability was positively associated with most secondary BAs in the caecum of high-fat fed mice [33]. A high-fat diet also promotes translocation of LPS into the circulation and subsequent provocation of systemic inflammation [8].

LPS, a component of the outer membrane of Gram-negative bacteria, can be liberated even from destroyed bacteria, and has been connected to inflammation and identified as a molecule occurring early in the development of metabolic diseases. LPS also has the capacity to trigger the secretion of pro-inflammatory cytokines. LPS subcutaneous infusion to mice has been shown to increase their body weight to the same extent as a high-fat diet [36]. Typically, a high-fat diet-induced gut phenotype is described as a decreased ratio of the two dominant phyla, Bacteroidetes/Firmicutes, both in obese humans and mice [37,38].

Besides the gut microbiota, high fat consumption may affect intestinal barrier function by reducing expression of tight junction proteins (zonula occludens-1/ZO-1 and occludin). Intestinal barrier function is essential in maintaining a stable environment in the gut by preventing invasion of potential harmful components. Dysfunction of this barrier can be recorded by analysing mRNA or protein expression of tight junction proteins in the gut tissues, or by quantifying the levels of bacterial components or endotoxins such as LPS in blood or indigestible sugars such as mannitol and lactulose in urine. This dual sugar test is commonly used in clinical studies, and increased lactulose/mannitol ratio has been found in type 1 diabetic and obese patients [39,40]. Being a non-invasive method, the lactulose/mannitol test has been suggested as an indicator of intestinal permeability in rats [41,42].

Receiving 70 - 75% blood supply from the portal vein, the liver may be potentially exposed to a surplus flux of microbial products if the intestinal barrier is disrupted. Increased intestinal permeability has been indicated in 39% of patients with NAFLD [43]. High-fat diets increase levels of succinic acid [44,45], a microbial and intermediate metabolite that is elevated in faecal, serum and liver samples of NAFLD patients [46]. It should be mentioned that the portal vein also connects the gastrointestinal tract with the spleen, an important organ hosting neuro-immune communications [47]. Systemic inflammation, infection, injuries, autoimmune and neurodegenerative diseases can lead to increased BBB permeability [48]. For example, BBB interruption or dysregulation has been found in patients with common neurodegenerative disorders like AD, Parkinson's disease and multiple sclerosis, and is suggested to occur in the early stages, promoting disease

progression. These links highlight how well an unbalanced diet accelerates whole-body metabolic reactions and related diseases. An overview of how high-fat diets contribute to metabolic disorders while fibre-rich foods suppress these adverse outcomes is presented in Figure 1.

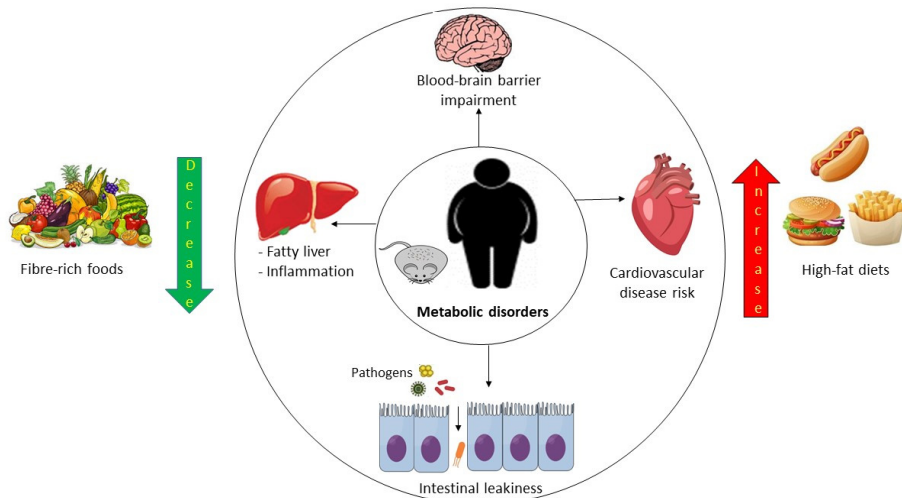


Figure 1. Overview of protective effects of fibre-rich diets against metabolic disorders caused by high-fat diets.

Apolipoprotein E – cholesterol transporter

ApoE, originally identified as arginine-rich protein, is an essential lipid transporter comprising 299 amino acids and encoded by the ApoE gene located on human chromosome 19. It is integrated with low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and HDL lipoproteins at the lipid-binding carboxy-terminal domain and facilitating the uptake of triglycerides, phospholipid, cholesteryl esters, and cholesterol into cells via receptors. The binding of ApoE to receptors occurs at amino-terminal domain, with high affinity for the low-density lipoprotein receptor (LDLR) family. ApoE is produced by many cells in the peripheral systems, such as hepatocytes, adipocytes and macrophages, and in the brain mainly by astrocytes, while neurons can produce ApoE only under stressed conditions.

The transport of ApoE in and out of the brain is restricted by the BBB. As a lipid transporter, ApoE delivers cholesterol between neuronal and non-neuronal cells in the brain and imbalance of cholesterol homeostasis may play a role in

neurodegenerative diseases. ApoE deficiency and elevated plasma cholesterol contribute to greater leakage of the BBB in mice [7], so ApoE has become a potential target for combating lipid-related disorders. Studies have indicated the prevalence of the ApoE allele epsilon-2 ($\epsilon 2$) in lipid disorders such as type III hyperlipoproteinaemia, coronary heart disease, and type 2 diabetes mellitus, whereas ApoE $\epsilon 4$ is considered as a genetic risk factor for neurodegenerative diseases such as AD, vascular dementia, and Parkinson's disease.

The degree of variation in the ApoE gene provides an interesting evolutionary story of how diet shapes the human genome and associated health outcomes. Genetic studies show that there are three common alleles of ApoE due to variation in its sequence, namely $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$ with a population prevalence of 8%, 77% and 15%, respectively. The resulting products of this variation are the three corresponding isoforms: ApoE2, ApoE3 and ApoE4. ApoE $\epsilon 4$, containing arginine (Arg) at both positions 112 and 158 in the sequence, is considered to be an ancestral gene due to its largely close relation to the chimpanzee ApoE gene [49]. Over the past 200,000 years, the most prevalent ApoE $\epsilon 3$ diverged from ApoE $\epsilon 4$ by substituting cysteine (Cys) for Arg at position 112.

This genetic transition is possibly related to diet shift from a mainly low-cholesterol (hunter-gatherer societies) to high-cholesterol and lipid-rich diet (modern societies with advanced technology), selectively favouring occurrence of meat-adaptive genes as ApoE $\epsilon 3$. Humans bearing the ApoE $\epsilon 3$ are less vulnerable to cardiovascular diseases and AD [50]. In contrast, ApoE $\epsilon 4$ increases the risk of hypercholesterolaemia and AD.

The least frequent allele is ApoE $\epsilon 2$, different from ApoE $\epsilon 4$ with Cys at both 112 and 158 positions. It has been suggested that people who are homozygous of the ApoE $\epsilon 2$ are sensitive to the development of familial type III hyperlipoproteinemia and atherosclerosis. However, the impact of diet on diseased conditions greatly outweighs genetic effects. For example, consumption of a diet low in meat and meat products (a feature of the Mediterranean diet with high intake of fruits and low intake of meat) leads to greater brain volume and better cognitive performance in cognitively healthy elderly individuals [51]. Most obese patients continuously suffer severe complicated metabolic disturbances as an outcome of unbalanced diets, such as high-fat diets, during their lifetime, and genetic factors only account for a minor proportion (2 - 3%) of the obesity burden [3].

Nutritional effects of short-chain fatty acids

Dietary fibre-originated metabolites

Data collected over recent decades have repeatedly proved the effectiveness of dietary fibres in counteracting diet-associated diseases. By modulating the amounts of fat in relation to dietary fibres, this nutritional approach represents a simple, modifiable and more preventive way to maintain good health. Dietary fibre is a common term, grouping all indigestible dietary carbohydrates found in fruits and vegetables, such as bananas, pears, apples, plums, guavas and oranges, and cereals such as oat, barley, rye and wheat [52]. More specifically, dietary fibre is defined as indigestible carbohydrate polymers with ten or more monomeric units, which depending on the methodology used may also include non-carbohydrates such as lignin and other components closely associated with the dietary fibre, such as antioxidants. Inclusion of carbohydrate monomers with a degree of polymerisation between 3 and 9 is left to national authorities. In such cases, oligosaccharides found in legumes and breastmilk should also be included. But in fact, also disaccharides, such as lactulose and lactitol, are indigestible and reach the colon, and sugar alcohols (sorbitol, mannitol) are only partly absorbed in the small intestine.

Dietary fibres are indigestible, which means that they cannot be hydrolysed by the endogenous enzymes produced in the gastrointestinal tract but can be fermented by the microbiota found in the colon in humans and rats. This fermentation process gives rise to short-chain fatty acids (SCFAs). Several studies have shown that low-fibre diets are often associated with increased inflammatory disturbances [53], while adding fibres to a diet, such as to a high-fat meal, can counteract negative effects by decreasing inflammatory mediators both in humans and rats [25,44,54]. Increased fibre consumption has also been reported to improve cardiovascular health, reducing atherosclerosis and immune disorders [55,56].

Because levels of SCFAs are generally increased with consumption of a fibre-rich meal, extensive research has been conducted to investigate the SCFAs as the key metabolites responsible for the health benefits of dietary fibres. It can be important to distinguish between effects of SCFAs administered orally and those originating from fermentation in the colon. When SCFAs are added to the diet, gastric emptying is slowed down, as shown by propionate and other organic acids (lactic and acetic acids and vinegar), and satiety hormones are affected. The activity of starch degrading enzymes may be influenced, increasing the amounts of indigestible components reaching colon for fermentation [57,58]. It cannot be excluded that lipases act in a similar way.

The SCFAs formed in colon do not act in the upper part of the gastrointestinal tract. With regard to lipid metabolism, some SCFAs have been shown to inhibit

cholesterol synthesis enzymes in the liver and influence BA excretion or lipid absorption in the small intestine [59-63].

Formation

SCFAs are volatile, small monocarboxylic acids with fewer than six carbon atoms. They are mainly produced by anaerobic degradation (fermentation) of dietary fibre in the colon, with the dominant components being acetic acid (two carbon atoms, C2) propionic acid (C3), and butyric acid (C4) and, to a lesser extent, valeric acid (C5). Minor SCFAs like isobutyric and isovaleric acids are generated from protein fermentation [64]. Concentrations of SCFA are highest in the proximal part of colon (20-140 mM), with a typical molar ratio of acetic, propionic, and butyric acids of 57:22:21 in the human colon and in the caecum of rats [65,66]. This ratio may vary depending on the types of dietary fibre consumed and gut microbiota composition and sampling locations.

Both the amount and pattern of SCFAs formed are dependent on the type of dietary fibre consumed. As shown in rats, pectin gives rise to high amounts of acetic acid, and guar gum increases propionic acid, while a mixture of pectin and guar gum and fructo-oligosaccharides with a low degree of polymerisation help produce higher levels of butyric acid [67,68]. Mixtures of dietary fibre seem to promote butyric acid formation and a more favourable microbiota composition. Acetic acid can be generated by most gut bacteria, while propionic acid is limited mainly to the phyla Bacteroidetes, some Firmicutes (*Clostridium* clusters IX and XI) and Actinobacteria (*Propionibacterium*), and butyrate-producing bacteria belong mainly to the phylum Firmicutes (*Clostridium* clusters IV and XIVa) [69].

Cross-feeding between specific bacteria can also affect the overall SCFA formation. This phenomenon is illustrated in labelling studies showing that substrates such as fructo-oligosaccharides, resistant starches or lactate cannot be converted into butyrate by species in the genus *Roseburia* without the presence of *Bifidobacterium adolescentis* [70].

Modes of action

The SCFAs, especially butyric acid, are used as energy by colonocytes. High levels of formation of SCFAs have been associated with improved colonic health and may prevent colonic diseases in the long term. However, most of the SCFAs formed are rapidly absorbed through the colonic mucosa but some also reach the distal colon, where they can be absorbed before being excreted in faeces [71]. Once absorbed, SCFAs are shuttled to the liver via the portal vein, and then enter the systemic circulation where they can have effects on peripheral organs like the heart and brain [52].

Transporters

As weak acids, SCFAs can exist in either protonated or deprotonated form, depending on the pH value of the environment in which they are acting. In a solution with a neutral pH, such as blood (pH around 7.4), they mostly appear in deprotonated form and are denoted as acetate, propionate and butyrate. The protonated SCFAs are easily absorbed via diffusion through colonic cell membranes, whereas the deprotonated form requires transporters, including H⁺-coupled monocarboxylate transporters (MCTs) and sodium-coupled monocarboxylate transporters (SMCTs). These transmembrane transporters are expressed not only in the gut but also in a variety of tissues like the liver, skeletal muscle, heart, kidney, pancreas, kidney and brain [52,70]. Uptake of SCFAs into the liver is facilitated by organic anion transporters (OATs) with OAT7 specifically responsible for butyrate uptake [72]. Hepatocytes metabolise acetate and butyrate to acetyl-coA, which can be used as precursor of cholesterol synthesis or take part in the citric acid cycle for energy production, while propionate can be used for hepatic gluconeogenesis.

Receptors

Regulatory effects of SCFAs on metabolic and immune disorders are mediated by binding G-coupled protein receptors (GPRs) to the cell surface. Three important GPRs stimulated by SCFA formation are GPR41 (synonymous with free fatty acid receptor 3, FFAR3), GPR43 (FFAR2) and GPR109A (known as hydrocarboxylic receptor 2, HCA2). Activation of the GPR43 gene is mediated preferably with the optimum chain length of two to three carbon atoms (acetate and propionate) and, for GPR41, three to five atoms (propionate and butyrate), both with an effective concentration of approximately 0.5 mM [73]. Both GPR41 and GPR43 are found in adipose tissue, the pancreas, the spleen, bone marrow, small and large intestines.

Expression of GPR41 and GPR43 in enteroendocrine cells has been suggested to be involved in appetite regulation and energy homeostasis by stimulating secretion of glucagon-like peptide 1 and peptide YY, a peptide that reduces appetite/food intake, implying potential improvements for type 2 diabetes [74,75]. The involvement of SCFAs in obesity and metabolic diseases can be exemplified by increased GPR41 found in blood of obese and type 2 diabetes patients, potentially relating to reduced satiety caused by overproduction of leptin, a hormone that decreases food intake and body weight, in adipocytes [76]. SCFAs in particular play a major role in regulating inflammatory and immune responses due to strong expression of GPR43 in immune cells including monocytes and T regulatory cells and, to a lesser extent, GPR41 [77]. As an example, activation of GPR43 has been shown to reduce NF- κ B activity and subsequent pro-inflammatory cytokine production (IL-6 and IL-1 β), with specific mediation of β -arrestin 2, which belongs

to a protein family important for regulating activity and intracellular signalling of activated GPRs [78].

GPR109A is found mainly in immune cells (macrophages and neutrophils), adipocytes and in the rat brain, specifically in hypothalamic neurons [79-81]. The receptor is activated by SCFAs, mainly butyrate, monomethyl fumarate and vitamin B3/niacin [82]. Activation of GPR109A is associated with the recruitment of β -arrestins to the cell membrane, and is involved in the regulation of vascular inflammation, including atherosclerosis [79]. GPR109A has been found to increase in microglia of Parkinson's disease patients and contribute to the neuroprotective effects of dimethyl fumarate, an approved anti-multiple sclerosis drug [83,84].

Inhibition of histone deacetylase

The regulatory effects of SCFAs on expression of inflammation mediators can be achieved via inhibition of histone deacetylases (HDACs). These proteins are expressed mostly in immune cells and endothelial cells, and inhibition of HDACs theoretically accelerates a specific gene expression [85]. HDACs are divided into four classes: I (HDAC1-3 and 8), II (IIa, HDAC4, 5, 7 and 9; IIb, HDAC6 and 10), III sirtuins (SIRT1-7) and IV (HDAC11) [86,87]. SCFAs, mainly butyrate and propionate, are known as potent inhibitors of HDACs class I and IIa with an effective millimolar range [88]. Activity of NF- κ B, an important transcriptional factor mediating multiple pro-inflammatory genes, is suppressed via HDAC inhibition in the order butyrate > propionate > acetate [89].

Strengthened barrier function

The intestinal mucosal health is crucial in regulating absorption of nutrients and preventing passage of pathogens. The epithelial cells in the intestinal mucosa form a monolayer, and the effectiveness of this epithelial barrier is controlled by tight junction proteins. These proteins expressed at the most apical intercellular-forming complex comprise transmembrane proteins (such as occludins and claudins) and intracellular scaffold proteins (such as ZO-1, -2, and -3) [69]. In cases of disease or malnutrition, the intestinal barrier is disturbed, leading to hyperpermeability or increased paracellular influx of pro-inflammatory stimuli across the epithelium, a phenomenon commonly known as 'leaky gut'. Increased intestinal permeability and altered expression of tight junction proteins have been associated with several diseases that share a similar inflammatory feature, including inflammatory bowel diseases, some types of colon cancer, obesity and diabetes, celiac disease and food allergy [39,69].

SCFAs, especially butyric acid, have been shown to improve intestinal barrier function. *In vitro* studies showed that butyric acid (2 mM) reduced intestinal paracellular permeability by promoting redistribution of tight junction proteins (ZO-

1 and occludin), which resulted in decreased *Escherichia coli* translocation across the epithelia (3 and 50 mM) [90,91]. Higher concentrations of butyrate (20 to 100 mM) increased intestinal permeability in human colonic epithelial cell lines [91]. The barrier-protective effect of butyrate is mediated via several mechanisms, including inhibition of HDAC [92], activation of 5' adenosine monophosphate-activated protein kinase (AMPK, an energy sensor regulating pathways of lipids, glucose and protein metabolism) [90], and by stabilising the hypoxia-inducible factor (a transcriptional factor targeting genes critical for intestinal barrier function, mucin production and microbial defence) [93].

The BBB controls the metabolite flow in and out of the brain. The BBB is composed of a monolayer of endothelial cells, and its paracellular permeability is controlled by highly specialised intercellular proteins, including tight junction proteins (occludin and ZO-1). There is less evidence about the effects of SCFAs on the BBB, but the SCFAs may be able to act, directly or indirectly, via similar mechanisms as those seen in the gut and peripheral organs. The predominant SCFA, acetate, has been shown to reach the mouse hypothalamus and regulate appetite by suppressing neuropeptides, resulting in reduced food intake [94]. Despite a relatively low brain butyrate uptake (around 0.006% in female baboons), measured as levels of carbon-11 labelled butyrate by dynamic positron emission tomography (PET) scanner [95], increased brain butyrate levels (0.4 to 0.7 $\mu\text{mol/g}$) by *Clostridium butyricum* have been shown to protect mice against vascular dementia or ischemia [96,97].

Glycerol esters of short-chain fatty acids

Why glycerol esters?

SCFAs, especially butyric acid, are well known for their anti-inflammatory functions, as well as positive effects on diet-induced metabolic disorders [71,98]. However, their strong odour is generally perceived as very unpleasant and unacceptable, thereby inhibiting their use via oral administration. The aroma is often described as a bile or vomit odour for butyric acid and for valeric acid, and as rancid Parmesan or foot odour for isovaleric acid. In contrast, their glycerol esters have more neutral or even pleasant fruity aromas resembling the scent of apples or pineapples. Glycerol esters of SCFAs are currently used as feed additives because of their superior properties in relation to parent SCFAs, such as non-corrosive, non-volatile, heat-stable properties and, more importantly, an antibacterial function.

A common way to boost the formation of SCFAs is by consuming dietary fibres. However, the SCFA profile varies considerably, depending on the dietary fibre types and the status of the gut microbiota. In addition, higher ingestion of dietary

fibre may not be suitable for people with gastrointestinal problems, where dietary fibre-derived SCFAs can cause bloating, abdominal distention and bacterial overgrowth [99]. This is a known symptom in IBD patients, who are less tolerant and develop clinical complications following consumption of fibre-rich diets [100,101]. In cases of inflammation, certain types of fibre such as inulin, and subsequent increased levels of caecal butyrate, may have negative effects by potentiating colitis severity in mice [102]. Attempts to deliver butyric acid, usually in the form of sodium butyrate, via intraperitoneal injection or intravenous administration have been tested. However, this route is not preferable due to the safety concern regarding increased hypernatremia (serum $\text{Na}^+ > 145$ mmol/L).

Another concern is how to sustain effective levels of butyric acid in blood or deliver it to target organs like the colon. *In vitro* studies show that butyric acid was the most potent SCFA that could reduce LPS-induced TNF- α production in peripheral blood mononuclear cells, with effective concentration > 0.5 mM [89]. However, this desirable concentration was not achieved under *in vivo* conditions, where intravenous infusion of sodium butyrate resulted in peak blood concentration of butyric acid < 0.05 mM due to its very short half-life (the time it takes for the concentration of butyric acid to fall to half of its initial value), around 6 minutes in rats [103].

A peak concentration of butyric acid was seen at 2 - 3 hours in venous blood of young healthy people after intake of breakfast containing rye [104]. The rapid clearance of butyric acid is probably related to its cellular use as a preferable energy source, especially for colonocytes. In contrast, tributyrin has longer half-life than butyric acid (about 40 minutes), and administering 1 ml of tributyrin to fasted rats reached higher plasma butyrate of 0.34 mM at 30 minutes and subsequently declined to basal levels after 4 hours [103]. Similarly, oral administration of tributyrin (1 g/kg) one hour before LPS injection to fasted rats increased butyrate levels in the portal vein, to 2.4 mM at 1 hour and 0.7 mM at 2.5 hours, returning to pre-treatment levels after 25 hours [99]. Overall, the use of glycerol esters may overcome some unexpected sensory and biochemical properties of SCFAs.

Although the effects of glycerol esters have not been as extensively studied as SCFAs, accumulating evidence demonstrates that glycerol esters of SCFAs display potential protective effects against high-fat diet-induced metabolic complications. In a recent study on 12 overweight and obese adults given inulin-propionate ester (20 g/day for 42 days), insulin sensitivity was improved and the proinflammatory markers IL-8 reduced [105]. Another study from the same laboratory demonstrated that food intake was decreased in healthy overweight and obese subjects after 6-day consumption of palatable food products such as bread rolls and fruit smoothies containing 10 g inulin-propionate ester [106]. However, this effect was not related to changes in blood hormones or metabolites (SCFAs, non-esterified fatty acids and glucose). These findings open a very promising avenue for further exploration of

the use of glycerol esters of SCFAs as food supplements counteracting metabolic disorders.

Monobutyryn and tributyrin

Glycerol esters of butyric acid, such as monobutyryn and tributyrin, are minor components naturally occurring in the fat of dairy products. For instance, total monoglyceride content has been reported to be present in the fat portion (mM/100 g fat) of several dairy products such as fresh milk (0.077), pasteurised whole milk (0.145), homogenised milk (0.206), cream (0.119), butter (0.189) and blue cheese (0.354) [107]. Although appearing in trace amounts in normal Cheddar cheese, an increase in monobutyryn is indicative of lipolysis occurring in rancid cheese [108]. Tributyrin has also been found at levels of around 3 - 4 g/100g in butter [109].

Anti-inflammatory action of glycerol esters of butyric acid has been shown in several animal studies. When administered orally, tributyrin (1 g/kg) elevated plasma butyrate concentration in the portal vein, protecting the rat liver from LPS-induced toxicity and subsequent inflammatory responses by suppressing NF- κ B activation and TNF- α production [99]. In mice fed a high-fat diet, oral gavage of tributyrin (2 g/kg) attenuated activation of the inflammation-related signalling pathway JUN NH₂-terminal kinase (JNK) in the liver and skeletal muscle, resulting in reduction of pro-inflammatory mediators (TNF- α , IL-1 β , IL-6) by macrophages as well as mRNA expression of these mediators and macrophage markers (F4/80 and CD11c) in adipose tissue [110]. Tributyrin supplementation also reduced short-term and acute ethanol-induced increases in plasma aminotransferase and liver pro-inflammatory mediators (TNF- α , IL-1 β , CMP1, MIP2), but not after chronic ethanol exposure [111]. Considered as a HDAC inhibitor, tributyrin has been proved to be preventive against liver cancer, during initial or promoting phases, [112,113] and colon cancer [114] in rats.

Reduced inflammation by glycerol esters of butyric acid is usually coupled with improvements in lipid metabolism. For instance, in an acute sepsis rat model, oral tributyrin administration (1 g/kg, 1 hour before LPS) suppressed the increase in levels of plasma triglycerides, total cholesterol and LDL-c at 24 hours [115]. This effect was associated with upregulated expression of nuclear hormone receptors at basal levels (PPAR- α) and in endotoxemia (PPAR- γ), fatty acid oxidation associated genes (FATP, FABP) and fatty acid synthesis associated genes (SREBP-1c) in the liver, were observed already at 6 hours after LPS challenge. While PPAR- α enhances fatty acid oxidation, PPAR- γ modulates liver lipogenesis and has been reported to upregulate hepatic expression of FATP [116,117], notably tributyrin-induced upregulation hepatic protein expression of PPAR- α and PPAR- γ , and enhanced acetylation of histone H3 with and without LPS challenge (at basal levels and maintained this effect at 6 h after LPS injection). Tributyrin gavage is effective

in partially correcting dyslipidaemia by repressing serum non-esterified fatty acid and hepatic triglyceride accumulation in mice fed a lard-rich diet (59.1% total energy from fat) for 10 weeks [110].

Monobutyryl and tributyrin are currently used as commercial feed additives due to their ability to improve growth performance and intestinal health in chickens [118,119] as well as their antimicrobial action against pathogenic bacteria such as *Salmonella* Typhimurium and *Clostridium perfringens* [120]. When supplemented to chicken feed, monobutyryl and a mixture of 30% mono-, 50% di-, and 20% triglycerides of butyric acid have been shown to reduce abdominal fat ratio (abdominal fat weight/final body weight) and decreased levels of serum total cholesterol, triglycerides and HDL-c [121]. This effect was linked to enhanced fatty acid oxidation in the liver and reduced synthesis, storage, transportation of lipids in the jejunum.

Experimental data have reported promising effects of glycerol esters of SCFAs on gut barrier function. Dietary supplementation of tributyrin to standard diets (1 - 5 g/kg diet) have been shown to have ameliorating effects on intestinal injury or experimental colitis in animals like pigs and mice [122,123]. In piglets, these protective effects were accompanied by a reduction in systemic and intestinal inflammation (decreases in plasma creatinine, prostaglandin E2, inducible nitric oxide synthase), oxidative stress (malondialdehyde in plasma and colon), intestinal damage (colonic caspase-3), and enhancement in mucosal repair and integrity (i.e. on tissue level like increases in villus height/crypt depth ratio, goblet cells, as well as on molecular level as increased expression of claudin-1 and epidermal growth factor receptor/EGFR in the colon).

In mice, tributyrin protection was due to attenuation in mucosal damage (histologically evidenced by undamaged mucosal basal cells and an absence of ulcerations) by reducing immune cell infiltration such as neutrophils and eosinophils, oxidative stress (decreased hydroperoxide, increased activities of superoxide dismutase and catalase), and increasing anti-inflammatory cytokines (IL-10 and transforming growth factor β). These effects were associated with reduced intestinal permeability and increased percentage of regulatory (LAP+) T cells [123]. Tributyrin also resulted in increased level of activated macrophages, and the resultant cytokine IL-1 β . IL-1 β is an important mediator of pro-inflammatory responses, regulating several cellular processes such as proliferation, differentiation and apoptosis. The association between the increase in IL-1 β and the improvement in mucosal structure after tributyrin supplementation might indicate that IL-1 β exerts a cell proliferative role rather than as a pro-inflammatory mediator.

Tributyryl, delivered via oral gavage or mixed in liquid diet in dose 0.83 - 10 mM, has been shown to protect mice from ethanol exposure-induced gut injury by stabilising expression and co-localisation of tight junction proteins (ZO-1, occludin), increasing expression of butyrate receptor (GPR109A) and transporter (SLC5A8) in the ileum and proximal colon [111]. Delivering sodium butyrate (1

g/kg body weight per day) by gavage for three days decreased BBB permeability in germ-free mice by increasing protein expression of occludin in the frontal cortex and hippocampus [124]. Germ-free mice that cannot perform colonic fermentation to produce SCFAs due to lack of gut microbiota have increased BBB permeability compared with conventionally raised mice of the same strain.

Monovalerin and trivalerin

Valeric acid was named after its naturally abundant origin, the perennial flowering plant valerian. Because of its sedative effects, extracts from this plant have been widely used to treat pain, epilepsy, insomnia, anxiety and depression [125], but not much is known about health or dietary/nutritional effects of valeric acid esters. However, a recent study has demonstrated positive effects on intestinal health of supplementing broiler feed with a mixture of monovalerin and trivalerin. This was indicated by increased density of glucagon-like peptide-2 immunoreactive cells, which are involved in the maintenance of the gut barrier function in jejunal and ileal villi, and reduced necrotic enteritis [119].

In humans, higher faecal valeric acid concentration was found in healthy centenarians living in the region famous for longevity, Bama county, China (mean age 103) compared with healthy elderly people from other regions of longevity (mean age 87-88) [126]. Similarly, the valeric acid-producing bacterium *Oscillibacter valericigenes* abundance is predominantly enriched in faecal samples in healthy individuals compared with patients having Crohn's disease [127]. High concentrations of valeric acid have also been found in venous blood from microscopic colitis patients [104], and also in faecal samples from people suffering from celiac disease [128] or obesity [129]. Positive or negative effects of valeric acid should therefore be considered in specific conditions.

Beyond the gut, valeric acid has also been shown to possess neuroprotective effects against dementia in rats as an agonist of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) [125]. Valeric acid may also be involved in lipid metabolism, since administration of a valeric acid sodium salt was shown to reduce cholesterol synthesis in rat liver [130].

The rat model

The strength of a model for studying different issues depends on the question under investigation and must be evaluated by the availability of tools necessary for solving the problem.

The rat has been, and is, widely used as a model in biomedical research, in comparison to the mouse [131]. Although starting almost 30 years behind the mouse

because of technical limitations such as gene-editing techniques, recent development of powerful genetic techniques, such as zinc-finger nucleases, has enabled scientists to create transgenic rats. Consequently, the rat is back as a more dominant model for studying molecular nutrition. At present, over 40 rat genomes have been sequenced. Each rat genome contains 2.75 billion base pairs and is therefore more closely related to the human genome (2.9 billion) than the mouse (2.6 billion) [132]. Parallel with this technical development, outstanding databases for rat-based studies have been set up, such as the Rat Genome Database, providing a huge databank for exploring new insights into disease pathways or mechanisms from rat studies [131].

Another consideration regarding the reliability of a model organism is its relevance/applicability to human physiology and pathophysiology. The rat is the preferred animal model for physiological studies, especially in the area of cardiovascular diseases and neurobehavioral research. An advantage of the rat is its larger size compared to the mouse, about ten times by weight. This larger size allows larger collection of blood and tissues, thereby enabling more analyses and decreasing the potential of error/variability and limited detection of low-abundant metabolites caused by low volume samples. More metabolites can be analysed in the same sample, reducing the sample size, i.e. the number of animals can be reduced, in line with guidelines for animal research. For neuroscience studies, rats are considered to be more intelligent, and perform learning and memory tasks better and more reliably than mice [131]. This advantage is due to not only the larger forebrain size but also the sociability of the rat. In addition, rats are easier to handle, more docile and rarely bite, unlike mice.

Rats have been widely used to study diet-related diseases. For instance, the Zucker rat has long been used as a genetic model for studying obesity and hypertension. Importantly, rat models reflect similar patterns to those seen in human disease in that environmental factors such as diet and stress can modulate the disease. It has also been shown that fibre fermentability and colonic microbiota composition are very similar between men and rats [133].

It can be argued that humans are the best choice for studying such issues, but there are limitations concerning ethical issues and sampling locations, for instance access to the proximal colon in humans, the main site of fibre fermentation. It is also difficult to have complex experimental matrices and study many components at the same time in such a model. Recent techniques like stable isotope technology can track the route of colonic-derived SCFAs into systemic circulation [134], but a challenge remains regarding the study of health benefits of SCFAs, especially dietary interventions where long-term dietary intake can be very difficult to control. Consequently, in controlled experiments, the rat is still a proper model, better than the mouse, for dietary interventions because they have more complex food choices and can easily eat a wider variety of foods in different forms [135].

Aim

The purpose of this work was to evaluate whether dietary supplementation of glycerol esters of SCFAs would have similar nutritional/food-induced effects on health as those suggested for SCFAs formed by the microbiota from indigestible food components, with the overall aim to improve the host health.

The specific aims of the four studies were:

- To examine whether monobutylin and tributyrin, added to butter-based high-fat diets, could influence the SCFA profiles in different parts of the body (caecum, blood, liver and brain), microbiota composition, lipids (blood and liver), cholesterol-associated hepatic gene expression and inflammatory markers in conventional rats (**Paper I**).
- To investigate whether monovalerin and trivalerin in butter-based high-fat diets could have possible effects on SCFA profiles (caecum, blood, liver and brain), caecal microbiota composition, lipids (blood and liver), cholesterol-related hepatic gene expression and inflammatory markers in conventional rats (**Paper II**).
- To determine whether there was a dose-dependent effect of monobutylin on lipid profiles (blood and liver) and intestinal permeability in conventional rats, and whether the effects on lipid profiles were dependent on type of fat (**Paper III**).
- To explore possible effects of monobutylin and monovalerin, added in lard-based diets, on lipid profiles (blood and liver), SCFA profiles (blood and brain), intestinal permeability, gut-brain barrier integrity, and pro- and anti-inflammatory markers in ApoE-deficient rats. This is a model characterised with extreme dyslipidaemia and often used to study cardiovascular diseases and AD (**Paper IV**).

Materials and Methods

Test products

Glycerol esters of butyric acid, monobutylin (MB) and tributyrin (TB), and valeric acid, monovalerin (MV) and trivalerin (TV), were investigated. These glycerol esters were kindly provided by Perstorp AB (Perstorp, Sweden), and their composition was analysed by liquid chromatography-mass spectrometry (LC-MS) before delivery (see below).

MB and TB were tested in Paper I, and MV and TV in Paper II. Different doses of MB were considered in Paper III, and the type of fat was also investigated (lard versus butter). Based on results from these three papers, MB and MV were selected for the dietary interventions in Paper IV.

Monobutylin and tributyrin

Monobutylin is a glycerol ester of butyric acid, generated by attaching one butyric acid mostly to a stereospecific numbering 1 (sn-1) of a glycerol molecule. The composition (% weight/weight) of MB used in Paper I, III and IV comprised 46% monobutylin, 13% dibutylin, 1.1% tributyrin, < 1% butyric acid and 39% glycerol.

Tributyrin is another glycerol ester with three butyric acids bound to the glycerol backbone. The TB used in Paper I comprised < 1% monobutylin, 6.1% dibutylin, 91% tributyrin, < 1% butyric acid and < 1% glycerol.

Monovalerin and trivalerin

Esterification of glycerol with one or three valeric acids results in monovalerin or trivalerin, respectively. MV used in Paper II comprised 46% monovalerin, 22.4% divalerin, 1.6% trivalerin, 3.3% valeric acid, 1.6% ethylvalerin and 23.7% glycerol. MV used in Paper IV consisted of 47% monovalerin, 24% divalerin, 3.2% trivalerin, 0.03% valeric acid and 25% glycerol. Trivalerin (95%) is the main component of TV, followed by 2% divalerin and 0.04% water.

Diets

In principle, the same composition was used to make the experimental diets in all the studies. Casein (Sigma-Aldrich, St. Louis, MO, USA) was used as the main protein source, accounting for 150 g/kg of the total amount of all ingredients (dry weight basis). Butter (180 g/kg, Arla Foods, Stockholm, Sweden) was used as the main fat source of saturated fat in the high-fat diets (44% total energy from fat) used in Paper I, II and one of the experiments in Paper III (experiment 1 Paper III). In another experiment in Paper III (experiment 2 Paper III) and in Paper IV, lard (230 g/kg, Dragsbaek, Denmark) was used as fat source instead of butter. This replacement was because butter may contain some mono- or triglycerides of butyric acid, which could potentially interfere with the interpretation of results of the added MB. However, our LC-MS analysis showed that the content of MB and TB in the butter used was under detection limit (< 0.001% weight). This result is in accordance with previous studies showing that glycerol esters of butyric acid increased only in rancid dairy products [108].

For low-fat diets (15% total energy from fat), rapeseed oil (50 g/kg) was used in all diets. The carbohydrate source was sucrose (100 g/kg) and wheat starch. The amount of wheat starch (396 - 591 g/kg) was adjusted to obtain the same dry matter in all diets. The wheat starch used in the studies is known to be hydrolysed completely in rats [136], so SCFA production from wheat starch was very limited. The amounts of other components were kept the same in all diets, including cellulose (50 g/kg), mineral mixture (48 g/kg) and vitamin mixture (8 g/kg) (both from Lantmännen, Stockholm, Sweden in Papers I and II or Altromin, Lage, Germany in Papers III and IV), choline chloride (2 g/kg) and DL-methionine (1.2 g/kg) from Sigma-Aldrich.

The doses of test products used in Papers I and II were based on the commonly used dose in commercial feed (1 g/kg diet) and previous studies (5 g/kg diet) [118,123] as well as from pilot studies prior to the first experiment. In experiment 1 Paper III, different doses of MB (2.5, 7.5 and 15 g/kg diet) were used, based on the results from Paper I and the maximal safe dose of monobutyryl reported in rats (27 g monobutyryl/kg body weight/day) [137]. The dose (10 g/kg diet) of MB and MV used in Paper IV was selected based on the results from all three previous papers, considering hyperlipidaemia status in rats lacking ApoE protein.

Study design

All animal experiments were conducted at the Animal Facilities, the Biology Department, Lund University. The controlled conditions were set to 12 hours light/dark cycle and room temperature around 21°C. Animals used were male rats,

belonging to either Wistar (Taconic, Denmark) in Papers I, II and III or Sprague-Dawley strain (SAGE Lab Inc., Boyertown, USA) in Paper IV. ApoE-knockout rats are rarely available commercially, and at the time of preparation for Study IV, SAGE Lab was the only commercial supplier of this special model (based on Sprague-Dawley strain) on the market. All rats were conventional/healthy rats, except 40 rats in Paper IV that were healthy/transgenic (i.e. the rats lacked the ApoE protein). The rats were randomly divided into different test groups based on similar initial body weight. All experimental set-ups were designed to contain a high-fat and a low-fat diet without any glycerol esters, serving as control groups. A group fed glycerol-high-fat diet was included in Paper III/Experiment 1 and, in Paper IV, along with ApoE knockout rats, one group with conventional rats of Sprague-Dawley strain was also included.

Experimental time was three weeks in Papers I, II and III/Experiment 1. Due to assumed difficulties in measuring lipid changes in the tail vein, the second experiment in Paper III was prolonged for an extra week, resulting in a four-week experimental period. In Paper IV, the study was of a five-week duration for the same reason, together with the extreme lipid disturbance in ApoE-deficient (ApoE^{-/-}) rats. The sample size in this study was higher than in the other studies, ten rats per group (n = 7/group in the other experiments) because there was less experience of this rat model and potentially detrimental effects of high-fat feeding on ApoE^{-/-} rats. This made it possible to fulfil statistical criteria. The rats had free access to the assigned diets and water during the study time and until dissection. Body weight gain and food intake were measured weekly. Intestinal permeability test was carried out one week before the end of the studies (Paper III/Experiment 1 and Paper IV).

On the dissection day, blood was collected from the portal vein and the aorta, when the rats were anaesthetised by a subcutaneous injection of a solution (1:1:2) containing Hypnorm (Division of Jansen-Cilag Limited, Janssen Pharmaceutica, Beerse, Belgium), Dormicum (F.Hoffman-La Roche AG, Basel, Switzerland or Accord Healthcare, London, UK) and autoclaved Milli-Q Millipore water, at a dose of 0.15 ml/100 g body weight. Next, organs were harvested, weighed and snap-frozen on dry ice and then stored at -40°C (liver, spleen, heart, caecum, intestine) or -80°C (brain, ceacal contents for microbiota analysis). A summary of experimental design and analyses in all papers are presented in **Tables 1 and 2**.

Statistical evaluation and calculations

All analyses were performed in duplicate, except for the gene expression tests, which were performed in triplicate. Statistical evaluation was based on software such as GraphPad Prism (version 7, San Diego, CA, USA) or Minitab (version 17, AutoBVT Microsoft). Normality of the data was checked by either Grubbs' test or

D'Agostino-Pearson test, depending on the software used. To compare differences in means between a control group (high-fat diet without supplements) and the experimental groups, one-way analysis of variance (ANOVA) and post-hoc Dunnett's test were used. The same statistical approach was applied when a group fed low-fat diet was used as a positive control. To compare differences between only two groups, the 2-tailed unpaired t-test was used. Correlations were examined using Pearson's test for parametric variables, while Spearman's test was employed for non-parametric variables. To obtain an overview of all relevant results, multivariable data analysis was performed using Partial-Least-Squares-to-latent-structures-Discriminant-Analysis (PLS-DA) in SIMCA software (version 14 or 15, Umetrics, Umeå, Sweden). The confidence level in all statistical tests was set at 95%, with $p < 0.05$ being considered statistically significant. P values within the range $0.05 \leq p \leq 0.2$ were considered as a tendency and reported for some parameters of interest. Results are presented as means and their standard errors of the mean (SEM).

In some cases where actual weights might affect the results differently, the data were calculated as total amounts (pools) by multiplying the obtained concentrations with the organ weights. This calculation was applied for lipids in the liver, SCFAs in caecal contents and brain.

Table 1.
Summary of study design

Paper/ Duration	Test products (doses)	Number of groups (rats per group)	Type of fat
I 3 weeks	Monobutylin (1 and 5 g/kg) Tributylin (5 g/kg)	5 (n = 7)	Butter
II 3 weeks	Monovalerin (5 g/kg) Trivalerin (5 g/kg)	4 (n = 7)	Butter
III a) 3 weeks b) 4 weeks	Monobutylin a) (2.5, 7.5 and 15 g/kg) b) 5 g/kg	a) 5 (n = 7) b) 3 (n = 7)	a) Butter b) Lard
IV 5 weeks	Monobutylin (10 g/kg) Monovalerin (10 g/kg)	5 (n = 10)	Lard

Table 2.

List of analysed parameters

Analyses	Paper I	Paper II	Paper III	Paper IV
Lipids (1)				
Portal vein	X	X	X	X
Liver	X	X	X	X
Aorta			X	X
Tail vein			X	X
SCFAs				
Caecal content	X	X	X	
Portal vein	X	X		X
Liver	X	X		
Brain		X		X
Caecal microbiota	X	X		
Gene expression	X	X		X
Liver succinic acid	X	X		
Portal vein serum LBP	X	X		
Urinary lactulose/mannitol			X	X
Aortic IL-1 β , IL-10				X
Portal vein ALT				X
Intestinal histology				X

⁽¹⁾ Lipids refer to total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides

X indicates the parameter measured

SCFAs, short-chain fatty acids; LBP, lipopolysaccharides binding protein; IL, interleukin; ALT, alanine transaminase

Results and Discussion

When this project started, *in vitro* studies had shown that TB was degraded to MB, where MB survived gastrointestinal conditions [138]. There were also some studies showing that both MB/TB and MV/TV prevented gastrointestinal infections in chickens and pigs [119,138]. Since SCFAs are associated with a number of physiological effects, this encouraged us to carry out this project, to evaluate whether glycerol esters could increase SCFAs in different parts of the body or change the microbiota composition, and consequently have nutritional effects.

Results show, which was quite unexpected, that there was no increase of SCFAs in caecum and serum of rats to any great extent, except for some changes in caecal SCFA distributions and serum valeric acid, after adding the glycerol esters - MB, TB and MV, TV - to the diet. Effects were greater on many other parameters: the caecal microbiota composition, the small intestinal permeability, liver and blood lipids, tight junction proteins and SCFA receptors in small intestine and brain, liver inflammatory markers, and the plasma cytokines. Body weight and feed intake were reduced.

Significant effects of glycerol esters of butyric and valeric acids on conventional and ApoE^{-/-} rats are discussed separately below, in line with the specific aims. The results for each glycerol ester are presented first, followed by discussion with relevant mechanism-related explanations.

Lipid profiles

Lipid levels were mainly reduced in the liver of the rats when glycerol esters of SCFAs were added to a high-fat butter-based diet, while lipid levels in the blood were also reduced when the glycerol esters were added to a high-fat lard-based diet.

Liver

Cholesterol

Supplementation with 5 g/kg MB to a high-fat butter-based diet reduced total liver cholesterol by 51% (Figure 2a, $p < 0.0001$). Simultaneous reductions in concentration of LDL-c and LDL-c/HDL-c ratio (12% and 17%, respectively) were also observed (Figure 2c and d). Higher doses of MB (7.5 g/kg and 15 g/kg MB) showed similar results, although to a lower extent, where total liver cholesterol concentrations decreased by 8% and 11%, respectively, compared with rats fed the control diet without MB (Figure 2b).

TB displayed a similar ability as MB to reduce total cholesterol (24%) and LDL-c concentrations (10%) and the ratio of LDL-c/HDL-c (19%) at 5 g/kg (Figure 2e). TB also tended to increase liver HDL-c concentration ($p = 0.075$), which could not be seen with MB.

The decreasing effect of total liver cholesterol levels with MB and TB can be explained by several mechanisms. There was a downregulated expression of BA synthesising genes, as indicated by the positive correlations between total liver cholesterol and *Cyp7a1* ($r = 0.441$, $p = 0.024$) and *Cyp8b1* ($r = 0.546$, $p = 0.004$). In the liver, cholesterol is used for the synthesis of BAs, and is initiated by *Cyp7a1* in the classical pathway, while *Cyp8b1* regulates the BA composition. Previous studies have shown that mice lacking in *Cyp7a1* have reduced cholesterol absorption and increased faecal neutral sterol excretion [139]. Butyrate as well as tributyrin can inhibit *Cyp7a1* expression, most probably by increasing the expression of PPAR α [115,140], which is acting as a *Cyp7a1* repressor in human liver cell lines [141]. Increased hepatic expression of PPAR α in rats fed high-fat diets was reported after oral gavage of butyrate, while the triglycerides levels decreased [142]. In the liver, BAs (cholic acid/CA and chenodeoxycholic acid/CDCA) are conjugated with glycine or taurine to facilitate solubility. Once secreted into the small intestine, primary BAs are deconjugated and converted to secondary BAs (deoxycholic acid/DCA and lithocholic acid) by the enzyme 7 α -dehydroxylase produced by anaerobic bacteria. BAs in the small intestine can be reabsorbed and returned to the liver where they inhibit BA synthesis, as seen specifically with reduced expression of *Cyp7a1* by CDCA [143]. In the study published as Paper I, both MB and TB decreased *Cyp7a1* and *Cyp8b1* levels. It is suggested that MB and TB decrease liver cholesterol concentrations by reducing the BA gene expression.

SCFAs released from the glycerol esters may also contribute to the cholesterol-lowering effect of MB. Although no changes/increases could be seen for individual SCFAs in caecum of the rats, the ratios of acetic-to-butyric acid and acetic-to-propionic plus butyric acids were significantly higher in rats fed the high-fat diet supplemented with 15 MB g/kg, compared with those fed the non-supplemented control diet (Paper III). Acetic acid is thought to stimulate cholesterol synthesis [70], while propionic and butyric acids inhibit the synthesis [144]. A lower ratio of acetic-

to-propionic acid or butyric acid would therefore be preferable, contradicting the results in Paper III. A possible explanation for these non-consistent results is that it would be more relevant to measure the ratios in the liver.

However, there are also studies reporting lipid-lowering effects for acetic acid, suggesting involvement of other mechanisms, probably pH and satiety hormones. In one study on rats, when acetic acid was supplemented to a diet rich in cholesterol, serum total cholesterol was lowered, which was explained by inhibition of liver lipogenesis and promotion of faecal BA excretion [62]. A follow-up study in obese humans showed that ingestion of a beverage containing 15 - 30 ml of vinegar (750 - 1500 mg acetic acid) for 12 weeks reduced body weight, body mass index, visceral fat area, waist circumference and serum triglycerides [145]. It is noted that only approximately 0.1% of colonic-derived acetic acid was assimilated into plasma cholesterol and <15% into fatty acids in healthy subjects, using stable isotope technology [134]. These diverging findings reflect that SCFAs affect the lipid profile via multiple factors, and that the outcomes may be dependent on the investigated parameters.

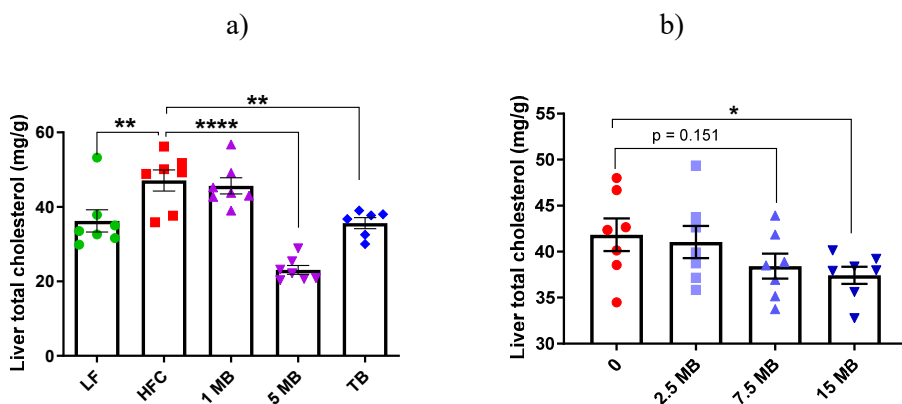
Influence of SCFAs on lipid metabolism can be achieved via their receptors. For instance, activation of GPR43 by SCFAs in the colon can stimulate the release of anorexigenic (appetite suppressing) hormones such as GLP-1 and PYY from enteroendocrine L-cells in human and rat gut [146,147]. Increases of these hormones have been seen in blood of humans following both rectal infusion of acetate or intake of indigestible carbohydrates from Swedish brown beans known to increase plasma SCFAs [148]. Such elevation has been shown to suppress hepatic accumulation of lipids *in vitro* [149] or decrease gastric emptying and small-intestinal transit time [150]. Similarly, supplementation of the diet with 5% butyrate was shown to decrease plasma concentrations of total cholesterol, triglycerides and fatty acids in mice fed high-fat diets, which was linked to the concurrent increase in plasma butyrate concentration [151].

SCFAs can also activate AMPK, an inhibitor of fatty acid and sterol synthesis, in several tissues including the liver in rats and men [152,153]. When activated, AMPK can promote fatty acid oxidation and decrease hepatic lipogenesis [154]. For example, activation of AMPK by acetic acid leads to suppression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-limiting enzyme for cholesterol synthesis [70].

Another interesting aspect in relation to SCFAs is that the conversion of acetic acid into butyric acid is the most dominant microbial interconversion in the proximal colon in humans [134] and in serum and faeces of rats [155]. In Paper I, butyric acid concentrations in the portal vein were, although insignificantly, higher with MB and TB diets (5 g/kg) compared with the control diet. It cannot be excluded that the differences may have reached significance with a longer experimental time. This result may indicate the existence of cross-feeding mediated conversion of acetic acid to butyric acid, leading to a gradual increase of butyric acid into the portal vein. In

this case, butyric acid might, in turn, suppress lipid release into the circulation by inhibition of intestinal lipid absorption [63]. Acetic acid was not distributed in the diet in the present study, but it is produced endogenously in different tissues and organs [70,156], so other mechanisms such as activation of AMPK may switch actions of SCFAs to lipogenesis or fatty acid oxidation [98].

Feeding the rats with MV and TV diets (5 g/kg) also reduced liver total cholesterol concentrations, although not to a greater extent than with MB, by 15% and 18%, respectively. This result seemed to be associated with valeric acid values, and there was a reverse correlation between serum valeric acid and liver LDL-c/HDL-c ratio. Addition of a valeric acid sodium salt or derivative to rat diets was also shown to suppress liver cholesterol synthesis, possibly via stimulating peroxisomal β -oxidation [157], rather than affecting HMG-CoA reductase [130]. Peroxisome proliferator-activated receptors (PPARs), particularly PPAR- α , are ligand-activated receptors crucial for regulating the expression of several genes involved in lipid and lipoprotein metabolism. Activation of PPAR- α and consequent increase in β -oxidation by valeric acid may therefore be related to its inhibition of HDAC [158]. Less is known about valeric acid formed in colon from indigestible fibre components on cholesterol metabolism, but it is known that very low amounts of valeric acid can be formed by the colon microbiota.



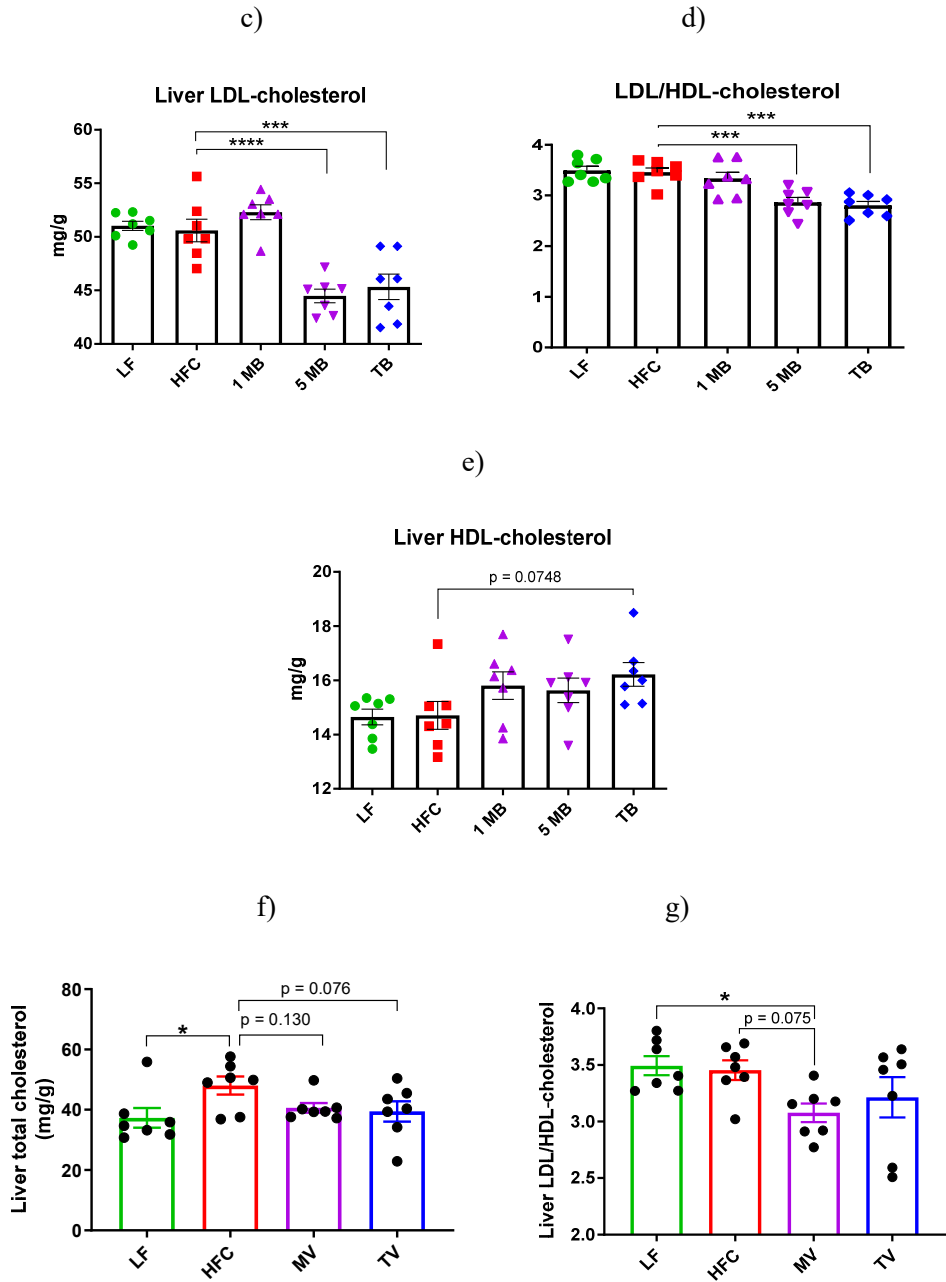


Figure 2.

Addition of glycerol esters of SCFAs to butter-based high-fat diets reduces liver lipids in rats. (a), (b) and (f) liver total cholesterol concentration (mg/g), (c) LDL-cholesterol concentration (mg/g), (d) and (g) LDL/HDL-cholesterol ratio, (e) HDL-cholesterol concentration (mg/g). LF, low-fat; HFC, high-fat control diet, pure or supplemented with monobutyryl (MB), tributyrin (TB), monovalerin (MV), or trivalerin (TV); LDL, low-density lipoprotein; HDL, high-density lipoprotein. Mean values were significantly different between two groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Triglycerides

In addition to reducing cholesterol in the liver, MB also reduced triglycerides (Figure 3). The two higher doses of MB (7.5 and 15 g/kg, Paper III) decreased liver triglyceride concentrations and tended to lower the TG/HDL-c ratio (for 7.5 g/kg MB). No effects could be seen with lower doses of MB (1 and 5 and 2.5 g/kg in Paper I and Paper III, respectively).

A reduction of liver triglycerides with tributyrin has also been reported by others, as seen with mice obtaining tributyrin by oral gavage (2 g/kg body weight) [110]. The time also seemed to be important for the reducing effects; triglyceride levels were reduced first after 10 weeks when mice were fed a high-fat diet (based on coconut and soybean oils) containing 5% butyrate [151].

SCFAs are able to influence triglyceride formation by several mechanisms, for example by promoting fatty acid oxidation or by inhibiting tissue lipolysis and non-esterified fatty acids in blood by activating their receptors or PPAR- α [98,159]. Like the cholesterol-lowering effects of SCFAs, activation of AMPK or GPR43 can inhibit activity of hormone sensitive lipase (HSL), an enzyme catalysing the ester bonds of triglycerides in adipocytes, resulting in reduced hydrolysis of triglycerides and release of free fatty acids into the blood [70]. In addition, activated AMPK may suppress the activity of enzymes that catalyse initial stages of glycerol-lipids and fatty acid synthesis such as glycerol-3-phosphate acyltransferase (GPAT) and acetyl-CoA carboxylase (ACC) [70]. Acetate administration, in particular, has been shown to directly activate AMPK in the mouse liver, by increasing AMP formation [160]. In the present study, decreased liver triglycerides levels might result from increased fatty acid oxidation and diminished fatty acid synthesis mediated by caecal acetic acid, as reported in rats by Fushimi *et al.* [62]. Monobutytrin has been shown to directly inhibit hepatic glycerol uptake, lowering the availability of substrates to produce triglyceride [161]. MB supplementation can attenuate deposition of triglycerides in the liver, with a dose-dependent consideration.

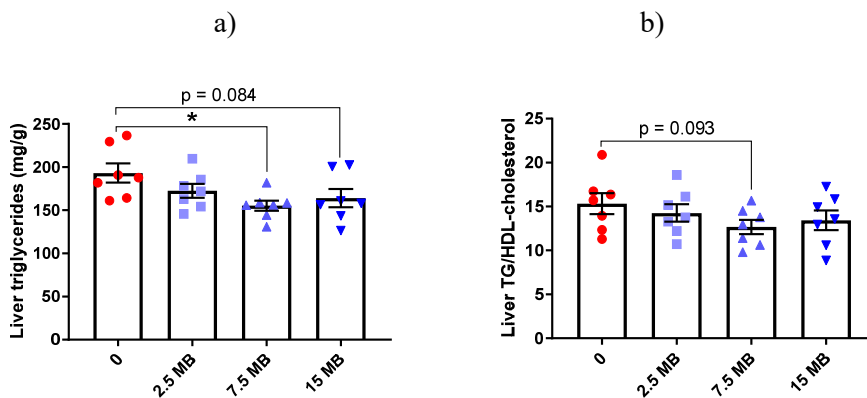


Figure 3. Monobutyryl (MB) supplementation decreases liver triglycerides in rats fed butter-based high-fat diets. (a) Triglycerides concentration (mg/g), (b) triglycerides/HDL-cholesterol ratio. TG, triglycerides; HDL, high-density lipoprotein. Mean values were significantly different between two groups: * $p < 0.05$.

Portal vein

No effects on lipids were seen in the portal vein when the glycerol esters were added to butter-based high-fat diets.

When the fat source of the high-fat diet was based on lard, no differences in liver total and LDL-c could be seen, unlike results obtained with butter-based diets. Adding MB, 5 g/kg, to the diet instead affected the lipid metabolism by increasing the amount of HDL-cholesterol in the liver (12.7%, $p < 0.05$), which is considered a positive effect (Figure 4a). Adding MB to the lard-based diets decreased serum concentrations of total cholesterol (7.7%, $p < 0.05$), as well as LDL-cholesterol (8.8%, $p = 0.118$) in the portal vein (Figure 4b and c).

The different outcomes from diets made of two types of fat is not surprising, and may be due to their different lipid composition. Lard has lower cholesterol content than butter (95 versus 219 mg/100 g; data retrieved from the USDA National Nutrient Database and [109]), which may be associated with the increase seen in liver HDL-cholesterol by MB. This indicates that MB together with lard accelerates a reverse cholesterol transport, as shown by the decrease of total and LDL-cholesterol portal serum concentrations.

There is another explanation as to why MB, incorporated into butter-based diets, did not change blood lipids. This could be due to the content of saturated fatty acids, which is higher in butter than in lard. Saturated fatty acids can suppress LDL receptors in the liver, causing elevated LDL levels in blood [162]. As a consequence, it is harder, or perhaps a longer experimental time is needed, to influence blood cholesterol levels with MB, as seen previously with butyric acid and its decreasing effects on blood cholesterol levels in mice that occurred first after 10 weeks [151].

The levels of saturated fatty acids at the stereospecific numbering position 2 (sn-2) of triglycerides is lower in butter than in lard; they are preferentially distributed to the liver and may have an effect there. This may be another explanation for the reducing effects of MB on total cholesterol and LDL-c seen in the liver of rats with butter-based diets but not with lard-based diets. Regardless of fat source, MB supplementation is effective in changing different parameters associated with lipid profiles at various locations in the body, indicating involvement of several mechanisms.

In ApoE^{-/-} rats, the concentration of HDL-cholesterol increased in the portal vein after consumption of a high-fat diet containing MV for 5 weeks (Figure 4d, $p < 0.05$). The concentration of triglycerides in the tail vein declined between week 2 and week 4 in some of the individual rats fed the MB (8 rats) and MV (6 rats) diets, but in none of the rats fed the control diet.

Serum HDL-cholesterol concentration was positively correlated with valeric acid in blood ($p = 0.0046$, $r = 0.41$) and brain ($p = 0.0006$, $r = 0.53$) and also with the expression of GPR109A ($p = 0.006$, $r = 0.49$) and ZO-1 ($p = 0.026$, $r = 0.41$) in the brain.

ApoE^{-/-} rats are generally known to have elevated blood lipids, which was also shown in this study, as evidenced by the 50% higher total cholesterol concentrations in these rats compared with conventional rats on the same diet. Supplementation with MV for 5 weeks increased HDL-cholesterol concentrations in the portal vein, which must be considered a positive effect (Figure 4d). Similar results were seen with female ApoE^{-/-} rats fed a Western diet (41% energy from fat and 0.21% cholesterol) [163], but when the rats developed a more human-like LDL-abundant lipid profile (at an age of about 20 weeks) the high HDL-cholesterol values disappeared.

The results in the present study suggest that MV have the capacity to enhance HDL-cholesterol and possibly also reverse cholesterol transport. A positive correlation was found between HDL-cholesterol and serum valeric acid, and they both increased with MV. This has been explained by enhanced β -oxidation in conventional rats. Another explanation may be that MV enhances HDL function by upregulating *Pltp*, a gene that promotes hepatic uptake of cholesteryl esters and phospholipid from HDL, as seen with valproic acid in mouse fatty liver [164]. This analogue of valeric acid also reduced the increase in cholesterol in neural stem cells of mice deficient in Niemann-Pick type C gene (a model of lipid storage disorder and neurodegenerative disease) [165]. Positive correlations between valeric acid and tight junction proteins and SCFA receptors in the brain (ZO-1 and GPR109A) further support the increase in HDL-cholesterol by MV as a positive effect.

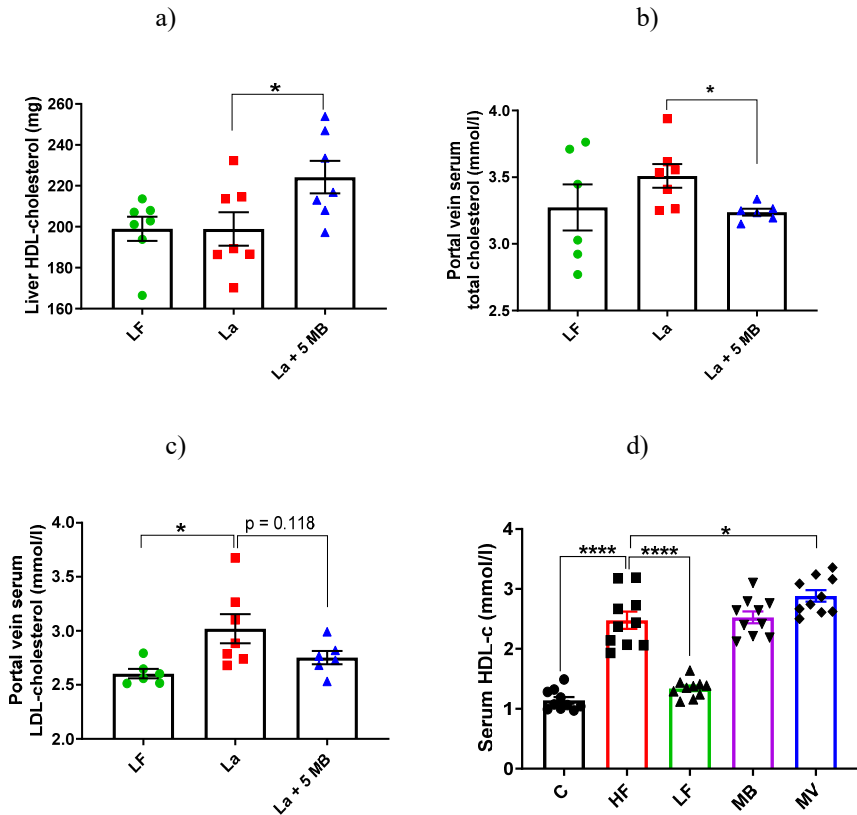


Figure 4.

Monobutyryn (MB) decreases lipids in rats fed lard-based high-fat diets. (a) Total amount of liver high-density lipoprotein (HDL)-cholesterol (mg), (b) portal vein serum total cholesterol concentration (mmol/l), (c) portal vein serum low-density lipoprotein (LDL)-cholesterol, in conventional rats fed a low-fat diet (LF) or a high-fat diet (La), pure or supplemented with 5 g/kg MB (La + 5 MB), (d) portal vein serum HDL-concentration (mmol/l) in conventional rats fed high-fat diet (C) or in ApoE^{-/-} rats fed a low-fat diet (LF) or a high-fat diet (HF), pure or supplemented with 10 g/kg of MB or monovalerin (MV). Mean values were significantly different between two groups: * $p < 0.05$, **** $p < 0.0001$.

Short-chain fatty acid profiles

Caecum

Supplementation of MB (5 g/kg) to a high-fat butter-based diet decreased caecal amounts of propionic and isovaleric acids, while the ratio of acetic-to-propionic acid increased compared with the control diet (Table 3, Paper I). Other doses of MB did not show any further effects, except higher ratios of acetic-to-butyric acid and acetic-to-propionic plus butyric acids with 15 g/kg MB (Table 2, Paper III). TB had

similar effects as MB, except for a decrease of acetic-to-propionic acid ratio. TB supplementation reduced total amounts of SCFAs and valeric acid compared with the control group.

MV and TV decreased the amounts of total SCFAs, acetic, propionic and valeric acids (only MV). These values were lower compared with the control group without any supplementation and compared with the low-fat group.

High-fat diets suppressed caecal formation of SCFAs compared to low-fat diets, as shown in several rat studies [44,166]. Supplementation of MB and TB to high-fat diets has not increased the amount of SCFAs extracted from the caecal content. This was somewhat unexpected and the reason for this is not known, but it could be that MB and TB were rapidly absorbed and metabolised in the caecum or even absorbed already in the upper parts of the gastrointestinal tract. The latter is in line with studies showing that triacylglycerol emulsions (including tributyrin) are quickly hydrolysed by gastric and pancreatic lipases, and freely absorbed into the digestive mucosa in hydrolysed or esterified forms [167,168]. It has been reported that monobutyryn is hydrolysed quickly *in vitro* by water-soluble hydrolases and esterases present in both plasma and tissues [169]. Similarly, tributyrin is rapidly converted to butyric acid in blood samples from rats given tributyrin (2 g/kg body weight) [170]. The peak concentration of tributyrin (2.2 μM) occurred at 25 minutes, while that of butyric acid was at 30 minutes (109.4 μM).

Caecal amounts of SCFAs were relatively lower with TB than MB supplementations, but these might be expected to be higher. This is because TB is degraded to MB, which survives *in vitro* gastrointestinal conditions [138]. It may be questioned whether MB and TB or their metabolites could reach the caecum, affecting the SCFA profile. Although the present study was not focused on the route of glycerol esters of SCFAs through the body, there is evidence that the glycerol esters are able to enter the caecum. For example, daily gavage of tributyrin (200 mg/100 g body weight) to rats for 9 weeks resulted in increased colonic tissue concentrations of butyric acid [114]. When encapsulated, tributyrin was detected in the caecum and colon content of rats, despite greater levels accumulated in the small intestinal walls [171].

Among the main SCFAs, MB and TB decreased caecal amounts of propionic acid. This is consistent with results in a study on humans, where oral supplementation of sodium butyrate (4 grams daily) for four weeks reduced faecal concentrations of propionic acid [172]. As a possible explanation, the authors discussed whether oral butyrate induced changes in the flux of acetic and propionic acids. Propionic acid is considered a substrate for gluconeogenesis, but the incorporation of colonic-derived propionic acid into glucose in humans (6%) is markedly lower than in mice (62%) [134,173]. There are no previous reports in rats, but blood glucose levels were not changed with supplementation of MB nor TB, so gluconeogenesis is of less importance for the decrease of caecal propionic acid. In contrast, the ratios of acetic-to-propionic acid, acetic-to-butyric acid and acetic-to-

propionic plus butyric acids increased with both MB (5 and 15 g/kg) and TB, indicating a redistribution of SCFAs and involvement of the microbiota. Transformation of acetate to butyrate (24%) is the most significant conversion by the human colon microbiota, but conversion of propionate to acetate (8%) or butyrate (5%) also appears at a lesser rate [134]. BAs may contribute to these transformations because they influence the gut microbiota composition.

Dietary supplementation of cholic acid altered the caecal composition at phylum level, characterised by an increase in Firmicutes and a decrease in Bacteroidetes in rats [174]. This compositional change is often seen with high-fat feeding in both humans and rodents [37,38]. The microbiota also affects the BA profile by producing enzymes required for BA deconjugation or formation of secondary BAs such as bile salt hydroxylases (BSH) and 7 α -dehydroxylase, respectively. Some *Clostridium* strains (phylum Firmicutes) have been reported to possess 7 α -dehydroxylating activity in the gut [175-177]. BSH activity is found in both Gram-positive (*Bifidobacterium*, *Clostridium*, *Enterococcus* and *Lactobacillus*) and Gram-negative (*Bacteroides*) gut bacteria [177,178]. The activity of these bacterial enzymes is responsible for the BA composition in the colon, which is predominantly enriched with unconjugated and secondary BAs [178]. Diet-microbiota interaction may lead to changes in caecal SCFAs [45,179], including propionic acid as seen in this study. Overall, MB and TB are able to affect the gut microbiota composition, directly as dietary substrates or indirectly by decreasing BA synthesis seen in the liver.

Caecal valeric acid in the rats was also decreased with MB and TB, although only TB reached significance. Less is known about potential nutritional effects of valeric acid, but valeric acid was concurrently increased in the caecum of piglets with increasing doses of a liquid analogue containing DL-methionine. It was also associated with improved growth performance and gastrointestinal morphology in the animals [180]. Under diseased states like obesity or celiac disease, high concentrations of valeric acid have also been found in human faecal samples [128,129]. MB and TB diminished caecal amounts of isobutyric and isovaleric acids. These fatty acids are products of protein fermentation, with a common source originating from intestinal sloughed cells independent of species, diet or age [181]. Increased faecal levels of isobutyric acid and isovaleric acid have been measured in obese people [129]. It is likely that the presence of MB and TB in the high-fat diet may limit protein breakdown and potentially contribute to an enhancing effect on gut integrity.

Portal vein

Valeric acid concentration in the TV group was twice as high as that in the control group without supplementation. Valeric acid was also higher in the MV and TV

groups compared with the low-fat diet, but a fall in concentrations of propionic and isobutyric acids was seen with MB and TB.

The increase in serum valeric acid concentration with MV and TV would be of interest. This result indicates that valeric acid derived from MV and TV is metabolised more slowly, particularly compared with butyric acid, and sustained in blood for a longer time. The increase may enable more valeric acid for other organs, for example the brain. High uptake has been reported in the liver and heart for valproic acid, an analogue of valeric acid used to treat epilepsy and mood disorders [95]. Valproate metabolites are considered to cause hepatotoxicity, and fatal cardiac malformation has been reported in neonates of epilepsy mothers given valproate during pregnancy [182]. Valeric acid is also higher in diseases related to the colon, as seen with high concentration in venous blood of microscopic colitis patients [104]. To draw a definite conclusion about health effects of valeric acid, it is necessary to compare its levels between healthy and diseased conditions, and probably between different sampling locations if applicable. For propionic and isobutyric acids, their decreased concentrations in the portal vein are probably related to the low amount in the caecum, as explained above.

In ApoE^{-/-} rats, MV supplementation led to increased levels of valeric acid in the portal vein ($p < 0.0001$, Paper IV), a phenomenon also seen in conventional rats (Paper II) and demonstrating an efficient release of valeric acid from the MV distributed. Valeric acid might be taken up by the liver and affect lipid metabolism, as previously reported for valproic acid [95]. In Paper IV, there was a positive correlation between portal serum valeric acid and HDL-cholesterol. The increased serum valeric acid was also reflected in the brain, and the concentration seemed to increase at the expense of isovaleric acid ($p = 0.017$, $r = -0.39$), which was also seen in conventional rats fed MV (Paper II). Isovaleric acid was negatively correlated with occludin in the brain ($p = 0.050$, $r = -0.43$), and expression increased with MV. Isovaleric acid has been linked to several undesirable effects, including depression, impaired neurotransmission, sleep disorder and encephalopathy [183-185] and is also involved in cholesterol synthesis [186]. These results suggest an improving role for valeric acid on lipid metabolism in diseased states.

Brain

MV and TV increased the amount of total SCFAs (51% for MV and 63% for TV) and acetic acid (52% for MV and 65% for TV), while MV reduced the total amount of isovaleric acid.

A correlation between valeric and acetic acids and the degradation of valeric acid seems to form acetic acid, as shown previously in the rat liver [187]. Like valproic acid, valeric acid may stimulate acetic acid formation by inhibiting the formation of succinic acid, an intermediary metabolite of the citric acid cycle [188]. Suppression

of succinic acid enhances levels of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), a metabolite reported to be reduced in the brain of Alzheimer's disease patients [189]. Valeric acid has also been shown to possess GABAergic properties in the rat brain, protecting against dementia [125].

The increase of acetic acid in the brain may represent potentially positive effects that cannot be fully reflected by limited SCFA uptake in the brain. Following injection into the carotid artery, uptake of acetic acid into rat brain was lowest compared with butyric (highest) and propionic acids [190]. Minimal brain uptake of SCFAs has also been seen in other species. For example, only about 3% of an intravenously injected dose of acetate was taken up into the mouse brain, whereas a gradual uptake (2%) occurred during 20 minutes at post-colonic infusion [94]. Using dynamic positron emission tomography (PET), butyrate brain uptake was less than 0.006% of the injected dose in baboons [95]. In healthy human brains scanned with PET, there was no measurable acetate uptake in the brain [191].

Low uptake in the brain does not necessarily mean that SCFAs are unable to have a positive effect, as suggested by Stilling *et al.* [52]. Indeed, acetate has been shown to reduce acute food intake considerably one to four hours after intraperitoneal injection in mice or intracerebroventricular administration in rats, despite low reported brain uptake [94]. This effect was further linked to inhibition of appetite by suppressing neuropeptides in the hypothalamus, already seen at 30 minutes after acetate administration and prolonged until 60 minutes. Our results are in line with this, since rats fed MV and TV diets had lower food intake compared with the control without supplementation. When considered as an HDAC inhibitor, a single dose of acetate has been shown to upregulate histone H3 and H4 acetylation in normal rat brain. After 28-day oral administration of acetate, neuroglial activation and protein expression of the pro-inflammatory cytokine IL-1 β were attenuated in the brain of rats subjected to LPS-induced neuroinflammation [192]. Acetic acid, naturally present in human diets in the form of vinegar and other fermented foods such as kimchi and sauerkraut, also have effects on obesity and gastric emptying [145,193]. Oral consumption of vinegar has been demonstrated to decrease blood lipids in rats fed cholesterol-rich diets and in obese humans, associated with lowered body weight and body fat mass [62,145]. MV and TV may therefore be used as promising anti-obesity food supplements.

The decrease of isovaleric acid in the brain may introduce a meaningful implication regarding isovaleric acidaemia (IVD). This is a rare, genetic recessive disorder caused by mutation of the enzyme isovaleryl-CoA dehydrogenase. As a result, the body cannot break down the amino acid leucine properly, causing isovaleric acid to build up. A distinct sweaty odour is characterised in individuals having this disorder. Although the prevalence of IVD is estimated to be low (0.19 to 0.79 per 100,000 cases in Western populations), its symptoms can constantly progress to serious health problems such as seizure, coma and possibly death [194]. Cognitive impairment was seen in IVD patients, especially those diagnosed later in

life [195]. Long-term treatments of IVD include increasing the conversion of potentially neurotoxic free isovaleric acid monomers to non-toxic conjugates that can be excreted in urine. These strategies can be achieved by reducing protein or leucine intake, in combination with L-carnitine and/or glycine supplementation.

Mechanisms linking the involvement of isovaleric acid in brain-related disorders have been described in several studies. Addition of isovaleric acid (0.5 to 10 mM) to supernatants and mitochondrial preparations from rat cerebral cortex was effective in reducing carbonyl production, an indicator of protein oxidative damage [196]. Isovaleric acid was shown to inhibit activity of Na⁺, K⁺-ATPase, an essential enzyme for maintaining normal neurotransmission, in synaptic membranes in rat's cerebral cortex [184]. Another isoform of isovaleric acid, isovaltrate, was also reported to interfere with sleep, by acting as a reverse agonist on adenosine A1 receptor in rat brain [185]. Despite most mechanistic actions of isovaleric acid in the brain being shown by animal studies, studies with depressed patients showed a positive relationship between faecal isovaleric acid and cortisol, as well as with depression-associated faecal bacteria [183]. These results, ours and others, highlight the influence of gut-derived SCFAs, both main and minor, on brain functioning. From a nutritional perspective, this connection is of interest since it can be positively and non-invasively modulated by supplementation of potentially bioprotective components like MV.

In ApoE^{-/-} rats, the amount of butyric acid was elevated in the brain tissue with MB in the diet compared with the control and the low-fat group ($p < 0.05$). MV also increased the content of butyric acid to a certain extent (insignificant, $p = 0.18$ versus the control) and decreased that of propionic acid ($p = 0.065$ versus the control and $p < 0.05$ versus the low-fat, respectively). The MV group displayed the lowest amount of isovaleric acid ($p < 0.05$ versus the control and $p < 0.01$ versus the low-fat, respectively), followed by the MB group ($p < 0.05$ versus the control). Both MB ($p < 0.05$) and MV ($p < 0.01$) increased valeric acid compared with the low-fat group.

The accumulation of butyric acid in the brain following consumption of MB is an interesting outcome. Uptake of butyrate into the rat brain was shown previously to be higher compared with acetic and propionic acids [156,190]. Elevated levels of butyrate in the brain of rats and mice have been shown to be protective against vascular dementia and cerebral ischemia [96,97,197]. It was reported that lack of ApoE gene compromises the BBB in mice [198]. This alteration might promote build-up of circulating metabolites, including SCFAs. There was a positive correlation between brain butyric acid and the expression of ZO-1 ($p < 0.0001$, $r = 0.78$) and GPR109A ($p = 0.0005$, $r = 0.72$) in the brain, indicating that butyrate may improve BBB integrity by upregulating the expression of tight junction proteins. The results also indicate the potential of suppressing inflammation, because GPR109A is known as an inflammation and tumour suppressor [82,199,200]. GPR109A is the receptor of nicotinic acid, a HDL-raising drug/vitamin used

clinically to treat dyslipidaemia [201]. As reported in the lipid section, serum HDL-cholesterol was positively associated with the expression of both GPR109A and ZO-1 in the brain. These connecting results emphasise an interactive effect of butyrate on lipid metabolism, BBB integrity and inflammation, and valeric acid increased in the brain with both MB and MV. Regarding A β neuropathology of AD, valeric acid, followed by butyric acid and propionic acid, was recently shown to potently inhibit the assembly of A β 1-40 and A β 1-42 peptides into soluble neurotoxic A β aggregates *in vitro* [202], suggesting a protective role of SCFAs in counteracting the onset of AD.

Intestinal permeability

The intestinal permeability decreased with MB in a dose-dependent manner in rats fed the butter-based high-fat diets. This was significant with the highest dose of MB, 15 g/kg, which exhibited the lowest urinary concentrations of mannitol and lactulose ($p < 0.05$ and $p = 0.101$, respectively). The glycerol-supplemented diet had no effect on intestinal permeability (Paper III).

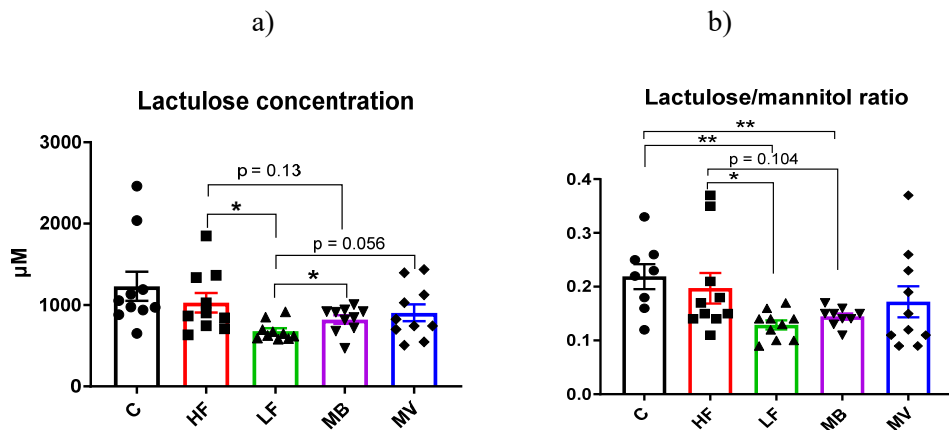
Increased intestinal permeability is associated with high-fat diet consumption in both humans and rats [39,203,204]. High-fat diet rapidly induced an increase in paracellular permeability in the ileum of rats as early as after 1 week, and in the colon after 3 weeks [205]. The increased permeability is connected to an altered distribution of occludin in the epithelial cells, indicating tight junction disruption [203]. However, Peng *et al.* showed that butyrate (2 mmol/l) accelerates redistribution of ZO-1 and occludin to the tight junctions, enhancing barrier function as evidenced by increased transepithelial electrical resistance in a human colonic epithelial cell line [90]. Dietary supplementation of tributyrin (5 g/kg standard diet) has been reported to be capable of reducing intestinal permeability and mucosal damage in mice chemically induced with colitis [123]. Mucosal infiltration of immune cells decreased with tributyrin supplementation, due to increased amount of regulatory T cells (Treg) and levels of anti-inflammatory cytokines (IL-10 and transforming growth factor β).

In a pig model of colitis induced by acetic acid, tributyrin supplementation (1 g/kg basal diet) increased claudin-1 protein and EGFR mRNA expression in the colonic mucosa [122]. EGFR is a receptor important for the regeneration of the mucosal epithelium or the repair of the small intestinal mucosa following damage, stabilising the gut internal environment. Tributyrin supplementation also increased goblet cell numbers in the colon, i.e. cells that are responsible for mucus production. The mucus layer acts as a lubricating barrier protecting the epithelial cells from interaction with the enteric bacterial and potentially toxic agents [69]. Acetate (5-100 mM, dose-dependent) and butyrate (5 mM) were shown to stimulate mucin

secretion in rat colon [206]. In the present study, caecal acetic acid was reversely correlated with urinary lactulose as well as the lactulose-to-mannitol ratio, indicating a suppressing role of acetic acid on intestinal permeability.

In ApoE^{-/-} rats, MB supplementation tended to lower the concentration of lactulose (Figure 5a, $p = 0.13$) in urine and lactulose-to-mannitol ratio (Figure 5b, $p = 0.104$) by 20% and 27% compared with the control, respectively. MV did not affect intestinal permeability. Greater mucosal thickness was observed in the duodenum and jejunum of ApoE^{-/-} rats fed MB ($0.001 < p < 0.01$) and MV ($0.001 < p < 0.05$) compared with the low-fat group (Figure 5c and d).

Obese humans have increased intestinal permeability [39] and are more vulnerable to the development of neuropathological disorders such as AD [3]. ApoE^{-/-} mice have been reported to have an impaired BBB [207]. We also found increased intestinal permeability in ApoE^{-/-} rats fed high-fat diets in Paper IV, which was consistent with the finding seen in conventional rats (Paper III). In contrast, incorporation of MB into the high-fat diet reduced urinary lactulose and lactulose-to-mannitol ratio to the level comparable with the low-fat diet. Mucosal thickness measurement also revealed that the proliferative/renewal ability in the duodenum and jejunum was greatly improved by MB and MV in comparison with the low-fat group. It is, therefore, proposed that the butyric acid released from MB was rapidly captured and metabolised by the intestinal epithelial cells, leading to improved intestinal health and integrity.



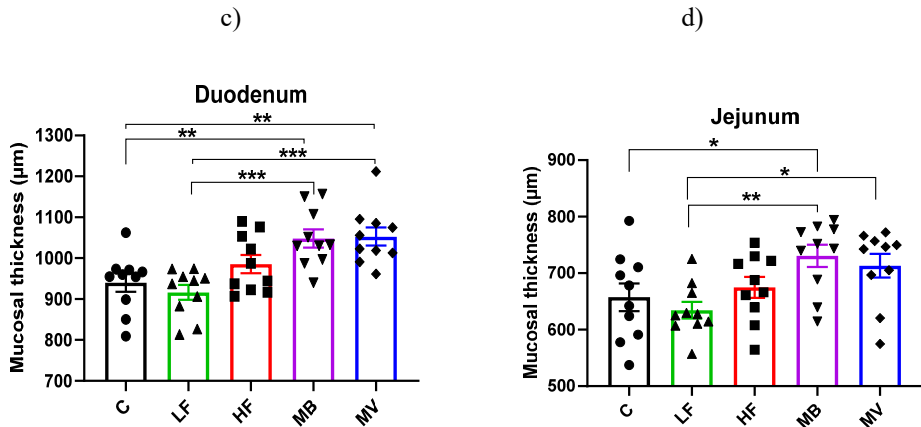


Figure 5.

Effects of monobutyryn (MB) and monovalerin (MV) on intestinal permeability and mucosal thickness in ApoE^{-/-} rats. C, conventional rats fed high-fat diet, ApoE^{-/-} rats fed a low-fat diet (LF) or a high-fat diet (HF), pure or supplemented with 10 g/kg of MB or MV. Mean values were significantly different between two groups: * p < 0.05, ** p < 0.01, *** p < 0.001.

Caecal microbiota composition

Supplementation of MB and TB completely depleted the phylum Candidatus Saccharibacteria (known as TM7) that was present in the control diet without supplementation. This change was also reflected at genus level, with the absence of an unidentified genus in the family *F16* in all the groups fed MB and TB. Both MB and TB decreased *Dorea* and *rc4-4* (both belonging the phylum Firmicutes) as well as an unclassified genus in the family *S24-7* (belong to the phylum Bacteroidetes). In contrast, *Mucispirillum* genus within the Deferribacteres phylum increased with MB and TB, but only MB attained significance.

MV supplementation increased the relative abundance of Bacteroidetes (p = 0.095) and decreased that of Firmicutes (p = 0.077), resulting in higher Bacteroidetes-to-Firmicutes ratio (p = 0.079) relative to the non-supplemented control group. A trend (p = 0.061) toward an increased relative abundance of Tenericutes was seen with MV. The phylum TM7 was completely depleted with TV and reduced with MV compared with the control group without supplementation (p < 0.05). At genus level, MV increased the abundance of some genera (*Bacteroides*, *Parabacteroides*, unclassified genera in the family *Rikenellaceae* and *RF32* order) compared with the control group without supplementation. In contrast, *rc4-4* tended to decrease with MV (p = 0.074). Both MV and TV suppressed/eradicated the relative abundance of an unclassified genus in the family *S24-7* and the representative genus of the TM7 phylum (an unclassified genus in the family *F16*).

An overview effect is that supplementation of glycerol esters of butyric and valeric acids in the diet modulated the caecal microbiota to a less obese and inflammatory profile. More effects were observed with the valerins, and the mono-forms seemed to have greater influence than their corresponding tri-forms. At phylum level, MV stimulated an increased ratio of Bacteroidetes-to-Firmicutes. An increased ratio of these bacteria is associated with a higher consumption of high-fibre diets, which is considered as positive [208,209], whereas a decreased ratio is commonly seen in obese individuals and in obese mice [37]. The phylum TM7 was totally absent (in MB, TB and TV) or very low (in MV) in the supplemented groups compared with the controls without glycerol ester supplementation. This finding supports a plausible anti-inflammatory property of the glycerol esters, since TM7 has been found in high abundance in patients with inflammatory bowel disease and in mice with chemically induced colitis [210,211]. Beyond the gut, TM7 is considered as an indicator of oral inflammation, and a higher abundance of TM7 in the human subgingival plaque has been found in sites with periodontitis than in healthy sites [212].

Results revealed that both MB and TB decreased the abundance of *Dorea* and *rc4-4*; MV supplementation also tended to decrease *rc4-4*. These two genera are enriched in rats with high-fat diet-induced obesity [45,205], and *Dorea* has been reported to be associated with total serum and LDL-cholesterol in high-fat induced hyperlipidaemic rats [213]. In mildly hypercholesterolaemic subjects, the abundance of *Dorea* decreased following consumption of β -glucan, which was related to reduced risk factors of cardiovascular disease, for example decreased body mass index, waist circumference and blood pressure, and increased serum HDL-cholesterol levels [214]. Improvement in lipid profiles in the liver was also seen with MB and TB (Paper I), and MV (Paper II), although to a lesser extent.

The abundances of *Bacteroides* and *Parabacteroides* have also been shown to increase with fibre-rich diets and are negatively associated with serum triglycerides [214]. These two genera were promoted by MV. *Bacteroides* was shown to positively correlate with faecal secondary BAs (CDA) and negatively with faecal SCFAs, implying that increased abundance of *Bacteroides* may promote the excretion of secondary BA, but not SCFA, into faeces [215]. This effect can be considered as protective because most secondary BAs have been suggested to increase the risk of colonic inflammation and cancer [216]. Besides effects on lipid-related bacteria, MB supplementation increased the abundance of *Mucispirillum*, a species known as an inhabitant, colonising the mucus layer of the gastrointestinal tract of rodents [217]. This genus has been reported to decrease rapidly under impaired colonic conditions such as colitis in mice, and was not reinstated until the mucus layer was fully regenerated [218]. Altogether, supplementation of glycerol esters of SCFAs created a caecal environment that favoured growth of potentially beneficial bacteria, which in turn diminished many adverse outcomes caused by high-fat diet.

Succinic acid

The concentration of succinic acid and the ratio of succinic-to-butyric acid in the liver decreased in all groups supplemented with glycerol esters of SCFAs and were significant for MV and TV compared with non-supplemented control group ($p < 0.01$). Lower values were also seen when considering the total amount of succinic acid in the liver ($p < 0.05$ for MV and $p < 0.01$ for TV, respectively, Paper II) and in relation to body weight ($p < 0.05$ for both MB and TB, Paper I).

Succinic acid is known as an intermediary metabolite of the citric acid/Krebs cycle, and may be considered as a signalling molecule by binding to its receptor GPR91. Succinic acid serves as a substrate for the microbial formation of propionic acid [219], and is not formed at significant levels under normal conditions. Higher amounts of succinic acid are found when the intake of dietary fat increases and after antibiotic treatment at the expense of especially butyrate [44,133,220]. The decrease of liver succinic acid by dietary MB and MV may be explained by the decreased amounts of propionic acid found in the caecum and portal blood.

Succinate in the liver stimulates the release of α -smooth muscle actin, a marker of fibrogenesis [221], which may eventually result in liver injury [222]. In this respect, it is important to mention that increased levels of blood succinate were seen in patients undergoing liver transplantation [223]. Similarly, elevated blood levels of succinate have been seen in animal models of obesity, diabetes and hypertension [224] and in obese subjects and those having cardiovascular diseases [223,225]. Accumulated succinate in the cells, as shown in ischaemia, can be rapidly transported to the bloodstream by the protein 'I'm not dead yet' (INDY, coded by life-extending genes) [226,227]. Succinate was also shown to increase with palmitate, a saturated fatty acid present in high-fat diets, and in the liver of mice subjected to non-alcoholic steatohepatitis [221]. LPS strongly stimulated succinate which in turn promotes IL-1 β production [228]. It was suggested that succinate causes cardiac hypertrophy through activation of HDAC5. Butyrate is known to inhibit HDAC class II, including HDAC5, indicating a negative relationship between butyrate and succinate, which has been shown previously in our lab [44,45]. In Papers I and II, succinic acid was found to decrease, while there was a simultaneous increase of butyric acid in groups supplemented with glycerol esters. These results indicate that glycerol esters of SCFAs can alleviate the increase in succinic acid, protecting the liver from potential damage mediated by high-fat diets.

Tight junction proteins and SCFA receptors

In the small intestine, MB had minor effects, while MV increased the expression of GPR109A compared with the low-fat group (Figure 6f, $p < 0.05$) and tended to increase the expression of ZO-1 compared with the control (Figure 6b, $p = 0.093$).

In the brain, MV had more pronounced effects on tight junction proteins, as seen with the upregulated expression of ZO-1 (Figure 6a, $p < 0.05$) and occludin (Figure 6c, $p < 0.01$) compared with both the control and low-fat group. MB also increased the expression of occludin ($p < 0.01$ versus the control and $p = 0.073$ versus the low-fat). GPR109A tended to be higher with MB (Figure 6e, $p = 0.081$) and MV ($p = 0.067$) in the diet compared with the low-fat group.

Expression of the tight junction proteins occludin and ZO-1 was upregulated in the brain by MB and MV, with MV having more pronounced effects. These proteins are essential for barrier formation and decreased levels have been linked to BBB leakiness in mice, subsequently leading to neurotoxicity and memory impairment [229]. In subjects with multiple sclerosis, the white matter disruption of occludin and ZO-1 has been shown to associate with BBB leakiness [230]. Interventions with sodium butyrate or butyrate-producing *Clostridium tyrobutyricum* demonstrated reverse BBB leakiness in germ-free mice [124]. Activation of the inflammatory pathway NF- κ B-matrix-metalloproteinase (MMP)-9 in brain cells (pericytes) was mechanistically responsible for BBB breakdown in mice expressing human APOE4 or lacking murine gene ApoE. Butyric and valproic acids have been proved to inhibit the activity of MMPs [231,232].

Combining these results, tight junction enhancement by MB and MV could be related to anti-inflammatory properties of butyric and valeric acids. This suggestion is further evidenced by upregulation of GPR109A by MB and MV, in both the jejunum and the brain. GPR109A is suggested as a suppressor of colonic inflammation [82,199]. Its activation is claimed to be responsible for the neuroprotective effects of an anti-multiple sclerosis drug (dimethyl fumarate), through mechanisms involving inhibition of HDACs and NF- κ B [84]. In Paper IV, upregulated expression of ZO-1 and GPR109A in the brain were strongly connected with increased amounts of both butyric acid ($p < 0.0001$, $r = 0.78$ and $p = 0.0005$, $r = 0.72$) and valeric acid ($p = 0.001$, $r = 0.68$ and $p < 0.0001$, $r = 0.78$). Consequently, dietary supplementation of MB and MV is effective in changing the SCFA profile in the brain to the extent that rescues components of the BBB blunted by high-fat diet.

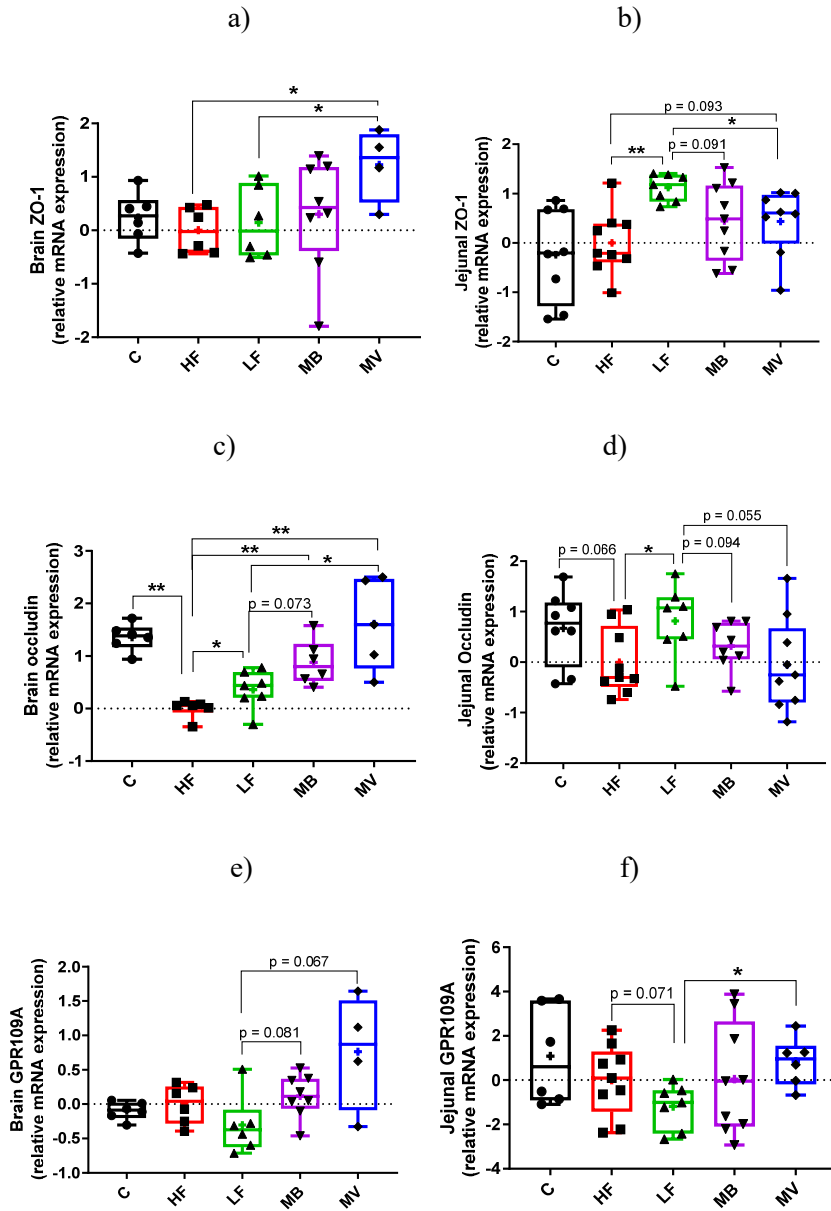


Figure 6.

Effects of monobutyryn (MB) and (MV) on brain and jejunal expression of tight junction proteins and receptors of short-chain fatty acids in ApoE^{-/-} rats fed high-fat diets. Conventional rats (C) were fed a high-fat diet or ApoE^{-/-} rats were fed a low-fat diet (LF) or a high-fat diet (HF), pure or supplemented with 10 g/kg of MB or MV for 5 weeks. Data are presented as logarithm 2 of fold change relative to the HF group. Mean values were significantly different between two groups: * p < 0.05, ** p < 0.01.

Cytokines

IL-10 levels were significantly lower in aortic plasma of ApoE^{-/-} rats fed the MB diet than those fed the control diet and low-fat diet (Figure 7a, $p < 0.05$). Concentrations of IL-1 β in the aortic plasma and brain tissue were similar in the low-fat group and lower than the control group, although insignificant (Figure 7b and c).

Decreased circulating levels of IL-10 in ApoE^{-/-} rats fed MB, at a dose of 10 g/kg, seemed to be in line with the results from a study in obese male subjects. In that study, peripheral blood mononuclear cells produced less IL-10 and other proinflammatory cytokines (IL-6 and TNF- α) after four-week oral butyrate supplementation (4 g/day) [172]. These findings imply that oral butyrate has a strong immune-modulating effect in cells, but it could be time-dependent for certain cytokines. For example, butyrate pre-treatment (0.5 mmol) resulted in higher levels of IL-10 (and lower IL-1 β and TNF- α) in human endothelial cells after 4 hours incubation with oxidised LDL [233], where IL-10 dropped after 24 hours.

In humans, the effects of SCFAs on systemic proinflammatory cytokines could be seen only after acute administration [156]. Plasma concentration of TNF- α decreased in hyperinsulinaemic female subjects after intravenous or rectal administration of acetate over the course of 60 minutes compared with the saline group [234]. In overweight or obese normoglycaemic men, fasting plasma levels of IL-1 β were reduced following rectal infusion of a SCFA mixture high in acetate (24 mmol sodium acetate, 8 mmol sodium propionate and 8 mmol sodium butyrate) compared with a SCFA mixture high in propionate (18 mmol sodium acetate, 14 mmol sodium propionate and 8 mmol sodium butyrate) [235].

IL-10 is an anti-inflammatory cytokine. This should be addressed only in specific experimental conditions, because in a mouse model of AD, overexpression of IL-10 has been shown to increase A β accumulation, reduce the memory and increase ApoE expression, especially in the plaque-associated insoluble cellular fraction, while microglial A β phagocytosis was diminished [236]. These data might have a translatable meaning for the non-effects of anti-inflammatory strategies on AD patients in clinical studies [237,238]. Considering the multiple actions of butyrate, targeting net outcomes on several biomarkers, rather than a single parameter, would be an effective approach to counteract high-fat and ApoE-induced metabolic diseases. As in Paper IV, improvements in different markers of gut-brain barrier were achieved, although plasma IL-10 decreased.

The decrease in plasma concentration of IL-10 could be explained by HDAC inhibition of butyric acid, which was highest in blood and brain tissue with MB. Transcriptional activation of IL-10 is controlled by the mediator HDAC6 and suppressor HDAC11 [239]. Expression of these two mediators has been shown to increase with butyrate treatment [240-242]. Because both HDAC6 and HDAC11 are

involved in the activation of IL-10, it is likely that butyrate might promote HDAC11 expression to a greater degree than HDAC6. Also, via HDAC inhibition, intraperitoneal injection of sodium butyrate (1.2 g/kg body weight for 6 weeks) was shown to restore associative memory and increase hippocampal expression of genes involved in memory consolidation in a mouse model of AD (APP^{PS1-21}), even at an advanced stage of disease progression [243].

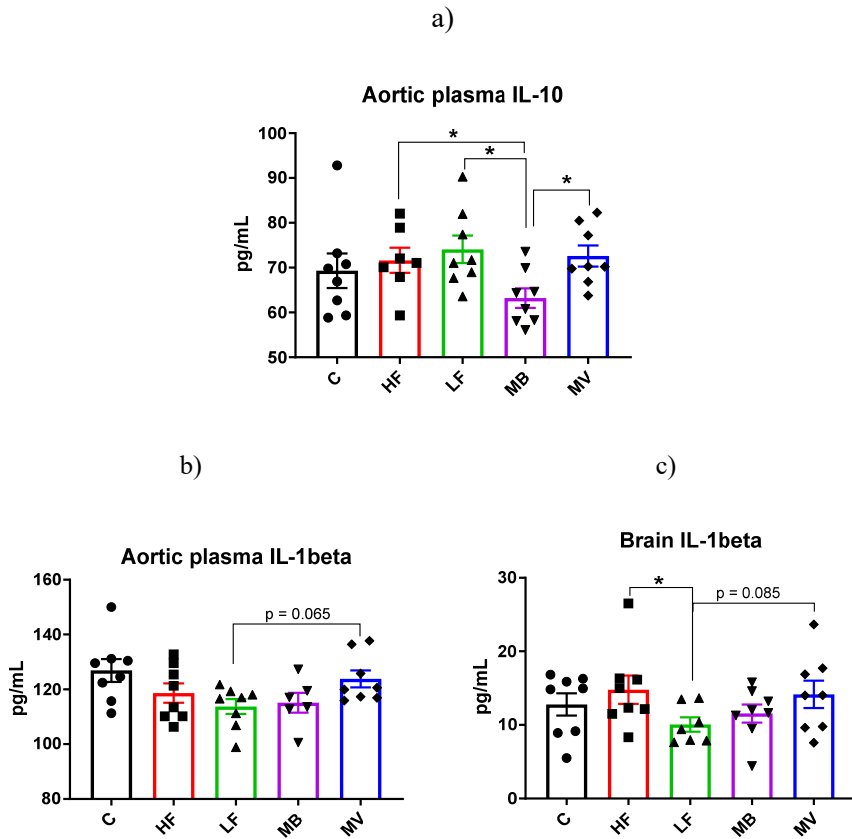


Figure 7. Effects of monobutyryn (MB) and monovalerin (MV) on cytokines in ApoE^{-/-} rats fed high-fat diets. Concentrations of (a) interleukin (IL)-10 (pg/ml) and (b) IL-1beta (pg/ml) in plasma, and concentrations of (c) IL-1beta (pg/ml) in brain tissue. Conventional rats fed high-fat diets (C), ApoE^{-/-} rats fed a low-fat diet (LF) or a high-fat diet (HF), pure or supplemented with 10 g/kg of MB or MV. Mean values were significantly different between two groups: * $p < 0.05$.

Where are the glycerol esters absorbed?

To our knowledge, there is currently no studies investigating hydrolysis or absorption of glycerol esters of SCFAs, except tributyrin. Studies in literature on tributyrin are not consistent and it has been suggested to be absorbed in the stomach and/or the small intestine, thereby increasing the butyric acid concentration in the portal vein [99] or the liver [112,113]. One *in vitro* study showed that tributyrin was extensively hydrolysed (60%) by gastric lipase [244] and when tributyrin was added to feed, butyric acid was detected in samples taken from the stomach, but not in the proximal colon [138]. Rats given tributyrin by gavage also increased colonic tissue concentration of butyric acid [114], but the dose in that study was comparatively high and the experimental time long. In the present study, the mucosal thickness of the small intestine was improved by MB and MV and there was also an indication that the comparatively low levels of MB/MV given to rats could reach the small intestine, caecum and colon [171]. *In vitro* studies showed that TB was hydrolysed to MB, which survived gastro-intestinal conditions. Diets used throughout the present project are high-fat diets, which contrasts with other studies. Build-up of long-chain fatty acids from high-fat diets can limit activity of gastric lipases in the stomach [245], partly protecting digestion of the glycerol esters, explaining why the glycerol esters survived further down in the gastrointestinal tract. One strength of this study is that we investigate both butyrins and valerins and know the exact composition of these components. Depending on the dose and means of administration, glycerol esters of SCFAs may be hydrolysed and absorbed in different parts of the gastrointestinal tract.

Conclusions

The results indicate that glycerol esters of butyric and valeric acids may counteract some of the adverse effects and metabolic conditions associated with a high-fat intake.

The specific conclusions for each paper are as follows:

- Dietary supplementation of monobutyrim and tributyrin to a high-fat diet decreased cholesterol, LDL-cholesterol and succinic acid and downregulated expression of bile acid synthesising genes in the liver, which associated with a less obese and inflammatory caecal microbiota profile. Monobutyrim seemed to have a greater effect than tributyrin (**Paper I**).
- Dietary supplementation of monovalerin and trivalerin decreased total cholesterol, LDL-cholesterol, and succinic acid in the liver, increased valeric acid in serum and acetic acid in the brain and modulated the caecal microbiota to lower abundances of bacteria related to the development of obesity and inflammation. Monovalerin seemed to have a greater effect than trivalerin (**Paper II**).
- Increasing levels of monobutyrim in high-fat diets led to a concurrent decrease in cholesterol and triglycerides in the liver and markers of intestinal permeability in urine. Monobutyrim consistently reduced total cholesterol and LDL-cholesterol in blood and raised HDL-cholesterol in the liver when the main fat source was based on lard instead of butter (**Paper III**).
- Supplementation of monobutyrim and monovalerin to the diets of ApoE^{-/-} rats improved blood-brain barrier function, as indicated by the upregulated expression of tight junction proteins in the brain. Monovalerin increased concentrations of HDL-cholesterol and valeric acid in blood, and decreased isovaleric acid in the brain. Monobutyrim tended to improve barrier function and stimulated accumulation of butyric acid in the brain (**Paper IV**).

A summary of results is shown in Figure 8.

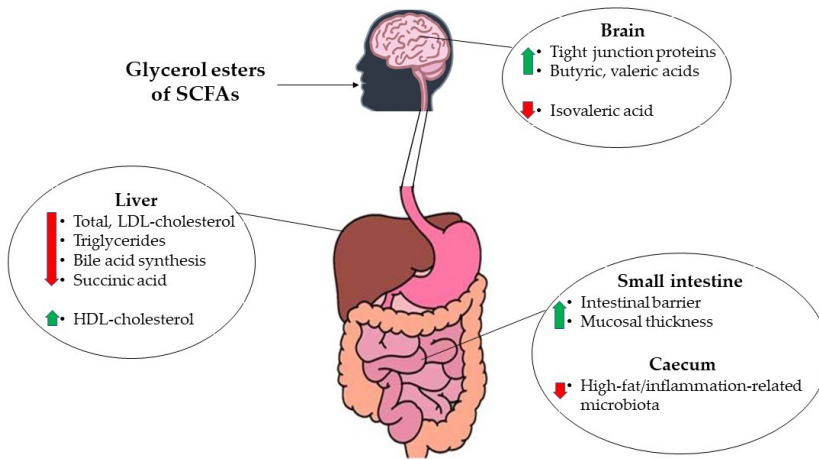


Figure 8.

Summary of effects of glycerol esters of short-chain fatty acids investigated in this project. Red arrows indicate decrease, green arrows indicate increase.

Future perspectives

The aim of nutritional research is to bring health benefits to humans by increasing knowledge about how diet-derived metabolites may affect the host metabolism. SCFAs are an interesting and attractive representative in this context, mostly due to their multiple functions that have been extensively investigated over recent decades. Several studies in humans and animals have supported that increased levels of SCFAs following consumption of fibre-rich diets may prevent and counteract metabolic disorders. Supplementation of SCFAs into high-fat diets may result in similar outcomes. The results from this project demonstrate that supplementation of high-fat diets with the glycerol esters of butyric and valeric acids attenuates markers for lipid disorders, intestinal permeability and systemic inflammation in rats. These effects are also evident on the caecal microbiota, reducing the abundance of bacteria associated with obesity and inflammation. The glycerol esters also have an impact on the liver and the brain, indicating SCFA-driven metabolic synchronisation across distant organs. The effects on the brain are promising, since current knowledge about a role for SCFAs in brain-related diseases is still insufficient and is just in the beginning to be explored. The following perspectives may be considered in future work.

Higher doses of the glycerol esters should be tested before conducting a human trial. In humans, the non-toxic maximal dose of tributyrin is 200 mg/kg, equivalent to 14 g tributyrin per day for a 70-kg adult. Our corresponding doses in rats are considerably lower. Assuming a rat weighing 270 g and consuming 20 g food daily, the dose of 5 g/kg of MB/TB would deliver 0.1 g MB/TB to the rat per day. Even with the highest dose (15 g/kg), the amount of MB/TB in food is only 0.3 g. With these doses, positive effects are achieved, and increasing doses seems to display stronger impact on certain parameters (barrier functions and liver lipids profiles). Butyrate has been included in mouse diets at a dose of 50 g butyrate/kg diet. Higher amounts of sodium butyrate (4 g) or glycerol esters of propionic acid (5 g) have also been used in human studies. Most studies in literature have used tributyrin. It is important to also test MB, since this compound gives more pronounced effects. The valerins have not been used at all in human studies and should be included in such studies.

Although this project has a preventive nutritional approach, consideration about stage of administration may be useful in clinical settings. The initial study with tributyrin in patients with advanced solid tumours showed no objective responses,

while later studies in rats showed that tributyrin is effective against liver cancer during initial or promotion stages. Prolonged treatment with sodium butyrate improved memory in mice, even when administered at a very late stage of AD. These studies might indicate that the experimental timing is specific for each disease, in which SCFAs may exhibit effectiveness or not. Depending on the type of disease investigated, further experiments with glycerol esters of SCFAs should explore specifically direct mechanisms of action. For AD, cognitive or memory tests can also be included.

Absorption studies may be needed to clarify where the glycerol esters of SCFAs are absorbed and the extent to which they are hydrolysed. Results from such studies are useful for designing means of delivery that target specific organs or diseases.

Co-supplementation of glycerol esters of SCFAs to foods requiring fortified folic acid, vitamin A or D is nutritionally promising, because combination of tributyrin with these components has been reported to strengthen the effectiveness against colon and liver cancers, at least in animal studies. Our results also highlight monobutylin and the valerins as new candidates for this application.

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